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Genetic Analysis of the yeast endo-exonuclease: Implications of its involvement in DNA double-strand break repair

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Abstract

Double-strand breaks (DSBs) are one of the most lethal threats to genomic integrity. In the yeast *Saccharomyces cerevisiae*, double-strand breaks are primarily repaired via the homologous recombination repair pathway. While the mechanisms underlying this pathway have been generally characterized, the early steps leading to commitment of DSBs to homologous recombination repair are as yet unclear. In particular, it has been shown that the exonuclease processing of DSBs is a critical step prior to strand invasion of a homologous template.

Endo-exonucleases represent a class of nucleases that have been linked to recombinational repair processes in various organisms. Early on, a degradative role was ascribed to the bacterial recBCD endo-exonuclease in processing DSBs in a 5' to 3' directionality prior to recA-mediated homologous recombination in *E. coli*. However, the nuclease responsible for this nucleolytic processing has yet to be identified in yeast. In this dissertation, I demonstrate that *RNC1* is a candidate gene that encodes a nuclease with characteristics similar to previously identified endo-exonucleases, and that *RNC1* is implicated in homologous recombination repair of DSBs.

While the *rnc1* null mutant is 10-fold more sensitive to persistent DSB induction than wild-type, a highly lethal *rad52* null mutation is suppressed in an *rnc1* knockout, indicating that RNC1 acts at an earlier step in the homologous recombination repair pathway than RAD52. Furthermore, in the absence of *RNC1*, the non-homologous end-joining pathway, mediated by the *KU80* gene product, appears to play a more dominant role in repairing DSBs. Moreover, a *ku80 rnc1* double mutant is hypersensitized to DSB induction.

Biochemical analysis of the purified RNC1 protein revealed that it has endonuclease activity on single-strand DNA as well as processive exonuclease activity on double-strand DNA with a 5' to 3' directionality. *In vivo*, this exonuclease activity was implicated in the processing of DSBs prior to repair. Our findings show that RNC1 possesses nucleolytic activities characteristic of previously identified endo-exonucleases, and confirm its role in the homologous recombination repair pathway. Intriguingly, RNC1 was also shown to exhibit methyltransferase activity which directs the modification of a specific base, m^5U_{54}, in elongator tRNA. While the biological significance of this activity is as yet unclear, it is attributable to a domain in the RNC1 protein separate from that engaging in DNA repair processes.

Résumé

Les cassures double-brin (CDB) sont une des menaces les plus potentes à l'intégrité génomique. En ce qui concerne la levure *Saccharomyces cerevisiae*, les cassures double-brin sont principalement réparées via le système de réparation par recombinaison homologue. Alors que les méchanismes à la base de ce processus ont été généralement caractérisés, les premières étapes conduisant de la CDB à la réparation par recombinaison homologue restent encore à definir. En fait, il a été démontré que la transformation des CDB par des exonucléases est une étape critique antérieure à l'invasion de la séquence homologue.

Les endo-exonucléases représentent une classe de nucléases liée aux procédés de réparation par recombinaison dans divers organismes. Dès l'abord, un rôle de dégradation fut attribué à l'endo-exonucléase bactérienne recBCD dans le cadre du traitement des CDB dans la direction 5' à 3' avant la recombinaison homologue médiée par rec-A dans la bactérie *E. coli*. Cependant, la nucléase responsable de ce procédé nucléolytic reste encore à être identifiée pour la levure. Dans ce document, nous démontrons que RNC1 est un gène qui encode pour une nucléase ayant des caractéristiques similaires à des endo-exonucléases précédemment identifiées et que RNC1 est impliqué dans la réparation par recombinaison homologue des CDB.

Alors que le mutant nul *rnc1* est dix fois plus sensible à une induction persistente de CDB que le type sauvage, une mutation nulle hautement fatale dans *rad52* est supprimée dans un knock-out de *rnc1*, indiquant ainsi que RNC1 agit dans une étape précédant l'entrée en action de RAD52 dans le méchanisme de réparation par recombinaison homologue. De plus, en l'absence de *RNC1* , la voie de ligation des

extrémités non-homologues, médiée par *KU80*, paraît jouer un rôle plus important dans la réparation des CDB. D'autre part, un double mutant *ku80* et *rnc1* est hypersensible a l'induction des CDB.

Des analyses biochimiques de la protéine RNC1 purifiée ont révélé qu'elle exhibe une activité endonucléase sur l'ADN simple-brin en plus de posséder une activité exonucléase processive sur l'ADN double-brin dans la direction 5' à 3'. *In vivo*, il a été démontré que cette activité exonucléase est impliquée dans le traitement des CDB avant la réparation. Nos résultats montrent que RNC1 possède une activité nucléolytic caractéristique d'endo-exonucléases précédemment identifiées et confirme son rôle dans le méchanisme de réparation par recombinaison homologue. Curieusement, il a aussi été démontré que RNC1 exhibait une activitée methyltransferase dirigeant la modification d'une base spécifique, m^5U_{54}, dans les ARN de transfert élongateurs. Alors que la signification biologique d'une telle activité reste encore non-définie, elle a été associée à un domaine de la protéine RNC1, distinct du domaine fonctionnel dans le réparation de l'ADN.

Acknowledgements

I would like to express my appreciation and gratitude to my supervisor Dr. Terry Chow, for his supervision and invaluable input to my research during my Ph.D. studies. I would also like to thank members of my Ph.D. thesis committee: Dr. Danuta Radzioch, Dr. Malcolm Whiteway, Dr. Dindial Ramotar and Dr. Claire Cupples, for all their support and guidance of my project.

Many thanks must be accorded to Dr. Alexandre Semionov, Dr. Harry Goldsmith and Dr. Nektaria Markoglou for their expert feedback and advice throughout my PhD, and to Jean-Philippe Belzile, for his help in translating my abstract into French. Also, I would like to thank my fellow colleagues and friends, for their support and friendship, over the years: Dr. Denis Cournoyer, Line Dufresne, Paul Kauler, Antonis Karatzas, Dr. Sylvain Letourneau, Fiona McIntosh, Rupa Narasimhadevara, Abraham Owusu, Jean-Pierre Paiement, Jean-Sebastien Palerme, Lakhbir Sandhu, Andreas Schwab, Hoi-Ying Shiu, Andre Simard, Chloe Villani, Ashley Webb, Chiaoli Yeh, and Bruce Ritchie, who will always be remembered. Special thanks to my sisters, for their encouragement and inspiration of my work throughout my studies.

This thesis is dedicated to my mother, whose personal sacrifices and unstinting support laid the foundation for my pursuits and dreams.

Preface

This thesis has been written according to the guidelines for a manuscript-based dissertation as outlined by the Faculty of Graduate Studies and Research of McGill University. These guidelines read, in part:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following: a table of contents, an abstract in English and French, an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature (in addition to that covered in the introduction to each paper) and a final conclusion and summary.

Additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers."

Chapter II has been published in Current Genetics (1998), Volume 34, pages 360-367 and is reproduced as printed. Chapter III has been submitted for publication to Mutation Research. The references in the General Introduction (Chapter I) and Discussion (Chapter IV) are given in the Bibliography (Chapter V).

Table of Contents

List of Abbreviations

ATP	Adenosine triphosphate
BER	Base-excision repair
β-gal	β-galactosidase
CBP	Calmodulin binding peptide
CHO	Chinese hamster ovary
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ds	Double-strand(ed)
DSB	Double-strand break
EE	Endo-exonuclease
GFP	Green fluorescence protein
GST	Glutathione *S*-Transferase
Gy	Gray
HR	Homologous recombination
IR	Ionizing radiation
Kb	Kilobase
MMS	Methyl methanesulfonate
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
PCR	Polymerase chain reaction
RNA	Ribonuclease acid

ROS	Reactive oxygen species
RPA	Replication protein A
RSS	Recombination signal sequences
SC	Synthetic complete
SCE	Sister chromatid exchange
SDSA	Synthesis-dependent strand annealing
ss	Single-strand(ed)
SSA	Single-strand annealing
SSB	Single-strand binding protein
tRNA	transfer RNA
UV	Ultraviolet
XRCC	X-ray-sensitive cell lines

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Contributions of Authors

1) In the work described in Chapter II, I performed all assays with the exception of the immunoblot assay, which was performed with assistance from Drs. Cournoyer and Lenhert, and the PCR analysis, which was performed by Paul Kauler. I constructed all strains except for the strain TC107a, constructed by Dr. Terry Chow.

2) In the work described in Chapter III, I carried out all the experiments except for the construction of the plasmid *GFP-RNC1*, which was performed by Ashley Webb, and fluorescence imaging of the nuclear localization of GFP-RNC1, performed by Dr. Dindial Ramotar.

Contributions to Original Knowledge

1) In chapter II, we demonstrated that an *rnc1* null mutation sensitizes the cell to DSB induction by HO endonuclease. Also, we identified an epistatic relationship between *RNC1* and *RAD52* corroborated in ionizing radiation studies. The manuscript details preliminary investigations of repair products in an *rnc1* environment via mating phenotype and PCR analyses.
2) In chapter III, we confirmed the genetic interaction between *RNC1* and *RAD52* via MMS toxicity assays, and showed that *KU80* and *RNC1* act in complementary DSB repair pathways. In-depth analysis of repair products via mating phenotype assays, PCR and sequencing revealed that non-homologous end-joining plays a more dominant role in DSB repair in the absence of RNC1. We demonstrated nuclear localization of RNC1 using a GFP tag, and provided *in vitro* and *in vivo* evidence of the nucleolytic activities of the RNC1 protein.

Chapter I

Introduction and Literature Review

1.1 Exogenous and endogenous sources of Double-strand break induction

One of the major challenges in maintaining genomic integrity lies in repairing double-strand breaks (DSBs), one of the most lethal forms of DNA damage. If left unrepaired, they lead to ruptured chromosomes and cell death. Improper repair of DSBs can lead to translocations, chromosomal aberrations, mutagenesis, and cancer.

Exogenous sources of DSB induction include ionizing radiation, free oxygen radicals, crosslinking agents and alkylating drugs. Treatment of cells with such agents has been known to cause a dose-dependent increase in frequencies of recombination, as a direct consequence of DSB induction.

While double-strand breaks are one of the most lethal and mutagenic DNA lesions, they also occur naturally in the eukaryotic cell as initiators of genetic rearrangement within the genome. Endogenous induction of DSBs serves to stimulate the recombinatorial mechanisms that are responsible for generating genetic variation and play essential roles in sexual reproduction.

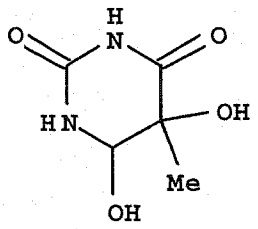
1.1.1 Ionizing Radiation

Ionizing radiation induces a plethora of DNA lesions, including single- and double-strand breaks, DNA-protein crosslinks and base damage (Kupiec, 2000; Norbury and Hickson, 2001). However, it is the incidence of DSBs that determines cell survival after ionizing radiation (Resnick and Martin, 1976). The biological effects of radiation result from either direct or indirect ionization of biological substrates. Charged particles such as α and β particles are directly ionizing; they disrupt the atomic structure of DNA and produce DNA lesions (Hall, 1994). Electromagnetic radiations such as x- and γ -rays

are indirectly ionizing. They do not produce chemical and biological damage themselves, but release energy that is absorbed by the material through which they pass (Hall, 1994). The absorption of energy leads to ionization, whereby high-energy particles called fast electrons are released through a transfer of photon energy into kinetic energy called the Compton process (Hall, 1994). These particles frequently ionize other atoms and create free oxygen radicals.

1.1.2 Reactive oxygen species/ Oxygen metabolism

Reactive oxygen species (ROS) or oxyradicals formed through the reduction of O_2 either directly oxidize DNA bases (Figure 1), or attack lipid or protein molecules to generate intermediates that react with DNA to form adducts (Marnett, 2000; Ward *et al.*, 1987). The resulting DNA damage blocks DNA replication, and causes DNA double-strand breaks (Johnson *et al.*, 1996b). Bleomycin is an anticancer drug that generates DSBs by producing oxidative free radicals (Chu, 1997; Hall, 1994). In bleomycin-induced DNA degradation, hydrogen is abstracted from C-4 of deoxyribose, probably by a transient high-valence iron-oxo complex of bleomycin (Burger, 1998). About half of the resulting C-4 free radicals react with O_2 to form peroxyl radicals, which in turn decompose to produce backbone cleavage (Burger, 1998).



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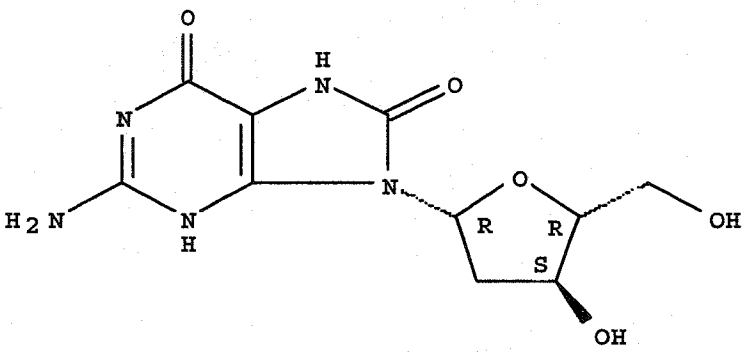

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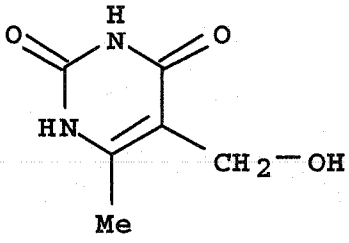
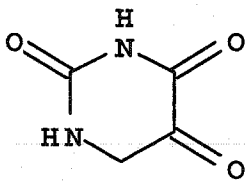


5-hydroxymethyuracil



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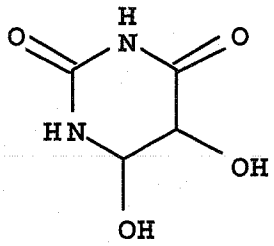

uracil glycol

Figure 1. Examples of oxidized DNA bases (Marnett, 2000).

1.1.3 Alkylating agents

A large number of chemical agents can alkylate DNA, and are widely used as laboratory mutagens. They decompose within a cell to produce an alkyl-diazonium ion which attacks nucleophilic sites within DNA to produce a variety of N-alkylated and O-alkylated nucleotides (Lawley, 1968). Methyl methanesulfonate (MMS) is an alkylating agent that is usually referred to as an "IR-radiomimetic" drug, because it has an effect very similar to that of ionizing radiation. MMS mainly methylates bases in DNA to produce 7-deoxyguanine and 3-methyladenine (Kupiec, 2000). These alkylation lesions do not by themselves cause double-strand breaks, but may eventually lead to DSB formation when processed by the base-excision repair (BER) pathway. In BER, a DNA glycosylase removes the damaged base, followed by cleavage at the abasic site by an apurinic/apyrimidinic (AP) endonuclease (Xiao and Chow, 1998). However, strand breakage may occur at abasic sites, resulting in DSB formation (Povirk, 1996). An alternate theory suggests that alkylation lesions cause replication blocks, and passage through S-phase eventually leads to the formation of DSBs (Kupiec, 2000).

1.1.4 Crosslinking agents

Crosslinking agents such as psoralens, mitomycin C, and cisplatin have the potential to react with two different nucleophilic centers in DNA resulting in intrastrand and interstrand crosslinks (Kupiec, 2000). These agents are extensively used in chemotherapeutic treatment of cancer as they have been found to selectively block DNA replication and transcription in rapidly dividing cancer cells. Treatment of yeast cells with crosslinking agents results in DSB induction mediated by products of a group of

genes called the *PSO* genes. These gene products appear to be highly specific for crosslinks, however their mechanism of action remains to be elucidated (Meira *et al.*, 1992). Interestingly, cisplatin treatment of both prokaryotic and eukaryotic cells sensitizes them to killing by ionizing radiation (IR), presumably by inhibiting DSB repair after IR (Dolling *et al.*, 1999).

1.1.5 Topoisomerase inhibitors

DNA topoisomerases catalyze the relaxation of supercoiled DNA through a mechanism of transient DNA strand cleavage (Fiorani and Bjornsti, 2000). However, they are subject to inhibition by a class of antitumor drugs, such as camptothecin, m-AMSA, adriamycin and VP-16, which cause double-strand breaks by interacting with and preventing the resealing of the topoisomerase-DNA complex, and thus initiate SSBs and DSBs (Li and Liu, 2001).

1.1.6 Endogenous sources of DSB induction

In yeast, DSBs act as initiators of meiotic recombination and mating type switching. In many species, including primates and rodents, diversity in immunoglobins and T cell receptors is generated by V(D)J recombination induced by site-specific DSBs.

Meiotic recombination in yeast differs from mitotic recombination in that there is a higher degree of association between meiotic gene conversions and crossover events than is seen in their mitotic counterparts (Malkova *et al.*, 2000). Furthermore, meiotic recombination occurs 100 to 10,000 times more frequently than spontaneous mitotic recombination. Initiation of meiotic recombination is dependent on the induction of DSBs

at meiotic recombination hotspots. DSB formation requires the gene products of at least 9 genes: *SPO11, MEI4, MER2, REC102, REC104, REC114, MRE11, RAD50* and *XRS2* (Keeney *et al.*, 1997). Deletion of any of these genes blocks DSB formation and meiotic recombination, resulting in chromosomal nondisjunction at the first meiotic division. The gene product of *SPO11* was identified as the catalytic factor responsible for DNA cleavage (Keeney *et al.*, 1997). Two Spo11 monomers cleave both DNA strands via a topoisomerase-like transesterase mechanism, rather than by endonucleolytic hydrolysis (Romanienko and Camerini-Otero, 1999).

V(D)J recombination, unlike the aforementioned recombination processes, proceeds along a nonhomologous end-joining mechanism following induction of DSBs. Gene shuffling between the V (variable), D (diversity) and J (joining) gene segments encoding immunoglobin and T-cell receptor proteins bestows a rich diversity in antigen receptors. Such diversity arises from two sources. The first, combinatorial diversity, results from the effect of shuffling the many different V, D and J gene segments to yield diverse receptor specificity (Fugmann *et al.*, 2000). The second source of diversity arises from the imprecise joining of the V, D and J gene segments, or junctional diversity.

V(D)J recombination is initiated by the recombination activating genes *RAG1* and *RAG2*, which are only coexpressed in cells of the B and T lymphocyte lineage, and are responsible for induction of site-specific DSBs (Schatz and Leu, 1996; Schatz *et al.*, 1992). The RAG proteins recognize recombination signal sequences (RSSs) that immediately flank each gene segment, and catalyze the cleavage of the DNA strands. The RSSs consist of a highly conserved heptamer and nonamer that are separated by a poorly conserved spacer whose length is either 12±1 bp or 23±1bp (Fugmann *et al.*, 2000).

Spacer length therefore defines two types of RSSs, termed the 12-RSS and the 23-RSS. Efficient recombination occurs only between a 12-RSS and a 23-RSS, a restriction known as the 12/23 rule (Fugmann *et al.*, 2000).

In the assembly phase of V(D)J recombination, RAG1 and RAG2, as well as the components of the non-homologous end-joining pathway, including the heterotrimeric DNA-PK protein, XRCC4 and DNA ligase IV, are essential. A deficiency in any of these factors results in defective V(D)J recombination, and an early block in lymphocyte development (Fugmann *et al.*, 2000).

1.2 The role of Recombination processes in Double-Strand break repair

Resnick and Martin (1976) initially proposed that double-strand breaks are repaired by a homologous recombination mechanism whereby sequence information at the site of a double-strand break may be faithfully replicated from a homologous sequence (Resnick and Martin, 1976). Extensive studies of targeting of plasmid integration in *S. cerevisiae* corroborated this hypothesis (Hicks *et al.*, 1979). It was reported by Hicks et al. (1979) that cutting a plasmid in a region of homology with a host genome greatly enhanced integration efficiency (Hicks *et al.*, 1979). These findings were supported in studies by Orr and Weaver (1981), who found that a plasmid with sequence gaps in the region of homology was integrated with the same efficiency as a plasmid that was merely cut; and that sequence information was reconstituted in the gapped plasmid from the chromosomal DNA (Orr-Weaver *et al.*, 1981). These and earlier studies by Holliday (1964) were the basis for early models that were proposed to describe the mechanisms of general recombination.

1.2.1 Conservative homologous recombination

In most organisms, there are several repair pathways that compete to repair double-strand breaks. In wild-type yeast cells, conservative homologous recombination (HR) predominates in repairing DSBs. The greatly enhanced sensitivity of haploid yeast cells to ionizing radiation when compared to diploid yeast cells attests to the fact that homologous recombination provides an efficient, high fidelity mechanism for the repair of DNA DSBs, since any coding information lost at the site of the DSB is restored using the undamaged homologue. Genetic studies in yeast and other fungi provided the opportunity to study the intermediates of homologous recombination in meiosis, and to test the theories on DSB repair proposed by Resnick and Martin (1976), and later elaborated by Szostak *et al.* (1983)-models based on earlier conceptions by Holliday (1964) and Meselson and Radding (1975).

1.2.1.1 The Holliday model for homologous recombination

In 1964, Holliday first proposed a model to explain recombination and aberrant segregation in the smut fungus *Ustilago maydis* (Holliday, 1964). This model, and subsequent variations based on it, formed the conceptual framework for much of the research efforts in understanding the molecular mechanisms of general recombination.

The precept that defines the Holliday model is the heteroduplex structure known as the Holliday junction, which is comprised of the association of two homologous duplex DNA molecules via reciprocal strand exchange (Holliday, 1964). Strand exchange is initiated by two single-strand scissions at identical sites on each homologous

chromatid. This results in a *chi*-structure that, by virtue of its ability to branch migrate, can generate long regions of heteroduplex which can account for the heterozygozity that is frequently observed in recombinant molecules (Figure 2) (Cox and Lehman, 1987). Gene conversion arises when mismatched alignment between two homologous sequences results in the unequal segregation of closely linked markers (gene conversion is defined as the nonreciprocal transfer of genetic markers from one DNA molecule to another) (Pacques and Haber, 1999).

Termination of the interaction by cleavage at either the crossed strands, or the non-crossed strands, results in DNA molecules with the parental configuration, or recombinant DNA molecules, respectively. The formation of Holliday structures *in vivo* was demonstrated in recombination intermediates of *Escherichiae coli* (Potter and Dressler, 1979).

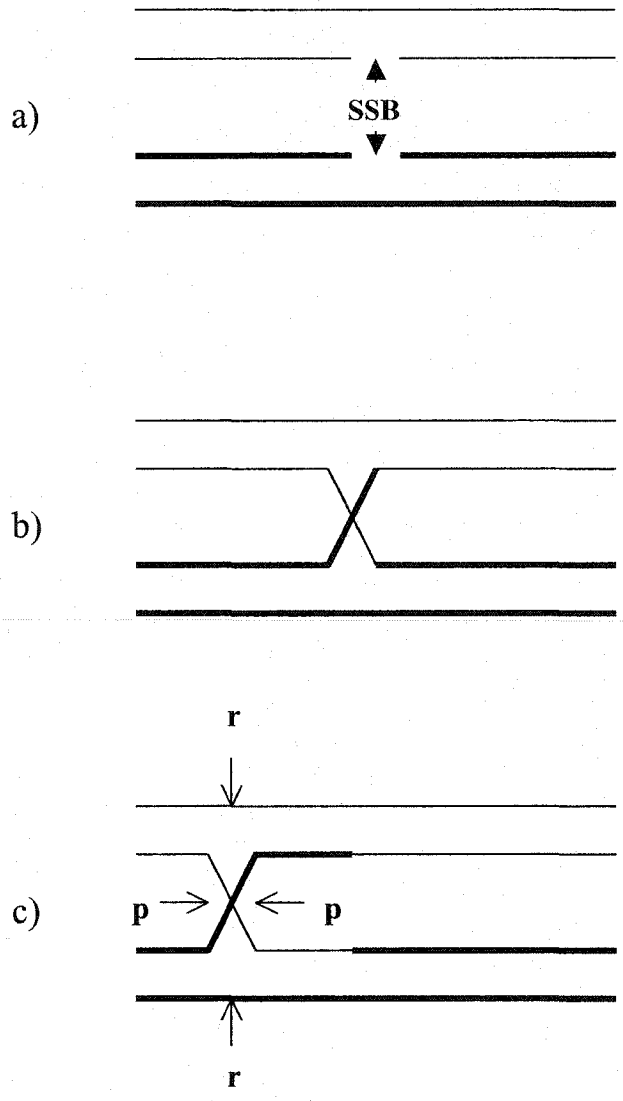

Figure 2. The Holliday model of homologous recombination (Holliday, 1964). **(a)** Single-strand scissions at identical sites on homologous chromatids lead to strand exchange **(b)** and the formation of a Holliday structure. The Holliday structure can migrate from the point of exchange (branch migration) prior to resolution. **(c)** Resolution occurs either at points marked **p** to yield DNA molecules with flanking arms in the parental configuration, or at points marked **r** to yield recombinant DNA molecules.

1.2.1.2 The Meselson-Radding DSB repair model

The DSB repair model formulated by Meselson-Radding (1975), differs from the Holliday model in that recombination is initiated by a single-strand (or asymmetric) transfer, which may, after isomerization, become a two-strand or symmetric exchange (Figure 3) (Meselson and Radding, 1975). The single-strand invasion of homologous duplex, hypothetically driven by the displacement of the invading strand from the break site by DNA synthesis, is the dominant feature of the model. The invasion of the homologous duplex results in D-loop formation. Eventually, the D-loop breaks and in a reverse reaction, the free ends from the broken D-loop invade the other duplex. The resulting Holliday structure may branch migrate to yield stretches of heteroduplex DNA prior to resolution. As with the Holliday model, resolution of crossed strands yields non-crossover products, while resolution of non-crossed strands results in crossover products.

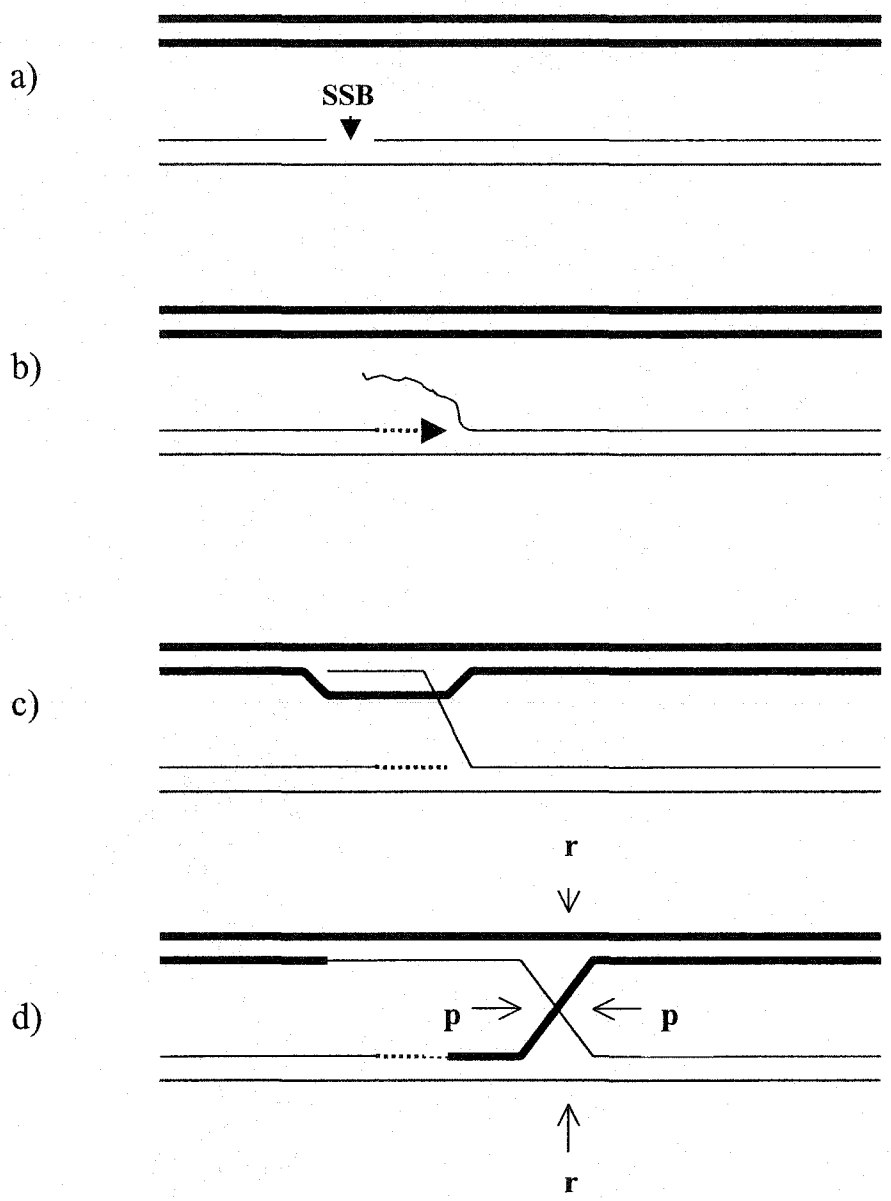

Figure 3. The Meselson-Radding model of recombination (Meselson and Radding, 1975). **(a)** Only one of the homologous chromatids acquires a single-strand break. DNA synthesis is primed by the 3'end of the single-strand break, resulting in a displaced strand **(b)** that invades the homologous DNA duplex **(c)** resulting in D-loop formation. Cleavage of the D-loop and its subsequent ligation into the other duplex results in a Holliday junction **(d)**, which may branch migrate prior to resolution at either **p** position for parental configuration, or **r** position to yield recombinant molecules.

1.2.1.3 The Strand Invasion model of DSB repair

A fundamental aspect of homologous recombination involves the intimate association of gene conversion and crossover events. The pioneering work of Fogel and Mortimer (1969) led to this key observation, and demonstrated that gene conversions exhibit polarity, whereby the probability that a nearby marker would be co-converted along with a specific marker decreases with the distance between the markers (Fogel and Hurst, 1967; Fogel and Mortimer, 1969).

Molecular models were proposed to account for the strong association of gene conversion with crossovers, culminating in the DSB repair model hypothesized by Resnick and Martin (1976), and later elaborated by Szostak *et al.* (1983). In the Szostak et al. DSB repair model, the recombinogenic event is stimulated by a double-strand break, following which a 5' to 3' exonuclease activity resects the DSBs to yield 3' single-strand overhang intermediates (Figure 4). These 3'ends are presumed to invade a homologous template and prime DNA synthesis in a manner similar to the recA-mediated recombination process that has been well-characterized in *E. coli* (Kowalczykowski *et al.*, 1994; Eggleston and West, 1996).

The primary feature of the Szostak et al. DSB repair model is the formation of two Holliday junctions. Resolution of the two Holliday junctions is mediated by a resolvase; cleavage of the crossed strands results in gene conversion without a crossover, while cleavage of the noncrossover strands results in gene conversion with an associated crossover event (Pacques and Haber, 1999).

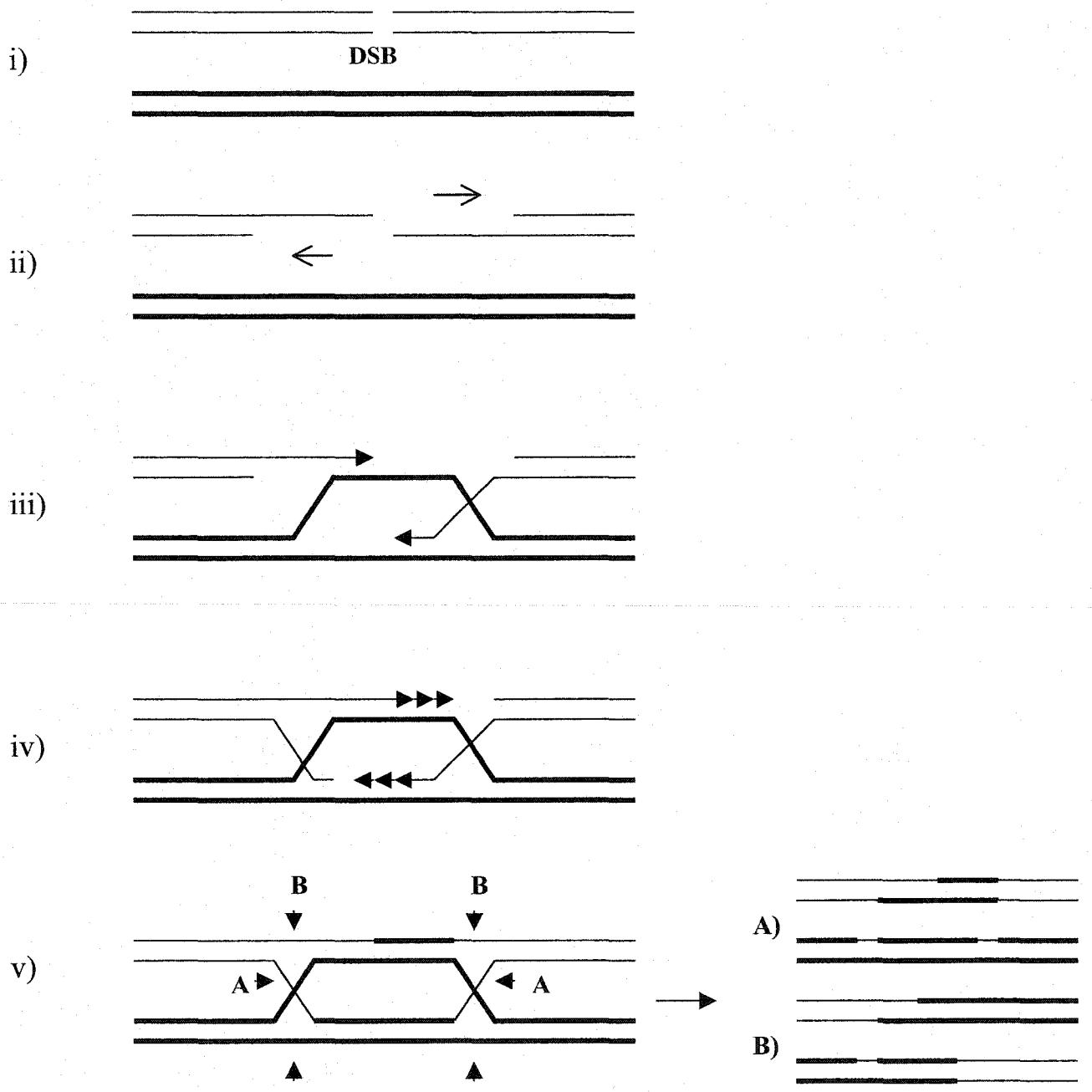

Figure 4. The Szostak *et al.* (1983) DSB repair model. **(i)** Following DSB induction, 5'→ 3' processing of DSB ends **(ii)** results in a 3'overhang intermediate that invades a homologous template **(iii)**, to initiate DNA synthesis **(iv).** The 2 Holliday junctions that result **(v)** can be resolved either by cutting the crossed strands, with no crossover (A), or by cutting the non-crossed strands resulting in crossover products (B).

1.2.1.4 The synthesis-dependent strand annealing model of DNA recombination

Gene conversions are infrequently associated with mitotic recombination events (Kobayashi, 1992). This has led to the latest adaptation of homologous recombination-repair models collectively known as the synthesis-dependent strand annealing (SDSA) models, which were first proposed by Nasmyth (Nasmyth, 1982; 1993; Ivanov *et al.*, 1996) and later elaborated by Hastings (1988) and Mcgill *et al.* (1989). The basic feature of SDSA is the displacement of newly synthesized strands from their template, allowing them to anneal to one another without gene conversion (Pacques and Haber, 1999). This is postulated to occur due to the actions of helicases or topoisomerases which disassemble the replication structure (Figure 5).

The SDSA model of recombination is supported by experimental observations of HO-induced recombination events. Sequence alteration accompanying DSB repair is exclusively found on the recipient molecule of homologous recombination, not the donor template (Nasmyth, 1993; Ivanov *et al.*, 1996; Strathern *et al.*, 1995). This supports a conservative DNA synthesis process rather than a semiconservative one as predicted in the Szostak *et al.* recombination model.

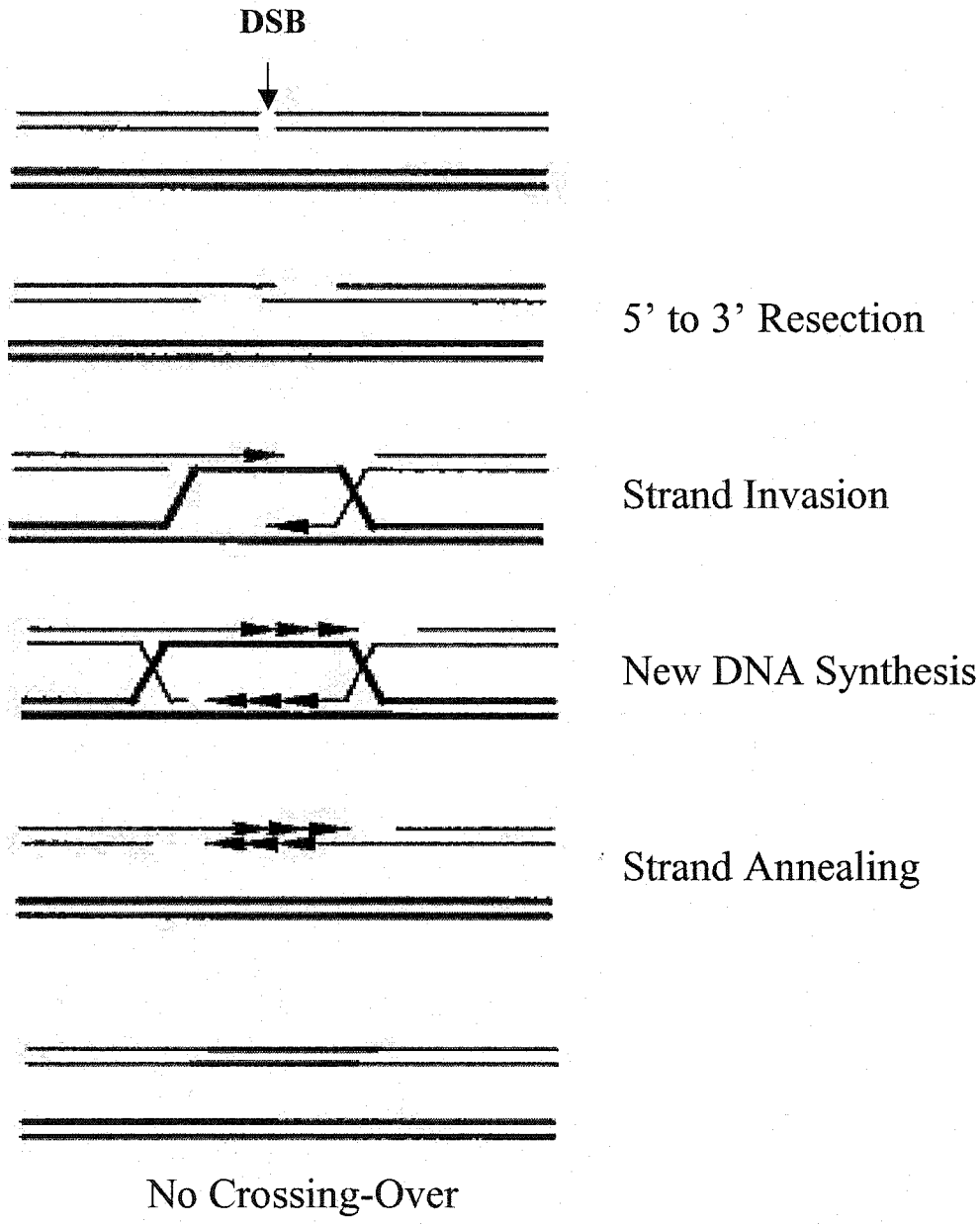

Figure 5. The standard synthesis-dependent strand-annealing (SDSA) model (Pacques and Haber, 1999). Variations of this model exist to describe branch or bubble migration, but generally they describe a process whereby both 3' ends invade the template to initiate DNA synthesis. The double HJ structure is resolved by strand displacement, rather than strand scission. The subsequent religation of newly synthesized strands and gap-filling results in a non-crossover repair event.

1.2.1.5 Homologous recombination in *E. coli*

The molecular basis of general recombination was first extensively examined in *E. coli*. Numerous screening methods have identified *E. coli* mutants with reduced recombinational proficiency. The most common screening process involved analyzing the ability of mutagenized F^- cells to produce recombinants when mated with lawns of *Hfr* cells in order to isolate rec^- mutants. In this manner, the archetypal *recA* gene was first identified (Clark and Marguiles, 1965; Mahajan, 1988).

The recA protein

The 38.5 kDa gene product of the *recA* gene is the most extensively studied protein implicated in recombination. It has gained the title of a recombinase protein based on its ability to catalyze the strand-exchange reaction between two homologous sequences critical to general recombination processes in *E. coli* (Cox and Lehman, 1987).

The recA-catalyzed DNA strand-exchange reaction has been divided into at least three distinct phases (Menetiski and Kowalczykowski, 1985). In the first phase (pre-synapsis), recA nucleates into long protein filaments. This reaction is potentiated by the presence of single-strand DNA (Cox and Lehman, 1987). At neutral pH, recA polymerizes onto ssDNA assisted by the tetrameric SSB protein (Cox and Lehman, 1987). This process is conditional on the binding of ATP (Menetiski and Kowalczykowski, 1985). Stoichiometric measurement of the binding of recA to ssDNA showed that one recA monomer is required to bind 4 nucleotides, based upon the ratio of recA protein to single-stranded ØX174 DNA in saturated recA protein-ssDNA complexes isolated by ultracentrifugation (West and Cassuto, 1981).

During the second phase, synapsis, the ssDNA and a homologous DNA molecule are brought together by recA. This phase is initially characterized by a homology search and the rapid "zippering up" of the homologous strands to form a DNA duplex (Cox and Lehman, 1987). This property of recA was initially demonstrated when it was found to stimulate the renaturation of a (+) circular ØX174 DNA strand with its (-) linear counterpart to generate RFII molecules (Bryant and Lehman, 2001). The processes that underlie the homology search are as yet unclear. However, it was demonstrated to be very rapid, requiring just a few minutes and needing less than 50 nucleotides of sequence homology (Conley and West, 1990; Hseih *et al.*, 1992) while discriminating in excess of 200,000 non-homologous sequences (Honigberg *et al.*, 1986). Non-Watson-Crick hydrogen bonding interactions, enabled by the transient "breathing" of the dsDNA duplex, leads to a transient 3-strand-containing structure (Stasiak, 1992; Camerini-Otero and Hsieh, 1993) and eventually to the formation of a D-loop (West, 1992).

The final phase takes place following sequence alignment, when strand exchange occurs. The (+) strand of the DNA duplex is displaced, and supplanted by the strand brought in by the nucleoprotein filament (Cox and Lehman, 1987). This step is followed by branch migration; a process that requires the hydrolysis of ATP, unlike spontaneous non-recA-mediated branch migration. Branch migration occurs at a rate of 10 to 20 bp/sec and progresses in a 5' $\rightarrow$ 3' direction relative to the invading strand, identical to the direction in which recA assembles on ssDNA (Cox and Lehman, 1987). Branch migration can occur for at least 7 kb and can traverse up to 1300 bp heterology in DNA duplexes (Bianci and Radding, 1983).

The SSB protein

The primary role of SSB (single-strand binding protein) in homologous recombination appears to be to bind ssDNA and stimulate recA-mediated strand exchange. SSB binds to ssDNA as a tetramer of 18 kDa (Cox and Lehman, 1987; Kowalczykowski *et al.*, 1994). As a result, it may serve to denature secondary structure in the ssDNA. It is then competitively displaced by recA protein to form a contiguous recA nucleoprotein filament. Studies by Radding *et al.* (1984) have demonstrated that recA binding to ssDNA is impeded by DNA secondary structure; this inhibition is abolished by addition of SSB (Muniyappa *et al.*, 1984).

The recBCD enzyme complex

The recBCD enzyme was originally classified as exonuclease V when it was first isolated from E. coli (Cox and Lehman, 1987). It is a multifunctional enzyme possessing both helicase and nuclease activities. The nuclease activities are discussed in depth in the section presented later on endo-exonucleases and their roles in DSB repair. The heterotrimeric protein consists of 3 subunits encoded by the *recB*, *recC* and *recD* genes. The recB subunit was found to be critical for the ATP-dependent helicase activity necessary for unwinding linear duplex DNA (Hickson *et al.*, 1985).

Table 1. Proteins involved in homologous recombination in *Escherichiae coli*

Protein	Activities
recA	DNA strand exchange; DNA renaturation; DNA-dependent ATPase
recBCD	Has dsDNA-dependent ATPase, ATP-dependent dsDNA and ssDNA exonuclease, and ATP-stimulated ssDNA endonuclease activities; has ATP-dependent helicase activity on linear dsDNA; during unwinding makes single-stranded nicks near Chi sites; has D-loop cleaving activity.
recE	ATP-independent dsDNA 5' → 3' exonuclease activity
recF	ss- and ds-DNA binding protein
recG	mediates branch migration, DNA helicase
recJ	ssDNA exonuclease, 5' → 3'
recQ	DNA helicase
recT	DNA renaturation
ruvA	Holliday-, cruciform-, and 4-way junction binding; interacts with ruvB
ruvB	Holliday junction branch migration; DNA helicase; interacts with ruvA
ruvC	Holliday junction cleavage; 4-way junction binding

SbcB	3'→ 5' ssDNA exonuclease
SbcC	Associates with SbcB to form ATP-dependent dsDNA exonuclease
SbcD	Associates with SbcB
SSB	ssDNA-binding protein
DNA topoisomerase I (*topA*)	type I topoisomerase
DNA gyrase (*gyrA, gyrB*)	DNA gyrase, type II topoisomerase
DNA ligase (*lig*)	DNA ligase
DNA polymerase I (*pol A*)	DNA polymerase, 5'→ 3' exonuclease; 3'→ 5' exonuclease
Helicase II (*uvrD, uvrE, recL, mutU*)	DNA helicase
Helicase IV (*helD*)	DNA helicase

Adapted from: (Kowalczykowski *et al.,* 1994)

Along with recBCD, there exists another helicase thought to function at the initiation step of recombination. This is the ATP-dependent recQ helicase; in the absence of recBCD, it is found to be critical for recombination processes in *E. coli* (Nakayama and Nakayama, 1984). SSB stimulates its helicase activity, presumably by binding unwound strands (Umezu and Nakayama, 1993). In absence of SSB, recQ is a relatively poor helicase.

1.2.1.6 Homologous recombination in yeast

Conservative homologous recombination is the primary process through which DSBs are repaired in yeast and occurs via the *RAD52*-dependent HR pathway. Genes involved in this DSB repair pathway were first identified as mutations rendering cells sensitive to X-rays but not to UV irradiation (Pacques and Haber, 1999). These genes were classified as the *RAD52* epistasis group. A null mutation in *rad52* renders the haploid yeast cell as sensitive to IR as a double mutation in *rad52* and any other member of the *RAD52* epistasis group (Pacques and Haber, 1999).

The *RAD52* epistasis group

The *RAD52* epistasis family of genes includes *RAD50-55, RAD57, RAD59, XRS2* and *MRE11* (Sung *et al.*, 2000). Some genes appear to be extraordinarily conserved from *S. cerevisiae* to the mammalian genome. The most highly conserved of these are *RAD51* and *RAD54*, as well as regions of extreme primary sequence conservation in *RAD52*,

MRE11, *RAD50* and *XRS2* (the mammalian homologue of which is known as *NBS1*) (Carney *et al.*, 1998; Bressan *et al.*, 1999).

Double mutants within the *RAD52* family of genes seldom, if ever, show an IR sensitivity greater than that seen in single mutants of *rad51, rad52* and *rad54*. Hence, they all belong in the same major epistasis group (with the sole exception of *RAD53*) (Game, 2000). The *RAD52* gene was identified as the most critical component for all recombinant events in yeast. The *rad51, rad54, rad55*, and *rad57* mutants have common phenotypes, appearing essential for some recombination events but dispensable for others. Mutants in *rad51, rad52* and *rad54* are deficient in both meiotic and mitotic recombination (Game, 2000).

RAD51

The *RAD51* gene in *S. cerevisiae* shares close sequence homology with the *E. coli recA* gene. Biochemically, Rad51 has been shown to exhibit properties characteristic of RecA. It forms nucleoprotein filaments on both ssDNA and dsDNA in the presence of ATP which are almost identical to the equivalent RecA-DNA nucleoprotein filament in overall dimension and structure (Ogawa *et al.*, 1993). Like recA, Rad51 has a DNA-dependent ATPase activity. Rad51 mediates homologous pairing and strand exchange during the presynaptic and synaptic phases, respectively.

In the presynaptic phase, Rad51 polymerizes onto ssDNA and dsDNA in the presence of ATP to form right-handed helical nucleoprotein filaments (Ogawa *et al.*, 1993). Formation of the Rad51 filament on ssDNA is stimulated by the heterotrimeric ssDNA binding factor Replication Protein A (RPA), whose *E. coli* homolog is the SSB

protein, whereas Rad51 assembly on dsDNA shows no dependence on RPA (Ogawa *et al.*, 1993). *E. coli* SSB protein is as effective as yeast RPA in stimulating ssDNA-dependent ATPase and the DNA strand exchange activities of Rad51. Since it is unlikely that Rad51 and SSB interact physically, the results suggest that the major functional role of RPA in presynapsis is to remove secondary structure in ssDNA (Sugiyama *et al.*, 1997). This is corroborated by the observation that homopolymeric DNA species devoid of secondary structure are more effective in activating ATP hydrolysis by Rad51 (Sugiyama *et al.*, 1997). However, although RPA is required for the assembly of Rad51 on ssDNA and is therefore an important co-factor in the strand exchange process (Sugiyama *et al.*, 1997), an excess of RPA can in fact suppress this reaction (Sung, 1997). This inhibitory effect has been attributed to competition of RPA with Rad51 for binding sites on the ssDNA (Sung *et al.*, 2000). Rad52, Rad55 and Rad57 can help overcome this inhibition (Sung, 1997).

During the synaptic phase, once the Rad51-ssDNA nucleoprotein filament is assembled, it is capable of mediating stable pairing with a homologous duplex molecule. As with the recA-mediated strand exchange, the search process for homologous sequences is poorly understood, but is thought to involve a random collision mechanism (Sung *et al.*, 2000). Strand invasion of the 3' ssDNA tail into the DNA duplex results in a D-loop formation, as outlined earlier in the Szostak *et al.* model of recombination, followed by branch migration.

Another *recA* homologue, *DMC1*, has also been isolated from yeast that seems essential for meiotic recombination. *dmc1* mutants are defective in recombination,

accumulate DSB recombination intermediates, fail to form synaptonemal complexes, and arrest late in meiotic prophase (Bishop *et al.*, 1992).

RAD52

RAD52 does not share any discernible homology with known prokaryotic recombination genes, and therefore appears to be unique to eukaryotes (Sung *et al.*, 2000). Rad52 protein from yeast and humans binds to DNA in a ring-shaped heptamer (Parsons *et al.*, 2000), showing higher affinity for ssDNA than dsDNA (Mortensen *et al.*, 1996; Shinohara *et al.*, 1998).

Rad52 is thought to promote assembly of Rad51 nucleoprotein filaments by direct contact with RPA and Rad51, forming a bridge between Rad51 monomers and the RPA-ssDNA complex (Gasior *et al.*, 1998). Consistent with the functional interactions of Rad52 and RPA in strand annealing/transfer, Rad52 has been found to interact physically with the central subunit of RPA (Shinohara *et al.*, 1998). Recruitment by protein-protein interactions may be necessary because Rad51, unlike recA, does not bind ssDNA more efficiently than dsDNA (Baumann *et al.*, 1996; Benson *et al.*, 1994; Shinohara *et al.*, 1992).

In vitro observations of Rad52, Rad55 and Rad57, have shown that they stimulate Rad51-mediated strand exchange by overcoming the competitive inhibition by RPA (Sung *et al.*, 2000; Gasior *et al.*, 1998). This stimulation of strand exchange proteins is thought to result at least in part from the ability of the accessory factors to allow initiation of assembly of Rad51 on RPA-coated ssDNA. Consistent with the idea that stimulation

occurs by nucleation of cooperative Rad51 assembly, Rad52 and the Rad55/57 heterodimer are found in lower cellular abundance in comparison to Rad51 (Sung, 1997).

rad51 and *rad52* mutations completely abolish mating type switching in yeast, a homologous recombination event that is initiated by the induction of a DSB by HO endonuclease (discussed next in the section on mating type switching) (Friedberg, 1988).

Genetic studies of Rad52 have revealed that it is also involved in the single-strand annealing (SSA) pathway. Its involvement in multiple pathways of recombination repair may be the reason why it appears to be the most critical factor in recombination events in yeast (Pacques and Haber, 1999). However, the *RAD52* homologue in vertebrates is not as essential in recombination, suggesting that it may not play such a crucial role in recombination processes, or that another recombination factor, perhaps a Rad52 homologue, provides a parallel function in other eukaryotes (Rijkers *et al.*, 1998; Sung, 1997; Yamaguchi-Iwai *et al.*, 1998).

RAD53

RAD53 has also been proven to be essential, but is not directly involved in DSB repair. Rather, it encodes a protein kinase that acts downstream of *MEC1* and *TEL1*, genes which are thought to be the functional homologues of the cell cycle checkpoint gene *ATM* in mammalian cells (Allen *et al.*, 1994; Friedberg, 1988; Pacques and Haber, 1999).

RAD54

The gene product of *RAD54* is believed to belong to a family of chromatin-remodelling proteins, including the transcription factors Swi2/Snf2 and Motl (Pacques and Haber, 1999). When DSB damage occurs in inaccessible chromatin structure, the Rad54 protein is important in stimulating a change in DNA conformation to promote heteroduplex DNA formation (Sung *et al.*, 2000). *In vitro*, Rad54 increases Rad51-mediated strand exchange. While Rad51 does not effectively form D-loops in vitro, the inclusion of Rad54 renders D-loop formation possible (Petukhova *et al.*, 1998). Like other members of the Swi/Snf2 family, Rad54 has an ATPase activity which is dependent on dsDNA (Petukhova *et al.*, 1998). Yeast two-hybrid screening as well as co-immunoprecipitation assays have shown that Rad51 and Rad54 interact (Pacques and Haber, 1999; Petukhova *et al.*, 1998).

RAD55* and *RAD57

Rad55 and Rad57 exist in heterodimeric form *in vivo* (Sung, 1997), and appear to promote Rad51-mediated strand exchange, as described above. They are thought to be the eukaryotic equivalents of the bacterial proteins recO and recR, which "load" recA onto ssDNA when it is in competition with SSB (Shan *et al.*, 1997).

However, while genetic data suggests that *RAD52* is absolutely required for all recombination events in yeast, *RAD55* and *RAD57* appear to play supporting roles and can be dispensed with (Pacques and Haber, 1999). Deletion of these genes confers a cold-dependent X-ray sensitivity. Cells are normal at 34 C but are defective in DNA repair at 18 C, when the formation of protein complexes is theoretically more difficult (Johnson

and Symington, 1995). Furthermore, over-expression of Rad51 compensated for the absence of Rad55 or Rad57 (Hays *et al.*, 1995).

RAD59

The *RAD59* gene was discovered as a result of studies on the *rad51* mutant; specifically, why a *rad51* mutation does not exhibit a defect in spontaneous homologous recombination between chromosomal inverted repeats (a 4-fold reduction) to the same extent as the *rad52* mutant (greater than 3000-fold) (Rattray and Symington, 1994; 1995). A systematic search for mutants in which *rad51*-independent recombination was abolished, resulted in the isolation of the *RAD59* gene (Bai and Symington, 1996). *RAD59* and *RAD52* were found to share considerable sequence homology, but *RAD59* cannot substitute for a *rad52* null mutation. A *rad59* mutation confers a modest defect in HR comparable to the *rad51* single mutant, but a *rad51 rad59* double mutation confers a defect in spontaneous recombination between chromosomal repeats that parallels that of the *rad52* mutant (Pacques and Haber, 1999). However, this synergy is not observed in spontaneous or HO endonuclease-induced recombination between plasmid-borne repeats: *rad51* has no effect, and *rad59* confers a 10-fold decrease, as does the *rad51 rad59* double mutant.

MRE11, RAD50 and *XRS2*

RAD50, MRE11 and *XRS2* appear to belong to a separate subgroup within the *RAD52* epistasis group based on their differing roles in meiotic recombination as opposed to mitotic recombination. In meiosis, Rad50, Mre11 and Xrs2 are postulated to be

necessary for both the introduction of meiosis-specific DNA DSBs and the processing of DNA ends (Ogawa *et al.*, 1995; Johzuka and Ogawa, 1995). However, they have not been indicated to play an important role in HR-mediated DSB repair in mitotic cells, while mutant phenotypes in any of these genes do display a slightly reduced ability to process DSB ends, resulting in a delayed completion of recombination (Ivanov *et al.*, 1992; Sugawara and Haber, 1992; Tsubouchi and Ogawa, 2000).

The difference between the *RAD50*, *MRE11* and *XRS2* and the *RAD52* subgroups is most distinctly seen in meiosis, specifically in the *spo13* genetic background, where two diploid spores are formed from a single division, rather than four haploid ones from the usual four divisions (Klapholtz and Esposito, 1978a). In repair-proficient strains, the *spo13* division is preceded by a normal round of meiotic recombination, but the division itself is equational; and as such, centromere and chromosomal non-disjunction normally seen during the first meiotic division is largely absent (Klapholtz and Esposito, 1978a; 1978b). Consequentially, meiotic recombination, which is usually an essential prerequisite to successful meiotic chromosome segregation, is not essential in the *spo13* genetic background (Malone and Esposito, 1981). Therefore, *rad50, mre11* and *xrs2* mutants, which normally produce dead meiotic spores, are able to produce viable diploid spores showing no recombination in a *spo13* background (Johzuka and Ogawa, 1995; Ivanov *et al.*, 1992). However, mutants in *rad51, rad52, rad55* and *rad57* are not "rescued" in meiosis by the *spo13* background and viable spores are rarely isolated (Malone and Esposito, 1981). This is probably because DSBs being processed through the recombination pathway accumulate in these mutants since recombination cannot be completed (Game, 2000). This implies that meiotic recombination is a potentially

catastrophic event in the above mutants. While the *RAD54* gene is classified as belonging to the same subgroup as genes implicated in meiosis recombination-deficient mutants, its *null* knockout confers at most a minimal defect in meiosis (Game, 2000).

In vitro, Rad50, Mre11 and Xrs2 have been found to associate strongly together. Rad50 and Mre11 are the respective structural homologues of *E. coli* SbcD and SbcC proteins. SbcD has double-strand exonuclease and single-strand endonuclease activity (Bishop *et al.*, 1992; Sharples and Leach, 1995). Initially, Mre11 was implicated as the nuclease responsible for the 5' → 3' resection of DSB ends prior to homologous recombination, due to a reduction in that activity in *mre11* mutants (Furuse *et al.*, 1998). It is confusing, however, that Mre11 has been shown to have a 3'→ 5' exonuclease activity on dsDNA. Furthermore, the nuclease activity of Mre11 is required for meiosis, but not for mating type switching; a process that requires mitotic homologous recombination (Moreau *et al.*, 1999; Weiner and Kleckner, 1994).

Besides a possible role in meiotic homologous recombination, the Rad50/Mre11/Xrs2 complex has been implicated in a bewildering array of repair processes including telomere maintenance (Kironmai and Muniyappa, 1997), homologous pairing (Weiner and Kleckner, 1994), suppression of mitotic interchromosomal recombination (Johzuka and Ogawa, 1995) and the non-homologous end-joining (NHEJ) pathway (Pacques and Haber, 1999). Also, Rad50/Mre11/Xrs2 is believed to mediate sister chromatid recombination by stabilizing sister chromatid interactions (Game, 2000; Petrini, 1999; Weiner and Kleckner, 1994). Beyond its role in mediating sister chromatid exchange, the Rad50/Mre11/Xrs2 complex has been implicated in chromatin remodeling in meiotic recombination. Yeast knockout strains of

MRE11, RAD50 and *XRS2* have been shown to undergo changes in chromatin structure that render meiotic hotspots inaccessible to DSB induction by Spo11 (Malkova *et al.*, 2000).

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Mating type switching and the HO endonuclease

Yeast has two haploid mating types, **a** and α. Haploid yeast cells of opposite mating phenotype will mate to form an **a**/α diploid cell that is capable of sporulation (Herskowitz *et al.*, 1992). **a**, α and **a**/α cells differ from one another in their transcriptional regulation of 4 different classes of genes. α -specific genes (αsg) are only expressed in α cells, such as genes for the α-factor precursor and the **a**-factor receptor; **a**-specfic genes (**a**sg) are only expressed in **a** cells. Haploid-specific genes (hsg) are only expressed in haploid yeast cells of both mating types; these include the genes for HO endonuclease and the *STE4* gene. Sporulation-specific genes (ssg) are only expressed in diploid cells, following nutrient deprivation (Herskowitz *et al.*, 1992), such as the *SPO11* and *SPO13* genes which are essential for meiosis (Klar *et al.*, 1981).

Expression of each gene set is regulated by the action of regulatory proteins encoded by the mating type locus, *MAT* (Figure 6). *MATα* encodes Matα1 and Matα2: Matα1 is a positive transcriptional regulator of α-specific genes while Matα2 represses the expression of **a**-specific genes (Herskowitz *et al.*, 1992). A complex of Matα1/Mat**a**1 is responsible for repressing the transcription of haploid-specific genes. These genes include *RME1*, an early repressor of early meiosis genes, and the gene encoding HO endonuclease (Haber, 1998). *MATα1* is also repressed, therefore both **a**sg and *α*sg are turned off, and heterothallic or diploid cells are nonmating. The *MAT**a**2* gene encodes a

polypeptide that corresponds to the last 119 amino-acid residues of Matα2 and possesses no known function (Herskowitz *et al.*, 1992). When both *MATα1* and *MATα2* are deleted, cells display *MAT**a*** phenotype, since α-specific genes are turned off and α-specific genes are not transcribed (Strathern *et al.*, 1981).

Homothallic mating type switching in yeast is a highly regulated gene conversion event involving a nonreciprocal transfer of information; a process that is strictly dependent on the homologous recombination-repair pathway. This occurs between the mating type allele expressed at the *MAT* locus and one of the adjacent *MATα* and *MAT**a*** alleles present on chromosome III (Hicks *et al.*, 1977). The *MATα* and *MAT**a*** alleles (or Yα and Y**a**) are located on the *HML* and *HMR* loci, respectively (Figure 6).

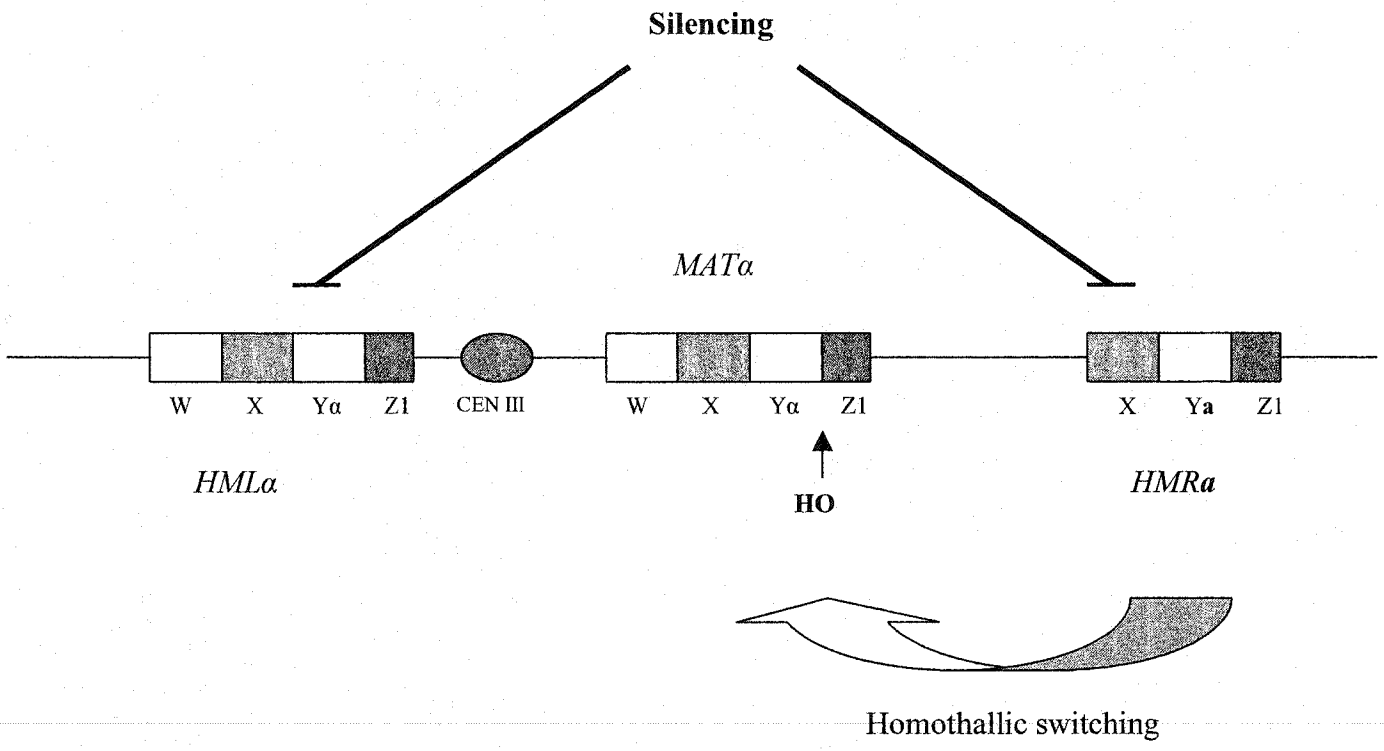


Figure 6. Organization of *HML, MAT* and *HMR* loci on chromosome III (Haber, 1998). During switching of *MATα* to *MAT**a***, approximately 750bp of the Yα region is replaced by 650bp of Y**a** DNA, copied from the unexpressed, silent locus *HMR**a***. The replacement occurs by a site-specific mitotic conversion event catalyzed by the site-specific HO endonuclease, which can cut the *MAT* locus near the Y-Z1 border but is prevented by the chromatin structures of the silent copies from cutting equivalent sites in *HMR* or *HML*. An analogous switching of *MAT**a*** to *MATα* occurs using the donor locus *HMLα* located on the opposite arm of the same chromosome.

These loci are homologous to *MAT* beyond the sequences that distinguish the *MAT**a*** and *MATα* alleles; the Y regions of *MAT, HML* and *HMR* are flanked by the X region to the left and Z1 to the right. Haploid *Mat**a*** cells switch to *Matα* via gene conversion between Y**a** from the *MAT* locus and Yα in *HML*. This conversion event is unidirectional; the *HML* Yα sequence is duplicated and replaces the *MAT* Y**a** sequence. The reverse is true for Matα cells; which utilize *HMR* as a donor of the Y**a** cassette. It was proven that *HML* and *HMR* serve as donors during *MAT* switching when it was demonstrated that a mutant *MATα* allele could be replaced by *MAT**a***, which then switched to a wild-type *MATα* allele (Hawthorne, 1963).

In addition to the directionality of the switching process, there exists a mechanism which dictates donor preference in the conversion event. Donor preference is not dictated by Yα or Y**a** content; replacing the *HMR* allele with *HML* does not alter the donor preference of Matα cells (Klar *et al.*, 1981; Weiler and Broach, 1992). This is thought to occur independently of Matα1, but is strongly dependent on Matα2 (Hicks *et al.*, 1977; Szeto *et al.*, 1997). Mat**a** donor preference does not depend on a functional *MAT**a*** gene, but operates through another as yet unidentified **a**-specific gene product.

Mating type switching is initiated by a double-strand break at the Y/Z1 junction. The DSB is induced by a site-specific endonuclease present only in homothallic yeast called the HO endonuclease (Kostriken *et al.*, 1983). In an actively switching culture of homothallic yeast cells, approximately 3% of cells will undergo cleavage at the Y/Z1 junction at any given time. The reason for the low cleavage activity of HO endonuclease resides in the fact that cells undergo mating type switching during the G1 phase of the cell cycle, and this is limited to haploid mother cells and daughter cells in their secondary

cell division cycle (Strathern and Herskowitz, 1979). Expression of HO endonuclease is therefore under tight regulation, most notably the repression of transcription of *HO* endonuclease gene by the Mat**a**1/Matα1 complex present in heterothallic cells (Herskowitz *et al.*, 1992).

HO endonuclease recognizes and cleaves a unique 24bp site at the Y/Z1 junction to yield a 4bp 3'-OH overhang (Figure 7). All three loci, *HML, MAT* and *HMR*, contain the HO endonuclease cut site, but only the site at the MAT locus is cleaved (Strathern *et al.*, 1982). Cleavage at *HML* and *HMR* is prevented by the same mechanism which suppresses the transcription of these loci. The silencing of the donor cassettes is accomplished by localized chromatin condensation. This is regulated by a pair of *cis*-acting silencer sequences, *HML*-E, *HML*-I, *HMR*-E and *HMR*-I that bracket the donor loci and interact with *trans*-acting factors including the histone proteins, Rap1, four Silent Information Regulator (SIR) proteins, and DNA replication Origin Recognition Complex (ORC) proteins and various transacetylases, deacetylases, and chromatin assembly factors (Haber, 1998). Together, these factors interact to create short stretches of heterochromatin (~3 Kb) that is transcriptionally silent.

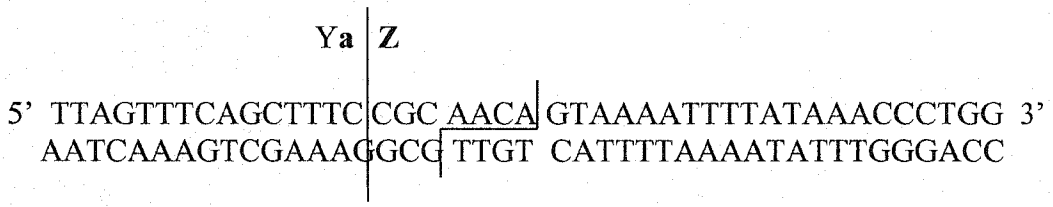


Figure 7. Recognition site for HO endonuclease (Strathern *et al.*, 1982). The HO endonuclease cleaves the *MAT* DNA strands at the site marked proximal to the Z junction, leaving a 3'staggered overhang of 4bp.

1.2.1.7 Homologous recombination in mammalian cells

Early observations had suggested that homologous recombination is not very robust in mammalian cells and therefore may not play a significant role in DNA repair. Characterization of mouse X-ray-sensitive cell lines (XRCC) has shown them to be defective in one or another component of the NHEJ pathway (Jeggo, 1998b). Also, gene targeting in mammalian cells is a relatively inefficient process. When DNA is transfected, it integrates into its homologous locus at much lower frequency compared to random integrations (Capecchi, 1989). However, studies in the last few years have challenged this notion, demonstrating that homology-directed repair has a major role in the repair of DSBs. Homologous recombination is stimulated at least 100-fold by introduction of a DSB at a genomic locus (Liang *et al.*, 1998; Rouet *et al.*, 1994; Richardson *et al.*, 1998). The relative contribution of HR in repairing DSBs was analyzed by incorporating a unique cut site for mitochondrial endonuclease I-*Sce*I in one of two directly repeated mutant *neo* genes integrated into the genome, and transiently expressing I-*Sce*I (Liang *et al.*, 1998). PCR and random clone analysis of colonies demonstrated that homologous repair accounted for a surprising high 30-50% of repair events.

The members of the *RAD52* epistasis group are highly conserved from fungi to higher eukaryotes. Human Rad51 is 68% identical to the yeast Rad51 (Shinohara *et al.*, 1993), while human Rad52 and Rad54 are less conserved at 30% (Muris, 1994) and 50% (Kanaar, 1996) respectively. The evolutionary conservation of the major components of the HR pathway is indicative of the relative importance of the homologous recombination in the maintenance of the eukaryotic genome.

Functional analysis of the human homologue of Rad51 has revealed compelling evidence that it plays an important role in embryonic development. While *rad51* knockout in yeast has little effect on cell growth, disruption of both alleles of *RAD51* in mice results in early embryonic lethality with arrest of the embryos at the egg cylinder stage (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). In addition, the embryos consistently exhibit chromosome loss as well as IR sensitivity, indicating a DNA repair defect. Transient inhibition of mouse *RAD51* expression with antisense oligonucleotides also results in increased IR sensitivity (Taki *et al.*, 1996).

Knockouts of *RAD52* in mouse and chicken do not confer increased sensitivity to IR or other mutagenic agents, and *rad52*$^{-/-}$ mice appear normal with respect to viability, fertility and immune system development (Rijkers *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1998). Mouse *rad52*$^{-/-}$ ES cells show slightly reduced gene targeting efficiency (Rijkers *et al.*, 1998).

Mouse *rad54*$^{-/-}$ cells are twice as sensitive to IR and mitomycin C (Essers *et al.*, 1997). Gene targeting efficiency is also reduced 5 to 10-fold, consistent with the essential role Rad54 may play in homologous recombination processes. Similarly, disruption of *RAD54* in chicken DT40 cells conferred 3-fold greater sensitivity to IR and 100-fold decreased targeted integration (Bezzubova *et al.*, 1997).

Similarly, there exist mammalian homologues of the yeast Rad50, Mre11 and Xrs2 proteins. As in yeast, there is evidence that they act in a multiprotein complex and have been implicated in DSB repair during mitosis(Dolganov, 1996). The mammalian homologue of Xrs2 is named p95, and is of the same size as Xrs2, but is highly diverged (Carney *et al.*, 1998). The human p95 variant, nibrin, is encoded by the gene *NBS1* and

has been implicated in the Nijmegen breakage syndrome, which is a variant of ataxia telangiectasia, characterized by spontaneous chromosome breakage, IR sensitivity, immunodeficiency, developmental defects and an increased risk of malignancies, specifically lymphoid cancer (Carney *et al.*, 1998). In mouse ES cells, the inability to recover *mre11*$^{-/-}$ mutants has led to the perception that mMre11 is essential for cell viability (Xiao and Weaver, 1997).

BRCA1 and *BRCA2*

Recently, a connection between homologous recombination and tumorigenesis has been realized with the characterization of the breast cancer susceptibility genes *BRCA1* and *BRCA2*. A germline mutation in either *BRCA1* or *BRCA2* confers a high risk for breast and ovarian cancer (Moynahan *et al.*, 1999; 2001). Interestingly, the gene products of *BRCA1* and *BRCA2* have been implicated in the HR pathway in a series of studies. Like *RAD51*$^{-/-}$, *BRCA1*$^{-/-}$ and *BRCA2* $^{-/-}$ knockout mice exhibit early embryonic lethality (Sharan *et al.*, 1997; Gowen *et al.*, 1996; Lim and Hasty, 1996) and murine ES cells derived from these knockouts are hypersensitive to IR and hydrogen peroxide, but not UV (Gowen *et al.*, 1998; Chen *et al.*, 1999). In addition, BRCA1 and BRCA2 colocalize with RAD51 to nuclear foci following radiation treatment in mitotic cells, and at synaptonemal complexes in meiotic cells (Scully *et al.*, 1997; Chen *et al.*, 1999). Direct interaction between BRCA2 and RAD51, as well as interaction between BRCA1 and BRCA2, has been demonstrated by their coimmunoprecipitation from extracts prepared from normal cells (Chen *et al.*, 1999). BRCA1 has also been found to associate

with the RAD50 repair protein, colocalizing with it upon IR damage to nuclear foci that are distinct from Rad51 foci (Gowen *et al.*, 1998; Zhong *et al.*, 1999).

It has been reported that *BRCA1*$^{-/-}$ ES cells have a 53-fold decrease in gene targeting frequency as compared with *BRCA1*$^{+/-}$ cells (Moynahan *et al.*, 1999). Homology-directed repair in the *brca2* mutant is also impaired, decreasing by 6 to 100-fold with the induction of specific DSBs (Moynahan *et al.*, 2001). A functional role has been ascribed to BRCA2 for regulating the transport of RAD51 to the nucleus in response to DNA damage, as BRCA2 was found to regulate the intracellular localization and DNA-binding ability of Rad51 (Davies, 2001). Transport of Rad51 is defective in cells carrying a cancer-associated *BRCA2* truncation.

1.2.2 Non-conservative Homologous Recombination

Many organisms possess a homologous DSB repair pathway separate from the HR pathway mediated by the RAD52 epistasis group. This DSB repair pathway is inherently non-conservative, typically occurring when DSBs are positioned between tandem repeats or when complementary sequences are exposed by exonuclease or helicase activity at DSB ends (Tomso and Kreuzer, 2000). The defining feature of this non-conservative homologous recombination pathway, more commonly known as the single-strand annealing (SSA) pathway, is the loss of at least one copy of homologous DNA, along with any intervening sequence that flank the break site (Figure 8).

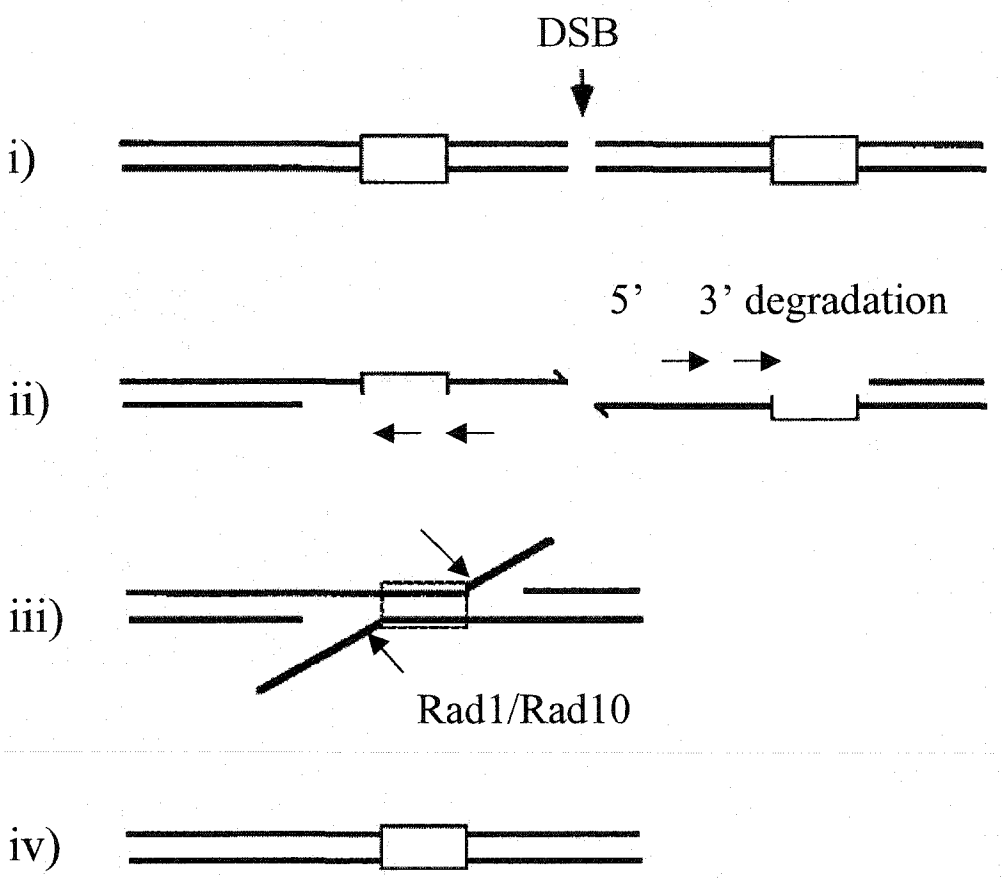


Figure 8. The single-strand annealing pathway (Ivanov *et al.,* 1996). SSA occurs when a DSB between flanking repeats **(i)** results in exposure of homologous sequences by the 5' to 3' resection of DSB ends **(ii)**. After excision of non-homologous 3' ends by the Rad1/Rad10 enzyme complex **(iii)** and gap-filling, ligation restores two continuous strands **(iv)**.

Transformation of linear dimer plasmids into *E. coli* was shown to sometimes yield monomer circular recombinants by a recA-independent pathway (Luisi-DeLuca and Kolodner, 1992). In yeast, SSA also occurs independently of the RecA homologue Rad51 (Ivanov *et al.*, 1996). Analysis of SSA in yeast has revealed several fundamental properties of this pathway. Induction of a DSB *in vivo* in one of two repeats results in repair either by SSA or by the RAD52-dependent HR pathway. Increasing the distance between the repeats decreases the efficiency of SSA in competition with gene conversion, consistent with the need for more time for 5' to 3' exonuclease activity to expose complementary homologies (Fishman-Lobell *et al.*, 1992; Haber and Leung, 1996). Efficiency of SSA is also dependent on the length of the flanking repeats and their degree of sequence homology (Sugawara and Haber, 1992; Sugawara *et al.*, 1997).

Factors implicated in the SSA pathway in yeast, include *RAD52* and the *RAD1* and *RAD10* genes, which play important roles in the nucleotide excision repair (NER) pathway. Absence of Rad52 results in the near elimination of SSA when the homologous sequences are 1 Kb or less in length, but larger regions of homology appear to compensate for the lack of Rad52 (Fishman-Lobell *et al.*, 1992; Rudin and Haber, 1988; Sugawara and Haber, 1992; Oettinger *et al.*, 1990). Deletion of *RAD50* and *XRS2* delays but only partially reduces SSA (Ivanov *et al.*, 1996; Sugawara and Haber, 1992). Deletions of *RAD51, RAD54, RAD55* and *RAD57* do not affect SSA (Ivanov *et al.*, 1996).

Rad1 and Rad10 interact stably *in vitro* (Bailly *et al.*, 1992; Bardwell *et al.*, 1992; 1994) and are thought to function as a complex during both NER and intrachromosomal mitotic recombination (Rodriguez *et al.*, 1996). Physical analysis of recombination intermediates in *rad1* and *rad10* mutants has suggested that Rad1 and Rad10 may

contribute to a nuclease activity that removes non-complementary 3' ssDNA ends from duplex DNA molecule during SSA (Fishman-Lobell and Haber, 1992; Ivanov *et al.*, 1996). *In vitro* studies have corroborated this observation in that purified Rad1 and Rad10 proteins exhibit nuclease activity that is not present when the proteins are assayed individually (Sung *et al.*, 1993; Tomkinson *et al.*, 1994). One endonuclease unit was shown to consist of one molecule each of Rad1/Rad10, and demonstrated junction-specific endonuclease activity that uniquely cleaves the 3' single-strand extension from a duplex/single-strand junction (Bardwell *et al.*, 1994).

1.2.3 The non-homologous End-Joining pathway

The non-homologous end-joining (NHEJ) pathway is the second major pathway of DSB repair in eukaryotic cells. It is defined by the rejoining of DSB ends with scarce need for homology, if any at all. In contrast to yeast, mammalian cells have abundant direct DNA end-joining, and repair of DSBs appears to occur primarily through the DNA-dependent protein kinase-mediated NHEJ pathway. The components of the DNA-PK-dependent NHEJ pathway of both yeast and mammalian systems share close homology. Since the former is more relevant to this dissertation, I will primarily focus on the yeast NHEJ pathway and its components.

Factors involved in DNA end-joining

DNA-PK is a nuclear serine/threonine protein kinase that comprises two subunits: a catalytic subunit (DNA-PK_{cs}) of ~465 kDa and Ku, a heterodimeric DNA end-binding protein composed of ~70 kDa and ~86 kDa subunits (Ku70 and Ku80 respectively) (Critchlow and Jackson, 1998).

DNA-PK_{cs}, a functional homologue for which has yet to be identified in yeast, mediates the catalytic activity of DNA-PK (Dvir *et al.*, 1993; Gottlieb and Jackson, 1993). DNA-PK_{cs} can also bind to and be activated by DNA ends in the absence of Ku (Hammarsten and Chu, 1998; Yaneva *et al.*, 1997), but Ku is likely to be required for the stabilization of DNA binding by DNA-PK_{cs} *in vivo* (Hammarsten and Chu, 1998) (Figure 9). *S. cerevisiae* does possess two proteins, Tel1 and Mec1, that share sequence homology with DNA-PK_{cs}, and are believed to be homologues of the kinases ATM and ATR, respectively. Possibly these proteins may play a functional role similar to that of DNA-PK_{cs} in yeast.

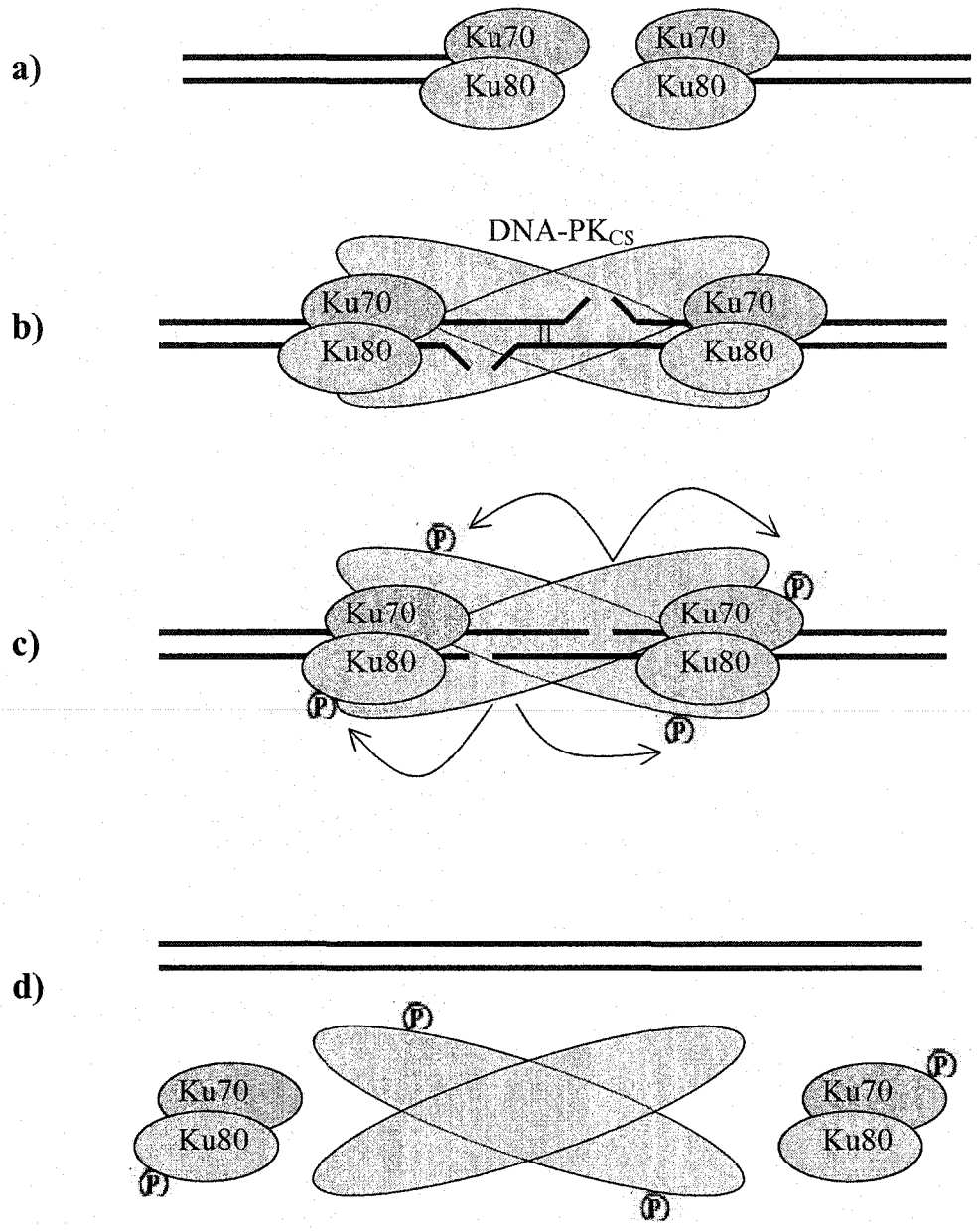


Figure 9. The proposed mechanism of action of the DNA-PK-dependent NHEJ pathway (Featherstone & Jackson, 1999). **(a)** The Ku heterodimer binds to DNA free ends and recruits catalytic subunits (DNA-PKcs). **(b)** The weakly processive helicase activity of Ku unwinds the DNA ends, permitting the annealing of the broken strands at sites of microhomology. **(c)** Non-homologous free ends may be trimmed by Rad50/Mre11/Xrs2 complex, and following gap-filling and ligation by DNA polymerase and DNA ligase IV; **(d)** the protein kinase activity of DNA-PK may autophosphorylate both Ku and DNA-PKcs, allowing them to dissociate.

The yeast homologues of *Ku70* (also called *Hdf1*) and *Ku80* have been identified and shown to play important roles in NHEJ. In yeast strains in which *Ku* is defective, repair of plasmids bearing 5' or 3' overhangs is reduced drastically. Moreover, the residual repair in these strains is inaccurate, due to a higher incidence of repair between short homology regions and deletion of intervening sequences (Boulton and Jackson, 1996a; 1996b). A *ku70* null mutant fails to display IR sensitivity, but a *ku70 rad52* double mutant shows greater IR sensitivity that a *rad52* single mutant, consistent with the notion that *Ku* operates in a DSB repair mechanism distinct from homologous recombination (Boulton and Jackson, 1996b; Milne *et al.*, 1996; Siede *et al.*, 1996). Collectively, these results raise the possibility that *Ku* functions in two general ways to potentiate accurate NHEJ: (1) to facilitate NHEJ itself; and (2) to suppress the alternative deletional (errorprone) pathway which is in all likelihood the SSA pathway (Boulton and Jackson, 1996b).

Moreover, a role has been suggested for *Ku* in telomere maintenance and telomeric silencing. Cells disrupted for *Ku* function are also debilitated in telomere-associated transcriptional silencing, also known as the telomere position effect (TPE) (Boulton and Jackson, 1998; Nugent *et al.*, 1998). Epistatic analysis defined a telomere-associated activity for the Ku end-binding complex, required in parallel with telomerase and Cdc13 (which binds the single-strand portion of the telomere) (Nugent *et al.*, 1998). Localization of Ku to the telomere does not depend on DNA-PK_{cs} (Hsu *et al.*, 1999).

More recently, using the yeast two-hybrid system, Sir4 was identified as a protein interacting with Ku70 (Tsukamoto *et al.*, 1997). Moreover, *sir2, -3*, and *–4* mutants have been found to be IR-sensitive in a *rad52* background and have defects in rejoining of

non-homologous DNA ends, similar to *ku70* (Jeggo, 1998a). Thus, Sir2, -3 and -4 have been concluded to play a role in NHEJ. In addition, they have been implicated in telomere silencing (Aparicio *et al.*, 1991).

The key enzymatic step of NHEJ is DNA ligation. *S. cerevisiae* possesses two ATP-dependent ligases: one homologous to mammalian DNA ligase I, the other homologous to mammalian DNA ligase IV (Critchlow and Jackson, 1998). Yeast ligase I is required for joining Okazaki fragments during replication and is involved in various forms of DNA repair (Tomkinson and Levin, 1997). Recently, the yeast homologue (Lig4) of DNA ligase IV was demonstrated to function epistatically with Ku in NHEJ (Schar *et al.*, 1997; Teo and Jackson, 2001; Wilson *et al.*, 1997). Furthermore, Lig4 interacts with ligase-interacting factor (Lif), a newly identified yeast homologue of human XRCC4, which also functions in NHEJ (Herrmann *et al.*, 1998).

In *S. cerevisiae*, Rad50, Mre11 and Xrs2 are known to participate in meiotic recombination, as described earlier. They have also been implicated in NHEJ. Disruption of the *RAD50, MRE11* or *XRS2* genes impairs NHEJ to an extent similar to that caused by disruption of *Ku70, Ku*80 or *Lig4* (Boulton and Jackson, 1996b; Tsukamoto *et al.*, 1996). Furthermore, studies in which *RAD50, MRE11* or *XRS2* have been disrupted, in either *ku70* or *lig4* backgrounds, have revealed that they all function epistatically in the same NHEJ pathway (Milne *et al.*, 1996).

1.3 The role of endo-exonucleases in double-strand break repair processes

Endo-exonucleases (EE) are a unique class of nucleases in their ability to process both single-strand and double-strand DNA substrates- a dual function which is critical in

recombination and recombinational repair, and is only now being appreciated in DNA degradative processes during apoptosis. Endo-exonucleases have ssDNA-specific endonucleolytic activity and a processive 5' → 3' exonuclease activity on both ssDNA and dsDNA to release 5' phosphoryl-terminated oligo or mononucleotides, with optimal activity in the neutral pH range (Chow and Fraser, 1983; Chow and Resnick, 1988; Koa *et al.*, 1990).

1.3.1 The *E. coli* recBCD enzyme complex

The recBCD nuclease (also known as exonuclease V) is a heterotrimeric enzyme consisting of 3 polypeptides of 134, 129 and 67 KDa in size, encoded by the *recB, recC* and *recD* genes of *E. coli*, respectively (Goldmark, 1972). It is the most well-characterized endo-exonuclease to date, having consistently been shown to be critical for recombinational repair of DSBs in *E. coli*. In wild-type *E. coli*, rejoining of the chromosomal DNA fragmented by gamma irradiation is completely blocked by only three mutations: *recA, recB* or *recC* (Sargentini and Smith, 1986). *In vitro*, recBCD rapidly degrades linear duplex DNA to oligonucleotides (Kuzmimov, 1999). *In vivo*, it degrades bacteriophage DNA cut by the host restriction systems, and in *recA* mutants, it degrades the entire chromosome after it was fragmented as a result of DNA damage.

The prominent activities of recBCD include DNA helicase, ATP-dependent dsDNA exonuclease and ssDNA endonuclease activities. The recBCD enzyme exhibits true ssDNA endonuclease activity as revealed by its ability to degrade ss-circular but not covalently closed ds-circular forms of bacteriophage and plasmid DNAs, including supercoiled and relaxed forms (Goldmark, 1972; Karu *et al.*, 1973). As a consequence,

recBCD does not promote plasmid DNA recombination in *E. coli*; a function possibly fulfilled by another nuclease (Smith, 1988). The endonuclease activity of recBCD is dependent in Mg^{+}, but not ATP (Goldmark, 1972). The recBCD nuclease degrades both ss- and ds-DNA exonucleolytically to release small 5'-P and 3'-OH-terminated oligonucleotides. Unlike most nucleases, it requires ATP hydrolysis for this degradative action (Goldmark, 1972; Karu *et al.*, 1973). The *rec* genes for each recBCD subunit were cloned and their gene products purified. The recombination activity of each purified subunit was only 20% in comparison to the trimeric holoenzyme, and only the recB subunit possessed a detectable ATPase enzymatic activity (Finch *et al.*, 1986; Hickson *et al.*, 1985; Masterson *et al.*, 1992). This implies that the activities of the recBCD enzyme are dependent on protein-protein interactions between each subunit.

In the presence of excess SSB, the ssDNA-binding protein, or in the presence of 1mM Ca^{+}, the nuclease activity, but not the ATPase activity of recBCD, is suppressed (MacKay and Linn, 1976; Rosamund *et al.*, 1979). In the presence of these nuclease inhibitors, recBCD exhibits the characteristics of an Mg^{+} and ATP-dependent helicase, unwinding duplex DNA at about 200 nucleotides per second to form twin ssDNA loops in dsDNA, appearing like rabbit ear structures in electron micrographs (Taylor and Smith, 1980; Telander and Linn, 1982).

A null mutation in *recD* results in a phenotype that includes a lack of nuclease activities and little or no ATP-dependent helicase activity, but surprisingly, the mutants are fully recombination proficient (Amundsen *et al.*, 1986; Chaudhury and Smith, 1984). This suggests that the major role of recBCD in recombination may not be so much

through its nucleolytic activity as its helicase activity, demonstrated to generate ssDNA regions (Fraser, 1996).

The recBCD enzyme was also found to selectively nick specific sequences in the E. coli genome called Chi sites. The octanucleotide (5' GCTGGTGG 3') Chi sites were first discovered in bacteriophage λ as mutations creating hot spots for *E. coli* recombination (Myers and Stahl, 1994). The *red gam* mutant λ cannot inactivate ExoV (*gam*) and lacks its own recombination system (*red*), and so its DNA is salvaged by recBCD after being linearized for packaging by a phage-encoded terminase (Enquist and Skalka, 1973). Linearization of Chi-containing DNA, instead of triggering its degradation, results in its recombining with other λ DNA molecules catalyzed, surprisingly, by the same recBCD enzyme. The paradox of the recBCD enzyme being able to carry out two mutually exclusive activities on linear DNA: complete destruction versus preservation through recombination, led to the proposal that the encounter of recBCD with a Chi site disables the dsDNA exonuclease activity of the enzyme, converting it into a recombinase by virtue of its helicase activity (Dixon and Kowalczykowski, 2001; Kuzmimov *et al.*, 1994; Stahl and Thomasen, 1990).

1.3.2 Eukaryotic endo-exonucleases

EEs isolated from eukaryotic cells display a diverse range of sizes and primary structures, but they essentially show the same basic enzymatic properties. They have true ss-specific endonuclease activity with linear and circular ssDNAs as well as with ssRNA. In contrast to recBCD, most eukaryotic nucleases have the ability to nick supercoiled ds-DNA and act slowly in the region of the nick to generate linear dsDNA,

which may then be processed via a dsDNA-dependent exonuclease activity (Fraser, 1996).

1.3.2.1 Mitochondrial endo-exonucleases

A 37 kDa mitochondrial nuclease, one of the earliest EEs isolated, was successfully purified and characterized at the molecular level from *S. cerevisiae* (Dake *et al.*, 1988). It was shown to display similar activities on both RNA and DNA as a 33 kDa EE previously isolated from the mitochondria of the fungus *Neurospora crassa* (Chow and Fraser, 1983). The gene encoding the *S. cerevisiae* mitochondrial EE was named *NUC1*, and is located on chromosome X (Vincent *et al.*, 1988). It shares extensive sequence homology with the C-terminal region of *recC* (Fraser *et al.*, 1990). The only observable phenotype for *nuc1* null mutants was a lack of mitochondrial nuclease activity, indicating that the mitochondrial enzyme was not essential for mitochondrial function (Zassenhaus *et al.*, 1988).

The yeast mitochondrial EE was found to be an Mg^{++}-dependent nuclease, although Co^{++} and Mn^{++} divalent cations could be substituted as activating co-factors. Its endonuclease activity was specific to ssDNA and ssRNA, and it possessed 5'→ 3' exonuclease activity on linear ssDNA and dsDNA (Dake *et al.*, 1988). Unlike the *N. crassa* EE, which is processive (Chow and Fraser, 1983), the exonuclease activity of the yeast mitochondrial EE is distributive.

A 44 kDa ssDNA-specific mitochondrial endonuclease was isolated from *Drosophila melanogaster* (Harosh *et al.*, 1992) which was similar to mammalian endonucleases isolated from mouse plasmacytoma cells (Tomkinson and Linn, 1986).

Endonuclease G, a 26 kDa endonuclease purified from bovine tissue mitochondrial extracts, was cloned and sequenced (Cote and Ruiz-Carrillo, 1993). The identified open reading frame was predicted to encode a polypeptide of 299 amino acids, with an estimated weight of 34 kDa. The lower weight yielded after purification suggested that endonuclease G undergoes proteolytic processing during purification.

All the mitochodrial nucleases isolated have been found to be associated with the inner membrane of the mitochondrion (Dake *et al.*, 1988; Cummings *et al.*, 1987; Fraser and Cohen, 1983). Despite differences in activities, specificity and size, they all seem to be immunochemically related. The 37 kDa *S. cerevisiae* mitochondrial endonuclease and the 37 kDa mouse nuclease both show cross-reactivity with a polyclonal antibody raised to the purified *Neurospora* EE (Dake *et al.*, 1988; Tomkinson and Linn, 1986). The same antibody had shown cross-reactivity with the recBCD nuclease from *E. coli* (Fraser *et al.*, 1990). The *Drosophila* nuclease was found to be cross-reactive with antibodies raised to both *S. cerevisiae* and the bovine heart mitochondrial nucleases (Harosh *et al.*, 1992).

Like the yeast mitochondrial EE, the mammalian mitochondrial endonucleases are not likely to play a role in the recombination of mitochondrial DNA as it is not known to undergo recombination. It has been postulated that the mitochondrial EE may be an important factor in the repair of oxidative damage, and replication of mitochondrial DNA (Low *et al.*, 1987).

1.3.2.2 Extra-mitochondrial Endo-exonucleases of fungi and yeast

The earliest extra-mitochondrial EE isolated was first partially purified from the mycelia of *Neurospora crassa* (Fraser *et al.*, 1976). It displayed an Mg^{++}-dependent ss-specific endonuclease activity with ssDNA and ssRNA, and exonucleolytic activity with dsDNA. All nuclease activity was inhibited by 0.1-0.5 mM ATP. The exonuclease activity was preferentially inhibited by 0.1-0.2M NaCl, Zn^{++}, Ca^{++}, EDTA, and could be abolished by trypsin, generating a ss-specific endonuclease comparable with the ss-specific endonuclease originally isolated from *N. crassa* (Chow and Fraser, 1983). A DNA-unwinding activity was also detected in the nuclease preparation.

The exonuclease activity lacks strand specificity and releases small oligonucleotides with 5'-P termini like recBCD nuclease, but it has strict 5'→ 3' polarity in its action, and was found to have the highest processivity of any EE reported to date (Fraser and Low, 1993). In the presence of a high concentration of Mg^{++}, the exonuclease activity generates long ss-tails in duplex DNA like recBCD, but unlike recBCD, the EE can also enter at nicks in duplex DNA and generate long ss gaps (Chow and Fraser, 1983).

Neurospora EE can be inhibited by two non-specific nuclease inhibitors: low levels of aurin tricarboxylic acid and heparin (Chow and Fraser, 1983). The activities of the purified EE, unlike assays with the partially purified enzyme, were not affected, and the ds-DNase activity was stimulated 2-fold, rather than inhibited by Ca^{++}.

Different sizes of active EEs have been detected in fresh extracts of *N. crassa* and *Aspergillus nidulans*, all of which are immunoprecipitable with antibody raised to the pure 31 kDa *N. crassa* EE (Ramotar *et al.*, 1987; Fraser *et al.*, 1986). Several different-

sized active polypeptides of 37, 43 and 76 kDa were found by activity gel analysis of nuclear fractions of *N. crassa*. The largest, 76 kDa, was associated with the nuclear matrix fraction. Since this polypeptide was also associated with the other active polypeptides in fresh sonicates of rapidly growing mycelia (Fraser *et al.*, 1986), it seems likely that the smaller active species were generated by limited proteolysis. In fact, the 31 kDa active EE purified from *N. crassa* is likely to be a proteolyzed fragment of this 76 kDa polypeptide.

Early attempts to purify and study the inactive precursor of the *N. crassa* EE (Kwong and Fraser, 1978) led to the isolation of trace amounts of a 93 kDa inactive precursor. The conversion of this precursor into active EE was found to be stimulated 20-fold by heat shock (Ramotar and Fraser, 1989). This led to the identification of a 24 kDa heat-resistant–specific inhibitor which appeared to form a stable complex with the enzyme, and was dissociated by proteolysis prior to EE activation (Fraser *et al.*, 1989).

The intracellular distribution of active and inactive forms of EE in *A. nidulans* was found to be the same as in *N. crassa*, but suborganellar localizations were not done (Koa *et al.*, 1990). Similar distributions of active and inactive EEs were also identified in *Flammulina velutipes* (Sen *et al.*, 1992) and *Schizosaccharomyces pombe* (Szankasi and Smith, 1992). The 42 kDa and 52 kDa forms of an ss-specific endonuclease, nuclease α, was purified from *Ustilago maydis* (Holloman and Holliday, 1973; Holloman *et al.*, 1981), which possesses properties similar to that of the mitochondrial ss-specific endonuclease originally isolated from *Neurospora* (Linn and Lehman, 1966). A 43 kDa EE was also isolated from the mushroom *Coprinus cinereus* (Lu and Sakaguchi, 1991), but its activities resemble more closely those of ss-specific exonucleases.

Using the *N. crassa* polyclonal antibody, an extra-mitochondrial EE was isolated from *S. cerevisiae* and purified by immunoaffinity chromatography (Chow and Resnick, 1987). The same antibody was used to screen a yeast DNA library in the λgt11 expression vector (Chow *et al.*, 1992). The *S. cerevisiae* EE gene first isolated was sequenced, and reported to encode a chimeric protein with a GTP-binding Rho-like domain in the N-terminal region and a presumed nuclease function in the C-terminal region of the protein. However, subsequent analysis revealed that the 4 Kb fragment actually encoded two separate genes, designated *RHO4a* and *NUD1* (Vliet-Reedijk and Planta, 1993). *RHO4a* encodes a 32 kDa protein which shares significant homology with other members of the *ras* superfamily of GTP-binding proteins. The originally proposed *RNC1* gene arose from a cloning artifact that resulted in the fusion of the *RHO4a* and *NUD1* genes and the deletion of an intergenic region as well as a 3'-terminal portion of the *NUD1* gene encoding 449 amino acids (Vliet-Reedijk and Planta, 1993). Recently, the gene encoding the yeast endo-exonuclease was reclassified as *RNC1*, and *NUD1* was used to denote another unrelated gene in the gene registry. The name RNC1 will be used to refer to the gene for the yeast endo-exonuclease for the rest of this introduction, as well as in the general discussion.

Sequence analysis of the *RNC1* gene revealed an ORF encoding 617 amino acids with a predicted mass of 70 kDa (Vliet-Reedijk and Planta, 1993) which correlated with an estimated size of 72 kDa for the immunoprecipitated RNC1 EE (Chow and Resnick, 1988). The sequence of *RNC1* was found to be unrelated to the mitochondrial *nuc1* EE.

Expression of *RNC1* was found to be directly influenced by *RAD52* expression. In the *rad52* mutant, which is deficient in recombination and DSB repair, expression of the

EE was observed to be at 10% of that in wild-type cells (Resnick *et al.*, 1984). Using strains of *S. cerevisiae* that exhibit high levels of synchrony during meiosis, an increase in nuclease levels was shown to correlate with the commitment to meiotic intragenic recombination (Chow and Resnick, 1988). A 10- to 20-fold increase in the levels of a single species of an Mg^{++}-dependent alkaline DNase that is cross-reactive with the *N. crassa* antibody was detected. It was shown directly, by immunoblotting extracts of cells with the same antibody, that a 72 kDa polypeptide corresponding to the purified *S. cerevisiae* EE was very poorly expressed in the *rad52* mutant (Moore *et al.*, 1993). It was also found that, while high levels of the *S. cerevisiae* EE were found in wild-type yeast cells during logarithmic phase, it was not detectably expressed in stationary phase cells, thus indicating a possible role for the EE in growth/DNA replication (Moore *et al.*, 1993).

The *RNC1* gene was subcloned into yeast multicopy plasmids and overexpressed in *S. cerevisiae* (Sadekova and Chow, 1996). Enhanced expression of the nuclear EE was confirmed through 10-fold increase in the corresponding mRNA levels and a 2.4-fold increase in nuclease activity. Increased expression of *RNC1* was associated with an increase in cell survival after irradiation treatment with gamma rays, and an increase in radiation-induced mitotic recombination frequencies between duplicated gene sequences. Recently, it was demonstrated that transiently expressing *RNC1* in Hela cells results in their increased resistance to ionizing radiation and cisplatin (Semionov *et al.*, 1999a). Additionally, transient expression of *RNC1* in mouse fibroblasts was found to increase the frequency of homologous recombination between two co-transfected linear plasmids by up to 62% (Semionov *et al.*, 1999b). These results confirm a role for *RNC1* in DNA repair and recombination processes in yeast.

Interestingly, RNC1 has also been implicated in the induction of mitochondrial "petite" mutations (mutations that result in respiratory deficiency and diminished growth on media containing a fermentable carbon source (Chow and Kunz, 1991) in that overexpressing *RNC1* results in increased frequency of spontaneous "petites" and an increased sensitivity to induction of "petites" with ethidium bromide (Chow and Kunz, 1991). The addition of ethidium bromide to the reaction mixture was also found to stimulate the activity of the RNC1 nuclease on double-stranded DNA

Recently, an alternate role has been suggested for RNC1 in the methylation of elongator tRNAs. Furthermore, the terminal carboxyl region of RNC1 bears 30% homology to the tRNA(m^5U) methytransferase-encoding gene *TrmA* in *E. coli* (Nordlund *et al.*, 2000). The presence of a 5-methyluridine at position 54 of the TψC loop of tRNAs is a ubiquitous feature of most eubacteria and in eukaryotic elongator tRNAs. However, *RNC1*-disrupted strains are deficient in the formation of this modified nucleoside (Nordlund *et al.*, 2000). While a definitive role has yet to be established for the presence of m^5U in most tRNA, the implications of the involvement of *RNC1* in this process are as yet unclear.

1.3.2.3 Extra-mitochondrial Endo-exonucleases of higher eukaryotes

A 33.6 kDa EE was first isolated and characterized from *D. melanogaster* which, like the *Neurospora* and *Saccharomyces* enzymes, displayed endonucleolytic activity with both ssDNA and RNA that was relatively salt-resistant (Shuai *et al.*, 1992). The exonuclease activity on dsDNA, was found to be salt-sensitive. In a manner characteristic of other EEs, the enzyme degrades dsDNA to release small oligonucleotides with 5'-P

and 3'-OH termini and converts supercoiled plasmid DNA to the relaxed and linear forms in a stepwise fashion.

The first mammalian EE was purified and characterized from monkey CV-1 cells (Couture and Chow, 1992). The enzyme, which was purified to near homogeneity, is a 65 kDa monomeric protein. The single-strand DNase activity is endonucleolytic and nonprocessive, whereas the double-strand DNase activity is exonucleolytic and processive. The enzyme also possesses detectable RNase activity assayed using poly-rA as a substrate. The same immunochemical approach mentioned earlier using the *N. crassa* polyclonal antibody was used to isolate cross-reactive proteins in Chinese hamster ovary cells, human HeLa cells and human ataxia telangiectasia cell lines (Liu *et al.*, 1995). Also, both active and inactive forms of an EE were identified in nuclear and extranuclear fractions of human leukemic CEM cells (Fraser *et al.*, 1996).

1.4 Summary

The first and most critical step in homologous recombination-based repair of double-strand breaks is the 5' → 3' resection of the DSB ends, which results in a 3' single-strand overhang intermediate that is critical for invading a homologous duplex DNA and priming DNA synthesis. Early on, the RecBCD enzyme was implicated in this exonuclease activity in *E. coli*. Given the similarities between RecBCD and other eukaryotic endo-exonucleases, it was anticipated that eukaryotic EEs would play a corresponding role in homologous recombination in eukaryotes.

As was outlined previously, the best evidence that implicates EEs in eukaryotic recombination and DNA repair comes from studies of the RNC1 endo-exonuclease in *S.*

cerevisiae. The findings of this thesis further corroborate the involvement of RNC1 in the HR pathway and provide the first systematic biochemical analysis of the purified protein *in vitro*.

PREFACE

The following chapter describes a series of genetic assays that were used to determine the response of the *rnc1* mutant (referred to as the *nud1* mutant up till the time of publication) when challenged with DSB-inducing agents. The genetic assays used include ionizing radiation and a specific DSB induction system utilizing HO endonuclease. In addressing the response of the *rnc1* mutant to DSB induction, we sought to establish an interaction at the genetic level between *RNC1* and *RAD52*, an essential component of the DSB repair pathway in *S. cerevisiae*.

The manuscript also describes our early investigations into characterizing the fidelity of DSB repair processes in the presence or absence of the *RNC1* gene product. This was performed utilizing the yeast mating type switching system as a model to study the repair dynamics following a specific DSB induction. Efficiency in repairing site-specific DSBs was analyzed by screening survivors of DSB induction for mating proficiency, followed by PCR analysis of the mating type locus.

Chapter II

Genetic analysis of the yeast NUD1 endo-exonuclease:

A role in the repair of DNA double-strand breaks

Abstract

Deoxyribonucleases (DNases) have been shown genetically to be important in the vital processes of DNA repair and recombination. The *NUD1* gene, which codes for an endo-exonuclease of *Saccharomyces cerevisiae*, was analyzed for its role in the DNA double-strand break (DSB) repair processes. While the *nud1* strain is only slightly sensitive to ionizing radiation, expression of the HO endonuclease to introduce a DSB at the *MAT* locus in that strain results in cell death. Cell survival is inversely proportional to the duration of HO endonuclease expression.

Analysis of the surviving colonies from the *nud1* strain indicated that many of the survivors are sterile and that the proportion of these sterile survivors increases with the time of HO-endonuclease expression. On the other hand, the surviving colonies from the isogenic *NUD1* strain are mating-proficient. Interestingly, double mutants of *nud1 rad52* are more resistant to ionizing irradiation than the *rad52* strain and have a cell-survival fraction of 32% for *rad52-1 nud1* and 9% for *rad52::URA3 nud1* following prolonged HO-endonuclease expression, indicating that *nud1* has a suppressor effect on the DSB-induced lethality in *rad52*. Polymerase chain reaction analysis showed that many of the *nud1* survivors contained small alterations within the *MAT* locus, suggesting that the survivors arose through the process of non-homologous end-joining. These results suggest that the endo-exonuclease acts at a DSB to promote DNA repair via the homologous recombination pathway.

Key words: Endo-exonuclease, *NUD1,* DNA recombination, DNA repair, Non-homologous end-joining.

Introduction

Deoxyribonucleases (DNases) have been implicated in several DNA repair processes, particularly in catalyzing the incision and excision of damaged DNA and thus providing a means for heteroduplex formation and processing in recombination. Despite their obvious importance, the role of DNases in eukaryotic DNA recombination is not well defined and the supporting data are mainly biochemical. For example, Holloman and Holliday (1973) described a nuclease from the eukaryote *Ustilago maydis* that appears to be required for both recombination and DNA repair. However, due to the complexity of its genetic control, the specific role of this nuclease in recombination was not established. Recently, Thelen *et al.* (1994) identified the *rec1* gene product of *U. maydis* and found it to be an exonuclease. An endo-exonuclease from *Neurospora crassa* has also been implicated in DNA recombination and repair (Chow and Fraser 1979, 1983; Ramotar *et al.* 1987). The phenotype of mutants with reduced or altered endo-exonuclease activity included meiotic sterility and sensitivity to ultraviolet light, X-rays, and/or alkylating agents (Fraser et al. 1980). Similar endo-exonucleases have been isolated from *Aspergillus nidulans* (Koa et al. 1990), *Coprinus cinereus* (Lu and Sakaguchi 1991), monkey cells (Couture and Chow 1992), and the mitochondria of *Saccharomyces cerevisiae* (Drake *et al.* 1988) and mice (Tomkinson and Linn 1986).

Previously, we isolated and characterized a *S. cerevisiae* enzyme that *in vitro* has an endonucleolytic activity with single-stranded DNA and an exonucleolytic activity with double-stranded DNA (dsDNA) (Chow and Resnick 1987). The mode of action with dsDNA was processive in the 5' $\rightarrow$ 3' direction (Chow and Resnick 1987). Expression of this *S. cerevisiae* endo-exonuclease was influenced by the functional state of the *RAD52*

gene (Chow and Resnick 1983, 1988), suggesting that the enzyme has a role in recombinational repair since *RAD52* is required for the recombinational repair of DNA double-strand breaks (DSBs) (Ho 1975; Resnick and Martin 1976).

In addition, the activity of the endo-exonuclease increases during meiosis in wild-type *S. cerevisiae* (Resnick *et al.* 1984 a, b) but not in a *rad52* strain (Chow and Resnick 1983, 1988). Overexpression of the gene (*NUD1*) encoding the yeast endoexonuclease (Chow and Kunz 1991; Chow *et al.* 1992; Moore *et al.* 1993; van Vliet-Reedijk and Planta 1993) increased the frequencies of spontaneous and radiation-induced recombination, as well as cell survival after irradiation (Sadekova and Chow 1996).

These observations suggest that the endo-exonuclease plays a direct role in recombinational repair. In *S. cerevisiae,* the DSB is an important intermediate during DNA recombination (Resnick 1976). It is the site for many competing DNA repair mechanisms including non-homologous end-joining (NHEJ) (Mézard et al. 1992; Mézard and Nicolas 1994; Moore and Haber 1996) and the homologous recombination mechanism known as single-strand annealing (SSA) (Fishman-Lobell *et al.* 1992). SSA involves the ends of the DSB being degraded by a 5' $\rightarrow$ 3' processive exonuclease to generate a long 3'-ended single strand of DNA containing complementary sequences for homologous pairing. Although the identity of the 5' $\rightarrow$ 3' exonuclease in SSA has not been determined, efficient SSA repair of a chromosomal DSB requires a functional *RAD52* gene (Sugawara and Haber 1992) but not other members of the *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD54*, *RAD55*, *RAD57*, or *XRS2*) (Ivanov and Haber 1995). On the other hand, the Rad50, Mre11, and Xrs2 proteins form a complex that has structural and functional similarities to the *sbcCD* proteins of *Escherichia coli* (Sharples and Leach

1995), and is required to produce a 5' ended single-strand intermediate during NHEJ (Sugawara and Haber 1992; Moore and Haber 1996).

Mating-type switching in yeast is initiated by the HO endonuclease generating a site-specific DSB at the mating type (*MAT*) locus (Haber 1992). This DSB is subsequently repaired by homologous recombination using one of two silent donors of mating-type information, *HML* or *HMR* (Haber 1992). However, when homologous recombination is prevented, for example by a *rad52* mutation, the cells are capable of repairing the DSB via non-homologous pathways of the NHEJ type (Kramer et al. 1994; Moore and Haber 1996). Using the repair of a HO-endonuclease-induced DSB in the *MAT* locus as a model, we investigated the genetic and molecular consequences of eliminating *NUD1* on DSB repair. We found that deletion of the *NUD1*gene sensitizes cells to a DSB and enhances the NHEJ repair process. Our results suggest that *NUD1* is required for the efficient repair of a DSB by the homologous recombination mechanism. In addition, deletion of the *NUD1* gene suppresses the DSB-induced lethality in the *rad52* mutant.

Materials and methods

Yeast strains. The strains used in this study are: TC106d (*MAT* α, *leu2-3,112, trp1-289, ura3-52, his1-7*), TC106dr52 (as for TC106d but *rad52::URA3*), TC107a (*MAT***a**, *leu2-3,112, trp1-289, ura3-52, his1-7, rad52-1*), TC111b (*MAT***a**, *leu2-3,112, trp1-289*), and TC111c (as for TC111b but *MAT* α). These are congenic strains derived from the SK-1 background (Game et al. 1980). The TC106dr52 was constructed by transforming the TC106d strain (Rothstein 1983) with a fragment (5.6 kb) from a *SalI* restriction endonuclease digest of p52Blast (a gift from Ed Perkins, NIEHS). This fragment contained the *URA3* gene flanked on both sides by the 5' upstream and the 3' downstream regions of the *RAD52* gene. The TC106dr52 is a *rad52* null-mutant strain. TC106dD, an isogenic derivative (*nud1*) of TC106d lacking *NUD1*, was constructed as follows: A 4-kb *Eco*RI-*Sal*I yeast chromosomal DNA fragment encompassing *NUD1* was ligated into the *Eco*RI-*Sal*I interval of YEplac112 (Gietz and Sugino 1988) to make YEpNUC200. The 1.5-kb *Kpn*I-*Bam*HI YEpNUC200 internal *NUD1* fragment was then deleted and replaced with the 3.5-kb YEp213 (Rose and Broach 1990) fragment containing the *LEU2* gene. The resulting plasmid, YEpNUC::LEU2, was digested with the restriction endonuclease *Eco*RV to release a 4.5-kb fragment containing the *LEU2* gene flanked at both ends by *NUD1* gene fragments. This fragment was then transformed (Schiestl and Geitz 1989) into TC106d to replace (Rothstein 1983) the chromosomal the deleted version of the *NUD1* gene.

Disruption of the *NUD1* gene with *LEU2* in several Leu+ transformants was confirmed by polymerase chain reaction (PCR) analysis using primers complementary to specific regions of *LEU2* and *NUD1*. Immuno-blot analysis (Fig. 1, see below) of a

putative *nud1* isolate (TC106dD) confirmed the disappearance of immune-reactive protein. TC106dr52D was constructed by transforming the TC106dD strain (Rothstein 1983) with the 5.6-kb *Sal*I restriction endonuclease-digested fragment of p52Blast (see above). TC107aD is an isogenic derivative (*nud1*) of TC107a. TC106d, TC106dD, TC106dr52, TC106dr52D, TC107a and TC107aD were then transformed with pGHOT-GAL3 to produce TC106dHO, TC106dDHO, TC106dr52HO, TC106dr52DHO, TC107aHO and TC107aDHO respectively. pGHOT-GAL3, a yeast centromere (*CEN4*) vector, is a pUC19-based plasmid containing a *GAL1::HO* transcriptional fusion and carries *TRP1*.

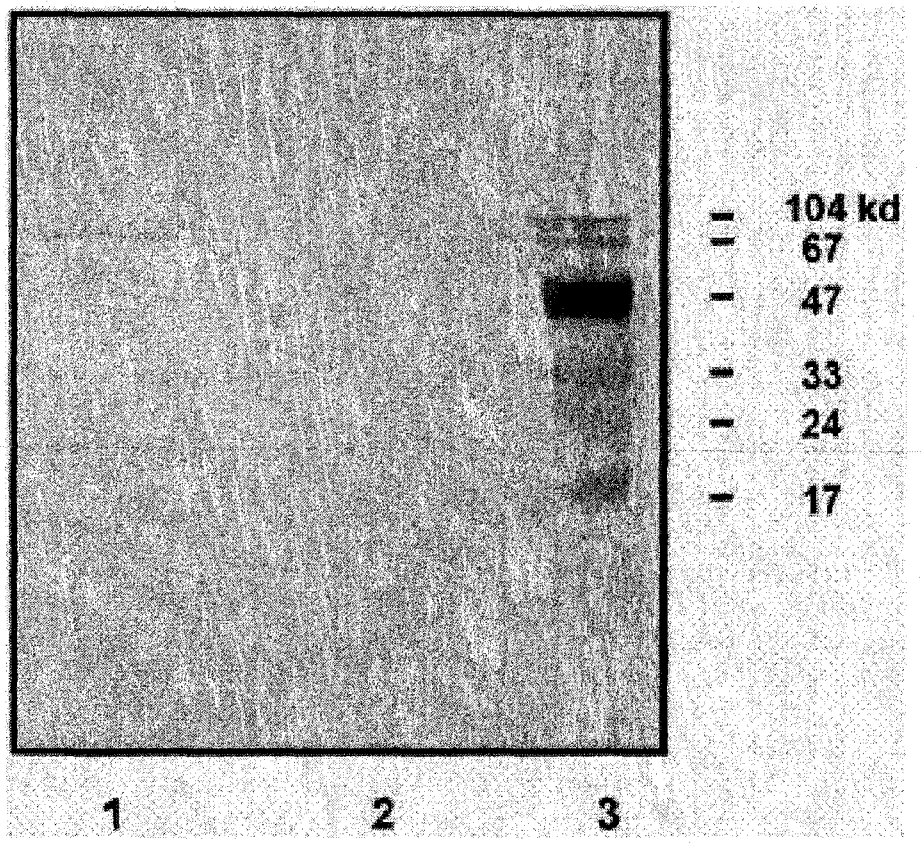


Figure 1. Immuno-blot analysis of *NUD1* inactivation in TC106d using anti-endo-exonuclease rabbit antibody; 100 μg of protein was loaded in each lane. *Lane 1*: TC106d (*NUD1*). *Lane 2*: TC106dD (*nud1*). *Lane 3*: protein standard.

Immuno-blot analysis. Exponentially growing cells in YPD media were harvested by centrifugation in a microfuge tube, washed once, and suspended (108 cells) in 0.02 M Tris-HCl buffer (200 ml), pH 7.5, containing 1 mM phenylmethysulfonyl fluoride. The cells were then lysed by treatment with Lyticase (0.05 U/ml final concentration) for 30 min at 37 °C; glass beads (170–180 mm, 0.4 gm) were added to the cell suspension and then mixed vigorously using a vortex mixer. The combined treatment of Lyticase digestion and of glass-bead mixing ensured a high degree of cell lysis. The cell lysates were collected by puncturing the bottom of the tube with a needle followed by centrifugation (1000 g) into a collecting tube. Each collected cell lysate was then transferred into another microfuge tube and centrifuged for 10 min at maximum speed. The protein concentration of the supernatant (crude cell extract) was determined as described by Bradford (1976). Proteins were then electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The presence of the endo-exonuclease was detected by a rabbit anti-endo-exonuclease antiserum (Chow and Resnick 1987) according to the method described by Liu *et al.* (1995).

Cell survival. Cells grown in synthetic complete (SC) media to a cell titre of 2–4′107 at 30 °C were irradiated with 60Co gamma rays (Theratron 780) with 50, 100, 200, or 400 Gy for *NUD1* and *nud1* strains, while the *rad52* and *rad52 nud1* strains received 50, 100, or 200 Gy. The cells were plated immediately after irradiation on SC agar plates to determine cell survival.

Induction of HO-endonuclease. Overnight-cultures of TC106dHO, TC106dDHO, TC106dr52HO, TC106dr52DHO, TC107aHO, and TC107aDHO were grown in tryptophan omission medium (SC-TRP) or leucine and tryptophan omission medium (SC-LEU-TRP), respectively (Sherman *et al.* 1986) with glucose as the carbon source (expression of the HO-endonuclease is repressed by glucose) to a cell titer of 1 X 10^7 cells per ml at 30 °C. The cells were collected by centrifugation, washed twice with sterile water, and then divided into two equal aliquots and re-suspended in glucose- or galactose-containing tryptophan-omission media (SC-TRP + GLU or SC-TRP + GAL) (1 X 10^7 cells per ml) to induce expression of the HO-endonuclease (Moore and Haber 1996). Aliquots were plated immediately on SC-TRP + GLU for the 0-h time point.

Induction of HO-endonuclease activity was confirmed using conversion of a covalently closed plasmid, containing the *MAT* locus as the substrate for incision by the endonuclease, to the relaxed linear form (data not shown). The remaining cells were incubated at 30 °C, and samples were withdrawn at 2 and 4 h and spread onto SC-TRP + GLU plates. Colonies were counted after 3 days of growth at 30°C. The fraction of viable cells was determined by dividing the number of colonies growing on SC-TRP + GLU plates after exposure to galactose-containing media by the number of colonies growing on SC-TRP + GLU plates that has only been exposed to glucose-containing media. For prolonged (>24 h) expression of HO-endonuclease the cells were spread immediately onto SC-TRP + GLU and SC-TRP + GAL plates. The fraction of viable cells was determined by dividing the number of colonies growing on SC-TRP + GAL by the number of colonies growing on SC-TRP + GLU.

Mating-type determination. Individual colonies emerging after HO-endonuclease treatment were picked and mated to *MATα* and *MAT***a** tester strains, TC111b and TC111c, respectively (Sherman *et al.* 1986). The resulting diploid cells were then plated onto sporulation-medium agar plates, incubated at 30 °C for 2 days, and examined under the microscope for tetrad formation.

Analysis of repair events. Initial analysis of repair events at the *MAT* locus was conducted by the complementation mating-phenotype test (Sherman *et al.* 1986) with TC111b and TC111c. These tests of mating ability were used to distinguish mating proficiency from the sterility phenotype. The sterile survivors and the "**a**-like" mating-phenotype survivors for the *nud1* strain were further analyzed by PCR using the primers L1 (TCTTGCCCACTTCTAAAGCTG) and L2 (TCGAAAGATAAACAACCTCCG) which flank the HO-endonuclease recognition site at the *MAT* locus and yield a PCR fragment of 0.8 kb. The PCR conditions were as described by Moore and Haber (1996) with the following modification. The samples were pre-heated at 95 °C for 3 min and at 80 °C for 5 min before the start of the PCR reaction. This "hot start" pre-treatment before the actual PCR reaction increases the specificity of primer binding to the genomic DNA. The PCR products were then precipitated, re-suspended in 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA, and visualized by electrophoresis on a 1.5% agarose gel with a 1-kb and a 100-bp marker ladder as the size standard.

Statistical analysis. The chi-square test was used to analyze several parameters. A *P*-value of less than 0.05 was considered significant.

Results

The effects of HO-endonuclease expression

In order to investigate the role of the yeast *NUD1* gene product in recombinational DNA repair, we initially tested the resistance of the *nud1* strain to ionizing irradiation (Fig. 2), which is known to induce DSBs. To our surprise, the *nud1* strain showed only a slight sensitivity to ionizing radiation but enhanced cell survival in a *rad52* background. It has been well documented that ionizing radiation induces many different types of DNA damage in addition to DSBs (Natarajan *et al.* 1993; Evans 1994; Lankinen *et al.* 1996; Roldan-Arjona and Sedgwick 1996; Kennedy *et al.* 1997). Therefore, cellular sensitivity to ionizing radiation may not necessarily point to a deficiency in a DSB when an alternative repair pathway is pre-dominant. For this reason, we sought another means of assessing the role of *NUD1* in the repair of DSBs.

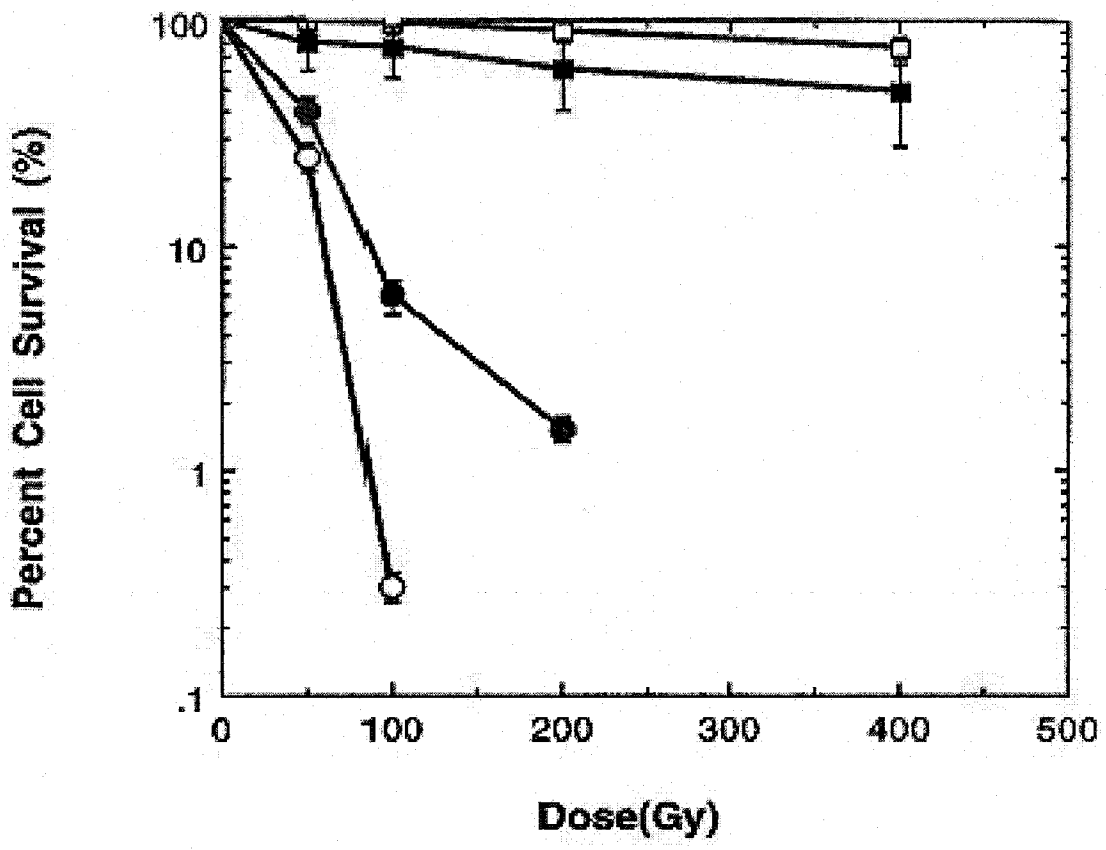


Figure 2. Survival response to gamma irradiation. The symbols are: □ *NUD1 RAD52*, ■ *nud1*, ○ *rad52*, and ● *nud1 rad52*. The error bars represent standard error of the means for four independent experiments.

The yeast plasmid pGHOT-GAL3, containing the selectable marker *TRP1* and the gene encoding the HO-endonuclease under the control of the inducible *GAL1* promoter, was introduced into the *NUD1*, *nud1*, *rad52*, and *rad52 nud1* strains. Transformants were then tested for the ability to grow under conditions of HO endonuclease expression. In agreement with many previous reports (for a review see Haber 1992), the expression of the HO endonuclease seemed to have little effect on the *NUD1* strain. The rate of increase in the number of viable cells of this strain was identical for glucose- or galactose-containing medium (Fig. 3). The surviving fractions of *NUD1* after 2-h and 4-h expression of HO endonuclease were 99% and 95% respectively (Table 1). The prolonged expression (24 h) of the HO endonuclease in the *NUD1* background caused only a small reduction (12%) in the number of colonies that emerged on medium containing galactose relative to the corresponding number on glucose-containing medium (Table 1).

Although the rate of increase in viable cells for the *nud1* strain in glucose medium was similar to that for *NUD1*, the number of viable cells of the mutant strain was markedly decreased in galactose-containing liquid medium (Fig. 3). Furthermore, the survival of the *nud1* strain was steadily reduced in proportion to the duration of HO-endonuclease expression and was 90% after 2 h, 81% after 4 h, and 8% after prolonged expression (Table 1). The effect of the *nud1* single mutant on the DSB repair process in terms of lethality was found to be much less severe than that of the *rad52* mutation (Table 1). Very few surviving colonies (<0.1%) were recovered from the *rad52* mutant strain when a DSB was introduced. Surprisingly, however, the double mutant *nud1 rad52* survived much better than the *rad52* mutant. The number of viable colonies when HO-

endonuclease is expressed in the double mutant was 32% for *nud1 rad52-1* but only 9% for *nud1 rad52::URA3*. This is in contrast to the survival fraction of < 0.1% observed with *rad52* when HO-endonuclease is expressed.

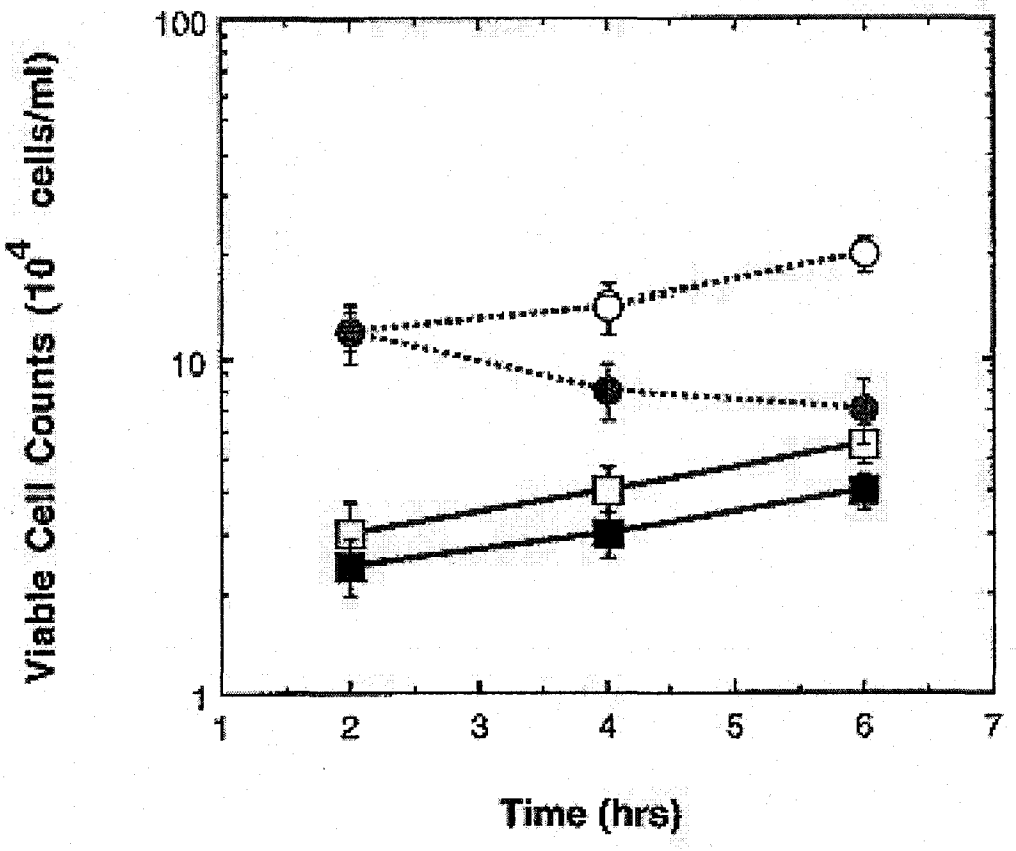


Figure 3. The rate of increase in viable cell counts of *NUD1* and *nud1* in glucose- and galactose-containing media. The values reported are the means ± standard error of three independent experiments. The symbols are □ : *NUD1* in glucose-containing medium; ■ : *NUD1* in galactose-containing media; ○ : *nud1* in glucose-containing medium; ●: *nud1* in galactose-containing media.

Table 1 Effect of short and prolonged periods of expression of HO-endonuclease on cell survival

Strain	Relevant Genotype	Expression period (hrs)	Surviving fraction[a] (±SE)
TC106dHO	NUD1	0	1.00
		2	0.99±0.02
		4	0.95±0.07
		>24	0.88±0.04
TC106dΔHO	nud1	0	1.00
		2	0.90±0.06
		4	0.81±0.06
		>24	0.08±0.02
TC106dr52HO	rad52::URA3	0	1.00
		>24	<0.001
TC107dr52ΔHO	rad52::URA3 nud1	0	1.00
		>24	0.09±0.01
TC107aHO	rad52-1	0	1.00
		>24	0.09±0.01
TC107aΔHO	rad52-1 nud1	0	1.00
		>24	0.32±0.05

[a] The values are the means (±SE) for four independent experiments

Phenotypes of the cells that survived HO endonuclease expression *NUD1* and *nud1* colonies that emerged following induction of HO-expression were characterized with respect to their mating phenotype and ability to sporulate in order to obtain an initial indication of the type of DSB repair event that might have occurred. There are several possibilities as follows (Moore and Haber 1996). The former *MATα* cells may: (1) mate with a *MAT***a** tester strain and the resulting diploid is able to sporulate, indicating that the treated cells retained the original *MATα* locus; (2) mate with a *MATα* tester strain and the resulting diploid is able to sporulate, indicating that the treated cells underwent a mating-type switch; (3) mate with a *MATα* tester strain but the resulting diploid does not sporulate, indicating that the treated cells have suffered a large deletion at the *MAT* locus to give an "**a**-like" mating phenotype; (4) do not mate with either *MATα* or *MAT***a** tester strains but are able to sporulate, indicating that the treated cells underwent a mating-type switch and subsequently conjugated with unswitched cells to form diploids; (5) not mate with either *MATα* or *MAT***a** tester strain and do not sporulate, indicating that the treated cells became sterile. For the same analysis in *MAT***a** cells, we have the following possibilities: (1) mate with *MATα* tester strain and sporulate: retain the original *MAT* locus; (2) mate with the *MAT***a** tester strain and sporulate: mating-type switched; (3) mate with *MATα* but do not sporulate: deletion at the *MAT* locus, and considered as sterile in our scoring (a small alteration or a large deletion can be distinguished by PCR analysis); (4) do not mate with *MATα* tester strain but sporulate: mating-type switched and subsequent conjugation with the unswitched strain to form a diploid.

Table 2 The effect of HO-endonuclease expression on the mating phenotype in *NUD1*, *nud1*, *rad52*, and *rad52 nud1* strains

Strain	Period of expression (hrs)	Mating phenotype			
		MATα	MATa	MATa-like	sterile
TC106dHO (NUD1)[a]	0	28	12	-	-
	2	14	25	1	-
	>24	38	-	2	-
TC106dΔHO(nud1)[a]	0	20	18	-	2
	2	14	20	-	6
	>24	6	-	4	30
TC107aHO(rad52-1)[b]	0	-	40	0	-
	>24	-	8	2	-
TC107aΔHO(rad52-1)[b]	0	-	40	0	-
	>24	-	2	38	-

[a] Original mating type: *MAT* α
[b] Original mating type: *MAT***a**

Of the colonies that we analyzed after short-term induction of HO-endonuclease expression in the *NUD1* strain, the fraction which had undergone a mating-type switch increased from 30% (12/40) to 63% (25/40) (Table 2). After prolonged expression of the HO-endonuclease, however, 95% (38/40) of the surviving colonies exhibited an ∝-mating phenotype and 5% (2/40) had an "**a**-like" mating phenotype (Table 2). Surviving colonies with a sterile phenotype were not detected. Although short periods of induction of HO endonuclease expression in the *nud1* strain resulted in slightly more colonies in the mating-type-switch category at 0 h (45%, 18/40) and slightly less after 2 h (50%, 20/40) compared to the *NUD1* strain, the differences were not statistically significant ($P>0.1$ for both comparisons).

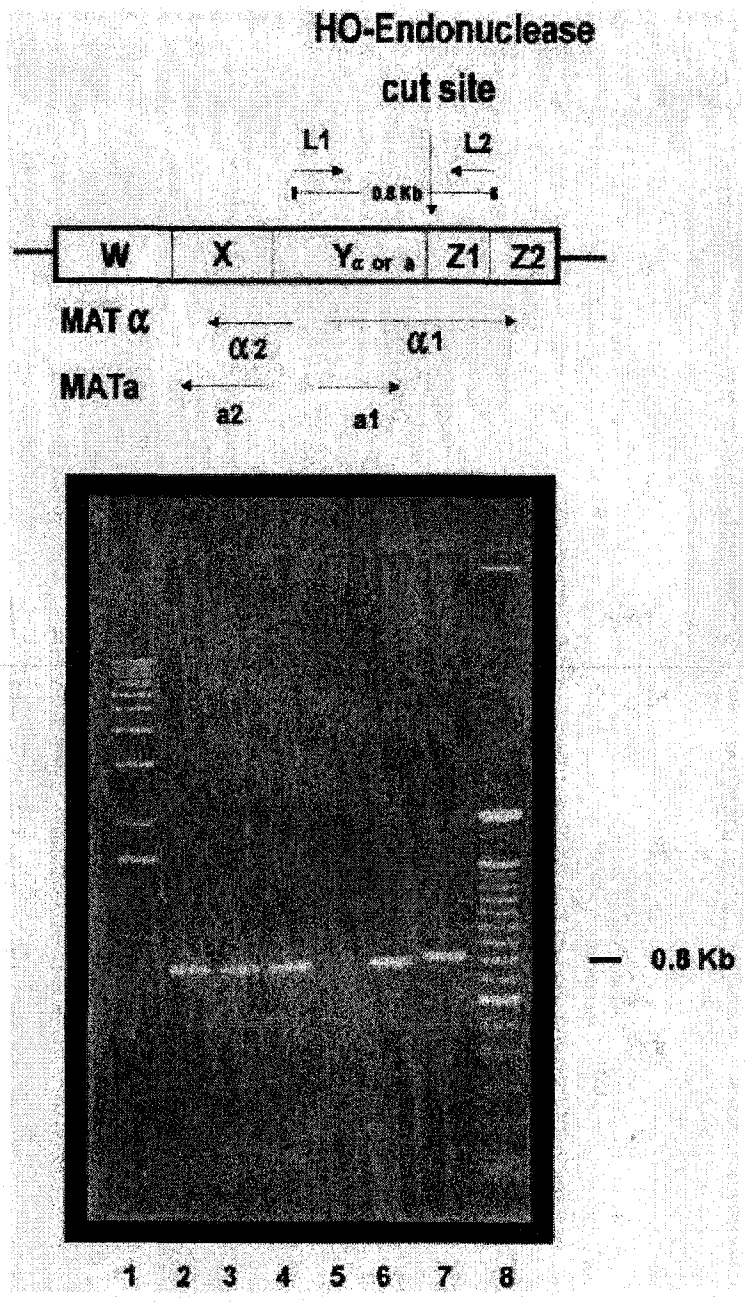
On the other hand, the number of sterile and *MAT***a**-like colonies in the *nud1* background increased with the length of HO-endonuclease expression with 5% (2/40) at 0 h, 15% (6/40) (<0.05) after 2 h, and 85% (34/40) ($P<0.001$) after prolonged expression of the HO-endonuclease. After prolonged expression, 15% (6/40) of the remaining colonies exhibited an ∝-mating type ($p<0.001$, compared to the *NUD1* strain), and 10% (4/40) had an "**a**-like" phenotype, although in this case the difference was not significant ($p>0.2$). We next characterized the mating phenotypes of the survivors after the prolonged induction of the HO-endonuclease in *rad52-1* and *rad52-1 nud1* strains. In these strains, the survivors were either of the **a**- or α-mating type. Since the *rad52* mutant is very sensitive to the induction of DSBs (very few survivors were obtained), we only characterized ten of these survivors with respect to their mating phenotype.

Of these ten survivors, eight mated as *MAT***a** with the resulting diploid cells proficient in sporulation (80%). The remaining two survivors were mated as "**a**-like" but

the resulting diploid cells did not sporulate (20%). On the other hand, a majority of the *rad52-1 nud1* cells that survived induction of HO-endonuclease were "**a**-like" in mating but the resulting diploid failed to sporulate (95%, 38 out of 40), and only 5% retained the **a**-mating phenotype with a proficient sporulation capability (Table 2).

PCR analysis of the *MATα* locus (Fig. 4) showed that the sterile colonies had small deletions, whereas the "**a**-like" survivors had large deletions very similar to the NHEJ events reported in the literature (Moore and Haber 1996).

Figure 4. PCR analysis of the *nud1* colonies that exhibited the sterile and the "**a**-like" mating phenotypes. The expected size of the PCR fragment from an intact *MAT* locus is 0.8 kb. *Lane 1*: 1-kb standard. *Lanes 2–4*: *6* four independent *nud1* sterile survivors. *Lane 5*: a *nud1* "**a**-like" mating-type survivor. *Lane 7*: *NUD1*. *Lane 8* 100 bp standard.


HO-Endonuclease
cut site
L1
L2
0.8 Kb
W
X
Yα or a
Z1
Z2
MAT α
α2
α1
MATa
a2
a1
0.8 Kb
1 2 3 4 5 6 7 8

Discussion

In *S. cerevisiae*, a DSB can be specifically introduced into the genomic *MAT* locus by expression of the HO endonuclease (Haber 1992). Thus, to determine whether the *NUD1* gene product plays a role in DNA DSB repair, we utilized the inducible expression of the HO-endonuclease and the mating-type switching system. The large fraction of mating- proficient *NUD1* cells detected following prolonged expression of the HO-endonuclease indicates that the recombinational repair process is very efficient in the *NUD1* background. In contrast, prolonged expression of the HO-endonuclease killed most of the *nud1* cells and altered the *MAT* locus in more than 85% (34/40) of the survivors (Tables 1 and 2). These observations argue that the capacity for recombinational repair is greatly reduced in the *nud1* strain and that the *NUD1* gene product is involved in the repair of DSBs at the *MAT* locus. The large surviving fraction in the *NUD1* strain when HO endonuclease is expressed, as compared to the much lower value (0.2%) reported by Moore and Haber (1996), may be due to the intact *HML* and *HMR* loci in our strains allowing mating-type switching events to occur. The strains used by Moore and Haber (1996) lacked these loci.

A large body of data suggests that the genes involved in the repair of DSBs elsewhere in the genome are also required for the mechanisms that repair the DSB at the *MAT* locus (Haber 1992; Kramer *et al.* 1994). These processes include the NHEJ (Mézard *et al.* 1992; Mézard and Nicolas 1994; Moore and Haber 1996) and SSA (Fishman-Lobell 1992) modes of DSB repair. The majority of colonies that emerged following prolonged expression of the HO-endonuclease in the *nud1* background were sterile, with the remainder exhibiting an α or "**a**-like" mating type (Table 2). This sterility

was due to a small deletion within the *MAT* locus (Fig. 4), reminiscent of the NHEJ events observed by Moore and Haber (1996). NHEJ events in wild-type *S. cerevisiae* are due to a minor pathway of DSB repair which only becomes dominant if the cell acquires a defect in the main recombinational repair pathway (Kramer *et al.* 1994). Thus, our results suggest that *NUD1* functions in the homologous mode of recombinational repair of DSBs.

Interestingly, following prolonged exposure of *NUD1* to HO endonuclease induction, no *MAT***a** colonies were detected whereas a 2-h exposure increased the fraction of *MAT***a** colonies by two-fold. This discrepancy may be due to experimental differences for the short versus long exposure time points. After short exposure to HO endonuclease induction, the cells were plated onto glucose-containing medium and formed colonies on this medium. On the other hand, cells that were exposed for >24 h to HO endonuclease formed colonies directly on galactose-containing medium. It is possible that, when cells were incubated in galactose-containing liquid medium for periods of time and then plated on glucose-containing agar plates, one round of mating-type switch occurs thus resulting in the recovery of *MAT***a** cells. In contrast, by plating the cells directly onto galactose-containing agar plates, the activity of the HO endonuclease may maintain the presence of a DSB at the *MAT* locus and therefore force repair of the break by a simple ligation process which may favor restoration of the original mating type. Such end-end ligation of the DSB was reported by Boulton and Jackson (1996a, b). If this scenario is correct, then the appearance of a large fraction of sterile colonies among the survivors after prolonged exposure of *nud1* to HO-endonuclease suggests that the *NUD1* gene product may be required for end-to-end ligation.

An intriguing observation with *nud1* is its ability to confer resistance to HO-endonuclease-induced lethality in *rad52* cells (Table 1) and, to a lesser extent, to reduce the ionizing radiation sensitivity of *rad52* (Fig. 2). The increase in survival of *rad52 nud1* mutants appears to be mainly due to an increase of NHEJ-like events in these cells. Almost all of the survivors (95%) were altered with respect to their *MAT* locus. The apparent suppression of *rad52* sensitivity to DSBs by *nud1* suggests that *NUD1* is acting at an earlier step in the homologous recombination repair of DSBs than is *RAD52*. The results presented here show that the *NUD1* gene product to be required for efficient DNA repair via the *RAD52*-dependent pathway. The lethality observed with the *nud1* strain in the presence of a DSB can be explained on the basis that the action of the *NUD1* gene product may be directed at the break itself. Thus, a DSB that has not been processed by Nud1 before proceeding to repair via the *RAD52*-dependent recombination pathway is only inefficiently repaired. In the absence of the *NUD1* gene product, the repair of the DSB is directed toward the NHEJ pathway. On this basis, the majority of *nud1* cells that survive exposure to the HO endonuclease would do so because the DSB is processed via another mechanism (perhaps the NHEJ process) that produces a deletion encompassing the HO endonuclease cutting site. These cells then survive subsequent exposures to the HO endonuclease since a DSB can no longer be introduced at the altered *MAT* locus. In this case, the price for survival is sterility.

Perhaps another interesting observation of our experimental results is the differing cell survival rates between *rad52-1 nud1* and *rad52::URA3 nud1* when HO endonuclease was induced (Table 1). The survival fraction of *rad52–1 nud1* is much higher than that of *rad52::URA3 nud1* (32% vs 9%). This difference may indicate that the Rad52-1 protein

retains some biological activity, which contributes to the higher cell survival. The exact nature of this biological activity is unknown but our results point to a possible role in the NHEJ process. Further dissection of the mechanism of action of the *NUD1* gene product in conjunction with the *RAD52* gene and other genes of the *RAD52* epistasis pathway or the NHEJ pathway will provide important additional information concerning the sequence of events (recombination or NHEJ) occurring during the processing of DNA DSBs. These experiments are currently in progress.

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PREFACE

In chapter II, we described a series of genetic assays aimed at determining the sensitivity of the *rnc1* mutant to DSB damage and specifically to identify interactions at the genetic level between *RNC1* and *RAD52*. We determined that the *RAD52* gene is epistatic to *RNC1*. This is evident in the manner in which lethality of DSB induction in the *rad52* mutant is partially suppressed in the presence of an *rnc1* mutation. The following chapter confirms the epistatic relationship between *RNC1* and *RAD52* via MMS survival assays and also examines genetic interactions between *RNC1* and *Ku80*, a component of the NHEJ pathway. We also set out to characterize the *in vivo* and *in vitro* enzymatic properties of RNC1 and interpret its role in DSB repair processes in *S. cerevisiae*.

Chapter III

Functional analysis of the yeast endo-exonuclease: Implications of its involvement in double-strand break repair in *Saccharomyces cerevisiae*

Abstract

The *RNC1/NUD1* endo-exonuclease was cloned into a calmodulin binding peptide (CBP) expression vector and purified on a calmodulin affinity column. The purified protein possesses properties characteristic of previously isolated endo-exonucleases as it has a highly processive 5' → 3' exonuclease activity on double-strand (ds) DNA, and endonuclease activity on single-strand (ss) DNA. We show that the endo-exonuclease is highly localized to the nucleus when expressed in yeast as a fusion protein with GFP.

The *rnc1/nud1* mutant is sensitive to DSB induction by HO endonuclease. This mutant phenotype can be rescued by expressing *RNC1/NUD1* on a multicopy plasmid. The *rnc1/nud1* mutant displays low sensitivity to MMS treatment. However, while the *rad52* strain is normally highly sensitive to DNA damage, the *rad52* lethality is suppressed in a *rnc1/nud1* background. Furthermore, we observed a synergistic effect for the lethality of double-strand breaks in the *rnc1/nud1* strain when the *ku80* gene is knocked out; whose gene product is critical in the non-homologous end-joining pathway (NHEJ). These results indicate that *RNC1/NUD1* acts in a competing repair pathway separate from NHEJ, and in the same homologous recombination pathway as *RAD52*. Based on their epistatic relationship, RNC1/NUD1 probably acts at an earlier step in the homologous recombination repair pathway than RAD52.

Keywords: *RNC1*, endo-exonuclease, DSB repair.

1. Introduction

Endo-exonucleases are nucleases with bifunctional roles *in vivo*. They display an exonuclease activity on linear double-strand DNA which may be vital for recombination and recombinational DNA repair, in addition to an endonuclease activity on single-strand DNA that may contribute to degradation of the genome during apoptosis. Furthermore, a protective function has been ascribed to the *Escherichiae coli* recBCD endo-exonuclease in destroying the DNA of invading bacterial viruses once they have been cleaved by host restriction endonucleases [1].

The recBCD nuclease (also known as exonuclease V), is the most well-characterized endo-exonuclease to date, having consistently been shown to be critical for recombinational repair of double-strand breaks (DSBs) in *E. coli*. *In vitro*, recBCD degrades linear duplex DNA to oligonucleotides at incredible speed [2]. *In vivo*, it degrades bacteriophage DNA cut by the host restriction systems, and in *recA* mutants, it degrades the entire chromosome after it was fragmented as a result of DNA damage.

The recBCD enzyme displays ATP-stimulated endonuclease activity on ssDNA [3,4] and exonuclease activity on dsDNA [2,3]. The purified B and C subunits have been implicated in DNA helicase activity while recD confers the nuclease activity of recBCD [5,6]. By virtue of this nuclease activity, recBCD is thought to catalyze the recA recombinase-mediated processing of DNA to the final products of recombinational exchange [7]. While recBCD does not generally have endonuclease activity on duplex DNA, it does cleave linear duplex DNA containing chi sites in *E. coli* and bacteriophage lambda DNA [8]. Other bacteria have very similar ATP-dependent recombination endo-exonucleases [3].

One of the earlier eukaryotic endo-exonucleases was isolated from the mitochondria of *Neurospora crassa* [9]. A 37 kDa mitochondrial nuclease was also isolated from *Saccharomyces cerevisiae* encoded by the *nuc1* gene [10]. This nuclease and several others from other species show cross-reactivity to a rabbit polyclonal antibody raised against the *Neurospora crassa* endo-exonuclease. These include nucleases from *Ustilago maydis* [11], *Aspergillus nidulans* [12] and *Coprinus cinereus* [13]. The same antibody shows cross-reactivity to recBCD nuclease [14].

The yeast endo-exonuclease was first isolated by immunoaffinity chromatography using the *Neurospora crassa* polyclonal antibody [15]. The gene encoding the endo-exonuclease was initially named *NUD1* in previous publications, but since then it has been classified as *RNC1*, as *NUD1* denotes the gene symbol of another unrelated gene in the gene registry. The same antibody was utilized to identify the gene from a yeast genomic DNA library in *λgt11* expression vector [16]. The gene was initially thought to possess a *rho*-associated GTP-binding domain, but subsequent analysis revealed this domain to be a result of a cloning error due to the deletion between adjoining genes *RHO4* and *RNC1*, resulting in a RHO4-RNC1 fusion protein [17]. The *RNC1* gene is located on chromosome XI and encodes a 70 kDa monomeric protein[16]. In the *rad52* mutant, which is deficient in DSB repair and homologous recombination, it was discovered that the endo-exonuclease was expressed at a level of only 10% of that observed in wild-type *S. cerevisiae* [15]. In addition, the activity of the endo-exonuclease increases during meiosis [18]. Overexpressing the *RNC1* gene on a multi-copy plasmid resulted in increased cell survival following gamma irradiation and an increase in spontaneous and radiation-induced mitotic recombination between duplicate genes [19].

Taken together, these results indicate a role for the *RNC1* endo-exonuclease in DSB repair and both mitotic and meiotic recombination.

Recently, evidence has come to light that the *RNC1* gene product exhibits methyltrasferase activity responsible for modifying elongator tRNA at position 54 (m^5U_{54}) . In the same publication, RNC1 is expressed and purified as a fusion protein with a 30 kDa glutathione-S-transferase (GST) tag. The fusion protein was demonstrated to show little or no nuclease activity. However, the GST moiety was not cleaved, and may have interfered with the *in vitro* activity of RNC1.

Here we report the purification of RNC1 as a fusion protein with a 4 kDa calmodulin binding peptide (CBP) tag in *E. coli*, and show that RNC1 possesses the enzymatic properties characteristic of previously isolated endo-exonucleases. We also present *in vivo* data which suggests that RNC1, like recBCD, is responsible for processing DSB ends during homologous recombination repair. Furthermore, MMS survival assays with the *rnc1* mutant in a *rad52* background corroborate previous studies that indicated that the lethality of DSB damage in *rad52* mutants can be suppressed in an *rnc1* background, whereas the opposite effect is observed in the *ku80 rnc1* double mutant; which is hypersensistive to DSB damage: a phenotype reminiscent of the *ku70 rad52* double mutant [20-22].

2. Materials and methods

2.1. Yeast strains.

The isogenic yeast strains used in this study are: *RNC1* or wild-type (*WT*) (*MATα leu2-3, 112, trp1-289, ura3-52, his1-7*), *rnc1* (identical to *WT* but the *RNC1* gene has

been disrupted with a *LEU2* marker gene), *ku80* (identical to *WT* but *Ku80::URA3*), *ku80 rnc1* (identical to *ku80* but *RNC1/NUD1::LEU2*), *rad52* (*RAD52::URA3*), *rad52 rnc1* (same as *rnc1* with *RAD52::URA3* knockout), TC111b (*MAT**a**, α leu2-3, 112, trp1-289*), and TC111c (same as TC111b but *MATα*). The *trm2* strain was constructed by disruption of the carboxyl terminal of the *RNC1* gene at the *BamH*I site with *HIS3*. The plasmid pRS316-*RHO4-trm2::HIS3* used for this construction was provided by Nordlund *et al.* [23]. All the above strains were derived from the SK-1 background [24]. The *ku80* strain was constructed by transforming the WT strain [25] with a 3.5Kb fragment from an *SphI* and *SpeI* digest of *pKu80::URA3* (provided by Steve Jackson, CRCI). This fragment contains the *URA3* marker gene flanked by the 5' upstream and 3' downstream regions of the *Ku80* gene. The *ku80 rnc1* strain was constructed in a similar fashion from *rnc1* [26]. The *mre11::HIS3* disruption strain was generated by transforming a 5.0 Kb fragment liberated from pCRscript*MRE11::HIS3* into the *WT* strain. The plasmid pCRscript*MRE11::HIS3* was obtained from Simon Boulton, CRCI, and has the ORF of the *Mre11* gene disrupted by insertion of the *HIS3* marker into the *Stu*I site [27]. The yeast strain DRY214 (provided by Dr. Dindial Ramotar, Maisonneuve-Rosemont Hospital) used for expression of the GFP-RNC1 fusion protein is isogenic to the parent strain FY86 (*MATα , his3Δ200, ura3-53, leu2Δ1*) but *Imp2::LEU2*. All strain knockouts were confirmed by hybridization and phenotypic analysis.

2.2. Media and growth conditions.

Yeast extract, peptone and dextrose (YPD) media and minimal synthetic complete (SC) media were used for growth of *S. cerevisiae* [28]. Medium for induction of HO

endonuclease contained 2% (w/v) galactose to replace glucose as a carbon source. SC medium without tryptophan (SC-TRP) was used to maintain the plasmid-borne *GAL1::HO* fusion gene.

2.3. Methyl Methanesulfonate (MMS) Survival.

Overnight cultures were grown to early log phase in YPD at 30 C. Serial dilutions of ~10^6 –10^2 cells/ml were plated onto YPD plates containing 0.005% MMS and grown a further 2 days at 30 C. Yeast strains bearing a *rad52* null knockout were plated onto YPD medium containing 0.001% MMS.

2.4. UV irradiation.

Cells were grown overnight in SC media and plated onto selective media at approximately 1 x 10^3 cells/plate. After drying for 10-20 minutes, plates were inverted over a UV box (312 nM (UVB) variable intensity, BioRad, CA, USA), aligned directly over the center of the UV lamp and irradiated with varying doses of UV radiation at a dose rate of 3 KJ/m^2/min. Following irradiation, plates were incubated under non-photoreactivating conditions over a period of 2-3 days at 30°C then subjected to a colony count.

2.5. Cell survival following induction of HO-endonuclease.

The strains under analysis were transformed with the plasmid pGHOT-GAL3 (provided by Michael Fasulo, Albany Medical College) bearing a *GAL1::HO* fusion gene. Cultures were then grown in selective media with glucose as the carbon source

(expression of HO endonuclease is repressed by glucose) to early log phase at 30°C. Cell titers were determined by cell count on a hemocytometer. The cells were collected by centrifugation, washed twice with sterile water, then divided into two equal aliquots and re-suspended into control glucose synthetic complete (SC) medium or galactose-containing SC medium to induce expression of HO endonuclease [29]. Aliquots were diluted and plated immediately onto glucose selective medium for the 0-hr time point. The remaining cells were incubated at 30 °C, samples were withdrawn at 2, 4 and 6 hour time points from cultures in galactose and control glucose media, diluted and spread onto glucose selective medium. Colonies were counted after 3 days growth at 30 °C. The fraction of viable cells was calculated by dividing the number of colonies growing following exposure to galactose-containing medium by the number of colonies growing following exposure to control glucose medium. For prolonged (>24hrs) expression of HO-endonuclease cells were spread directly onto selective medium with galactose as the carbon source and grown for a period of 3 days. Induction of HO endonuclease was confirmed using conversion of a covalently closed plasmid MATα-Ycplac33, containing the *MAT* locus as the substrate for incision by the endonuclease, to the relaxed linear form (data not shown).

2.6. Analysis of repair events.

Analysis of the fidelity of repair events was initially performed with the complementation mating-phenotype test [30]. Individual colonies emerging from HO-endonuclease treatment were picked and mated to *MATα* and *MATa* tester strains, TC111b and TC111c respectively. Diploid colonies were selected for in these crosses

using tryptophan and histidine omission SC medium. The resulting diploids were then plated onto sporulation medium agar plates, incubated at 30°C for 3 days, and examined under a microscope for tetrad formation. These tests were used to distinguish between mating proficient and sterile phenotypes [26]. The *MAT* locus of sterile survivors was amplified by PCR and sequenced by dideoxynucleotide chain termination method [28] using α-^{32}P end-labeled PCR (*f*mole; Promega). The primers used were MAT Z1 (TCGAAAGATAAACAACCTCC) and MATα2 (TCTTGCCCATTCTAAGCTG) to generate an 800 bp fragment. The $MgCl_2$ concentration used was 1.5 mM; the reactions were carried out for 34 cycles of 95°C for 1.5 minutes, 48°C for 2 minutes, and 72°C for 3 minutes [29]. The final cycle was followed by an extension step at 72°C for 7 minutes. The PCR products were visualized by electrophoresis on a 1.5% agarose gel with 1-kb and 100-bp marker ladders as the size standards, and purified before sequencing with the Prep-a-gene DNA purification matrix (Biorad).

2.7. Plasmids and cloning methods

The plasmid pGHOT-GAL3 was used for inducible expression of HO endonuclease. pGHOT-GAL3, a yeast centromere (CEN4) vector, is a pUC19-derived plasmid containing a *GAL1::HO* transcriptional fusion and carries a selectable *TRP1* marker.

For expression and purification of RNC1, a 2 kb fragment of *RNC1* cDNA [17] was subcloned into the pCAL-n-FLAG vector (Stratagene), with ligation-independent cloning (LIC) using the forward primer 5'-GAC GAC GAC AAG GTT TAG GAC GAA TTC ATT ATG TAC -3' beginning at position 1582, and the reverse primer 5'- GGA

ACA AGA CCC GTG TCG ACA TTT ACT CTA GAA AGA TAT ACA T–3' at position 3598 on the *RNC1* sequence. The pCAL-n-FLAG vector allows fusion of the CBP purification tag followed by the FLAG epitope, to the N-terminus of RNC1.

RNC1 was also cloned into a GFP vector bearing a *GAL1::GFP* transcriptional fusion (obtained from Dr. Dindial Ramotar); pGAL::GFP-RNC1 and a *URA* selectable marker. An *EcoRI* cut site was introduced by oligonucleotide-directed mutagenesis [31] at position 1582 using the forward primer 5'-GTT TAC GAC GAA TTC ATT ATG-3'. The same forward primer as noted above was used in conjunction with the reverse primer 5'-CTT TAC ATA GAG TCT TAA GTA-3' which is complementary to the *RNC1* sequence beginning at position 3605 and was downstream of an *Xba*I cut site.

2.8. Purification of the RNC1 endoexonuclease.

The CBP affinity tag is based on the high affinity for calmodulin (CaM), of a 26-amino-acid C-terminal fragment from muscle myosin light-chain kinase, at physiological pH in the presence of calcium. When calcium is removed from the environment, immobilized CaM undergoes a conformational change, releasing the affinity-tagged fusion protein. To express the CBP-RNC1 protein, the bacterial strain BL21-Gold (Stratagene) was transformed with the plasmid pCAL-RNC1. A 0.5 L volume of LB containing 100μg/mL ampicillin was inoculated with the transformed strain and grown to OD_{600} = 1.0 at 37°C, chilled on ice to 15°C prior to adding IPTG to a final concentration of 1mM. After induction for 4 hours at 15°C, the cells were harvested and lysed by treatment with lysozyme at a concentration of 10μg/ml of LB culture followed by 10 freeze-thaw cycles in an isopropanol/dry-ice bath. Cell debris was removed by

centrifugation at 48,000 x g for 10 min. The crude protein extract was bound at 4°C overnight to the CaM affinity resin. The resin was then washed extensively with binding buffer containing 2mM calcium chloride until there was no detectable A_{280} – binding material in the final washes. Subsequently, the fusion protein was eluted with elution buffer containing 2mM EGTA. Protein concentrations were determined using the Bradford assay [32]. Purification of the fusion protein was assayed SDS-PAGE analysis of the eluted protein (Fig. 1). SDS-PAGE was performed according to the method of Laemmli [33] with 4% (w/v) polyacrylamide in the stacking gels and 10% (w/v) polyacrylamide in the resolving gels. Proteins were stained with 0.5% Coomassie Brilliant Blue R-250.

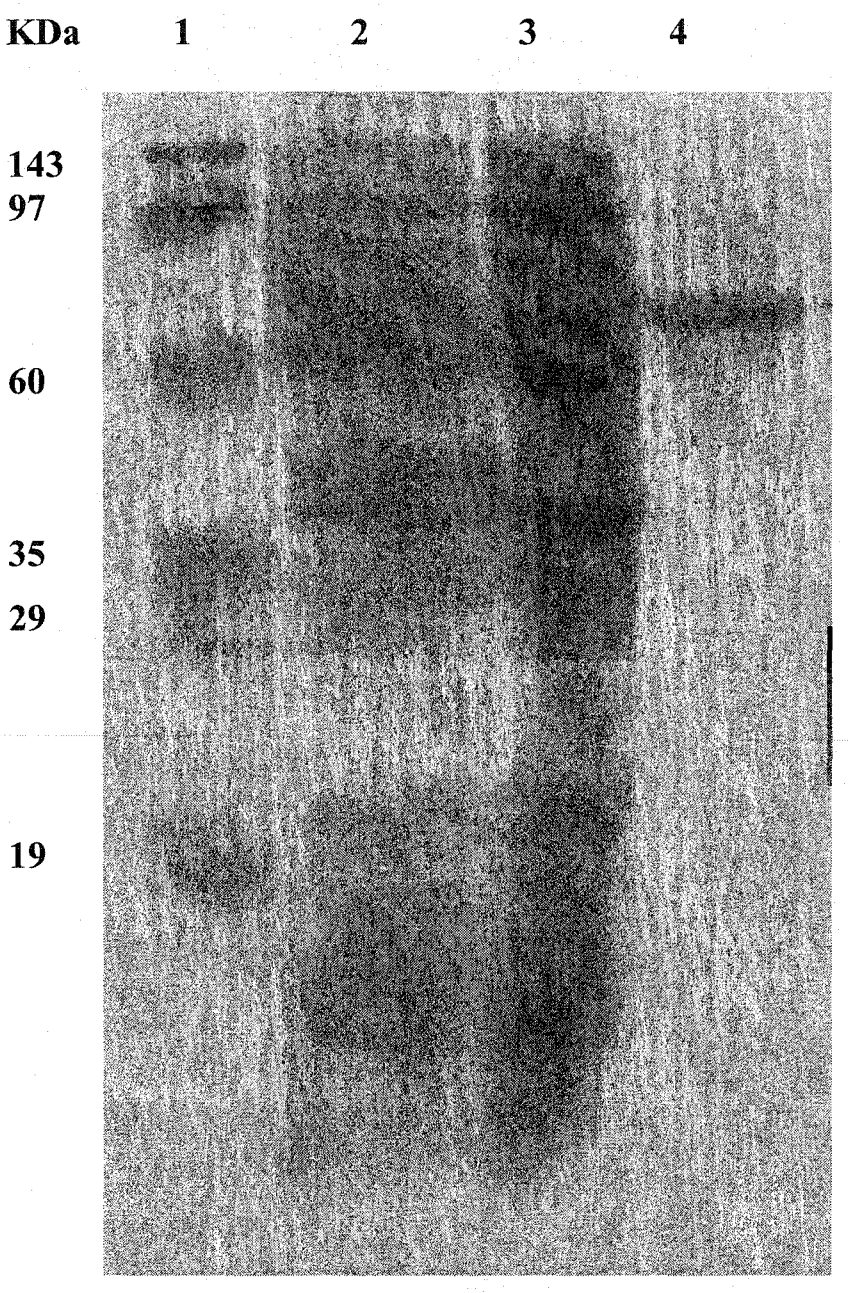


Figure 1. SDS-PAGE analysis of affinity-purified protein. Protein samples were electrophoresed on 10% SDS-PAGE and stained with Gelcode blue stain Reagent (Stratagene) for analysis. Lane 1: Molecular weight marker (Bio Rad). Lane 2: Uninduced BL21 cells boiled in SDS-PAGE sample buffer. Lane 3: Crude extract from BL21 E coli cells induced in 1 mM IPTG. Lane 4: 30 μg CBP-RNC1 protein purified via HPLC using calmodulin affinity resin.

2.9. Nuclease activity assay.

The ssDNase activity of RNC1 was assayed by measuring the release of acid-soluble radioactivity from heat-denatured α-^{32}P-labelled pUC19 DNA labeled to a specific activity of 2-3 X 10^4 cpm/μmole according to the method described by Chow and Resnick [15]. In a reaction volume of 600 μl of 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$ and 50 μg of CBP-RNC1, 2 μg of α-^{32}P-labelled pUC19 DNA was heat-denatured at 95°C for 5 min prior to being added to the reaction. The reaction was incubated at 37°C and samples of 100 μl were withdrawn at time intervals to be stopped with 150 μl of ice-cold trichloroacetic acid. The efficiency of DNA degradation was quantitated by measuring the release of acid-soluble radioactivity. The above experiment was also carried out using the GFP-RNC1 expressed and purified from the *S. cerevisiae* strain *rnc1* by immunoprecipitation using the GFP monoclonal antibody immobilized on protein A-sepharose and washed in 20 mM Tris-HCl pH 7.5, 1mM EDTA. 1 mM PMSF, 100μM pepstatin A, 10μM leupeptin and 10% glycerol.

Double-strand DNase activity was assayed with linearized plasmid DNA obtained by treating pUC19 DNA with either *Sma*I or *Eco*RI (Pharmacia Biotech) followed by phenol extraction and ethanol precipitation. 10μg of CBP-RNC1 was incubated with 5μg of linear plasmid DNA in a reaction volume of 60μl of 100 mM Tris-HCl, pH 8 and 10 mM $MgCl_2$. Degradation of unlabelled DNA substrate was visualized by directly running samples on 0.8% agarose gel and staining with ethidium bromide. Samples were prepared by obtaining 10μl aliquots from incubation times of 0, 10, 30 and 60 minutes and terminating the reaction by adding 2μl of loading buffer (100mM EDTA, 50mM Tris pH

8.0, 0.25% Bromophenol Blue, 0.05% SDS and 15% Ficoll). The same reaction conditions were used in assaying the degradation of 5'-labelled linear double-strand plasmid DNA. 5' end-labelling was performed using T4 polynucleotide kinase (Promega) and [γ-^{32}P] ATP. Samples were run under denaturing conditions in 10% acrylamide and 7 M urea. After the run, the gel was analyzed by autoradiography using Biomax-MR autoradiograph film (Interscience).

2.10. DNA slot blot analysis of ssDNA formation.

In order to monitor the *in vivo* activity of the *RNC1* gene product, it was necessary to quantitate the formation of ssDNA formation directly at the HO endonuclease cleavage site. This was performed by a DNA slot blot assay based on Southern hybridization analysis as described by Sugawara *et al.* [34]. Wild-type, *rnc1*, *rad52* and *rad52 rnc1* strains were transformed with the plasmid pGHOT. Overnight cultures were prepared, washed in sterile water, and resuspended in SC medium containing 2% galactose. Samples were obtained from time intervals of 0, 30 and 60 minutes, spun down and resuspended in 10X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate). After the 60-minute timepoint, the cultures were spun down and resuspended in an equal volume of SC medium containing 2% glucose solution. Additional samples were obtained from the 90 and 120-minute timepoints. Genomic DNA from the time course samples was prepared by the SDS-potassium acetate method of Sherman and colleagues. [35].

Time course genomic DNA was directly blotted onto nylon membranes without denaturation, using the Bio-Dot microfiltration Apparatus (Biorad). The membrane was probed with a 24-mer ssDNA oligonucleotide probe 5' GAA GTA GTC CCA TAT TCC

GTG CTG 3' which is specific for the 3' sequence of the *MAT Ya/Z* junction, proximal to the HO cut site. The right side of the DSB site was chosen as it was previously demonstrated that this is primarily where ssDNA formation occurs [34,36]. As positive controls, the same genomic DNA samples were denatured and bound to the membrane, using only 1/5 the amount used in the native samples.

3. Results:

3.1 Specific DSB induction using HO endonuclease.

Specific DSB induction was performed by the inducible expression of HO endonuclease. In order to control the period of induction in the strains that we were analyzing, we transformed them with the plasmid pGHOT-GAL3. Cells in early log phase were then tested for the ability to grow under conditions of HO expression in 4 independent experiments. Data for the wild-type and *rnc1* strains has previously been published [26], but have been included for comparison. The wild-type strain consistently shows a high level of efficiency in repairing DSBs over increasing durations of DSB induction; from 99% at 2 hours, 95% at 4 hours, 93% at 6 hours and 88% at >24 hours induction (Table 1).

Table 1. Percent Survival following HO induction.

Period of HO endonuclease induction (hrs)[a]	Strains				
	WT	*rnc1*	*rnc1*+ pRNC1	*ku80*	*ku80 rnc1*
2	99.0 ± 0.5[b]	89.8 ± 3	91.8 ± 4.4	86 ± 3	40 ± 3
4	95.1 ± 2	76.0 ± 3.6	85.8 ± 3.3	73.5 ± 1.3	6 ± 0.8
6	92.9 ± 1.3	57.2 ± 4	78.7 ± 3.6	60.5 ± 4.2	3 ± 0.8
>24	87.6 ± 4.5	9.1 ± 2.6	32 .0± 4.7	38.3 ± 3.2	0.12 ± 0.02

[a] HO endonuclease expression was induced by suspending cells in liquid galactose medium for 2 to 6 hours as indicated. To stop HO endonuclease induction, cells were plated directly onto glucose medium. At the >24 hours time point, cells were plated directly onto solid galactose medium, and colonies counted within 2-3 days once visible colonies were obtained.

[b] Values represented are the means (±SE) for four independent experiments.

Over a range of induction of 2 to 6 hours it is apparent that the *ku80* and *rnc1* mutants have similar survival rates; there is a trend of increasing sensitivity of the *rnc1* mutant with increasing persistence of DSB induction. A two-sample student's T-test analysis of the means from the two strains did not yield statistically significant differences in sensitivity to DSB induction from 2 - 6 hours of HO endonuclease induction, with p-values greater than 0.1 (A p-value of less than 0.05 was considered significant). However, at the >24hr time-point, the average survival rate of *ku80* is 38%, while that of *rnc1* is only 9% (Table 1). T-test analysis of these means showed that the survival of the *rnc1* strain is significantly lower than that of the *ku80* mutant at the >24hr timepoint ($p < 0.05$). The results seem to indicate that the *rnc1* strain displays a much higher increase in sensitivity relative to the *ku80* strain with increasing persistence of DSB induction. The *ku80 rnc1* double mutant shows sensitivity much higher than that of either the *ku80* or *rnc1* single mutants. At 2 hours induction, the strain exhibits a 40% mean survival rate, falling to 6% at 4 hours, 3% at 6 hours and 0.12% at >24 hours.

The *rnc1* strain was transformed with pGFP-RNC1 and transformants selected for in uracil and tryptophan (in order to maintain the HO-bearing plasmid) omission medium. As can be observed in Table 1, co-expression of GFP-RNC1 with HO endonuclease by induction in 2% galactose medium partially restores the wild-type phenotype of the *rnc1* strain. Full restoration of wild-type phenotype may not be possible; as it is probable that over-expression of GFP-RNC1 may have some deleterious effect on the cell as a direct consequence of its nucleolytic activity. In a separate experiment, *rnc1* mutants bearing the GFP-RNC1 plasmid were incubated 6 hours in selective galactose medium versus control selective glucose medium, then plated onto nutrient-rich glucose medium. Cells

which had overexpressed *RNC1* consistently showed a 12% reduction in growth (data not shown).

Previously, we reported that the *rad52* mutant in an *rnc1* (referred to as *nud1* in that publication) background shows a decreased level of sensitivity as compared to the *rad52* single mutant [26]. As such, it appears that *RNC1* is epistatic to *RAD52*. These findings, taken together with the fact that a *ku80* mutation hypersensitizes the *rnc1* phenotype, seem to indicate that *RNC1* operates in the same pathway, but in an earlier step than, *RAD52* and in a complementary pathway to *KU80*.

3.2. Analysis of repair events.

In order to examine the fidelity of the repair events occurring in the mutants, we analyzed the mating phenotype of the survivors following prolonged induction of HO endonuclease. Survivors from cells plated directly onto galactose medium were crossed with tester strains TC111b and TC111c. Survivors from 2 to 6 hour induction generally showed negligible sterility (data not shown). With persistent DSB induction (>24 hrs), however, the picture changed. As shown in Table 2, wild-type cells displayed a high fidelity of repair, while three-quarters of *rnc1* survivors analyzed were sterile. Sterility in *ku80 rnc1* and *rad52 rnc1* survivors closely paralleled that for *rnc1* single mutants, and *ku80* survivors showed minimal sterility, while *rad52* survivors showed the highest rate of sterility.

Table 2. Sterility rates in survivors of persistent DSB induction

Strain	Percentage of Sterility
Wild-type	1% (1/70)
rnc1	75% (45/60)
rad52	50% (20/40)
rad52 rnc1	60% (24/40)
ku80	9% (5/64)
ku80 rnc1	73% (34/47)

Sterile survivors from all strains were selected and the *MAT* locus was amplified. PCR products were analyzed by agarose gel electrophoresis and sequenced for mutations at the HO endonuclease cut site. 78% (14/18) of the *rnc1* mutants possessed discernible mutations- all but 2 involved small insertions/deletions at or near the HO endonuclease cut site (Table 3). A +CA insertion was most common among *rnc1* survivors. Of the *ku80 rnc1* mutants, 15/24 or 62% sustained direct mutations at the cut site, of which 14/24 involved base deletions and a mere 1/24 consisted of a base substitution. A single –G deletion was the predominant mutation (10/24) in *rnc1 ku80* mutants. Only 5 of 12 sterile *rad52* survivors possessed discernible mutations at the HO cut site. Sequence analysis of the *ku80* sterile mutants indicated that 71 %, or 10/14, possessed mutations directly at the HO cut site. Unlike the mutation spectra of *rnc1* mutants, all the mutations in the *rad52* and *ku80* mutants involved base substitutions. This indicated that an alternate mechanism of DSB repair is dominant in the absence of RNC1.

Table 3. Mutation spectra in sterile survivors.

Mutation Type		*rnc1*	*rad52*	*rad52 rnc1*	*ku80*	*ku80 rnc1*
Wild-type Sequence						
CTTCGCGCAACAGTATA[a]						
+CA	CGCGCAACA**CA**GT	7/18	0/12	1/18	0/14	0/24
+AA	CGCGC**AA**AACAGT	2/18	0/12	5/18	0/14	0/24
-GC	CTTCGCAACA	1/18	0/12	0/18	0/14	3/24
-G	CTTCCGCAACA	2/18	0/12	1/18	0/14	10/24
-T	CTCGCGCAACA	0/18	0/12	0/18	0/14	1/24
C → T	CTTCGCGCAA***T***A[b]	1/18	2/12	0/18	1/14	1/24
G → C	CTTCGC***C***CAACA	1/18	1/12	0/18	3/14	0/24
A → C	CTTCGCGC***C***ACA	0/18	2/12	2/18	2/14	0/24
GC → AA	CTTCGC***AA***AACA	0/18	0/12	0/18	4/14	0/24

[a] Sequence starts at the proximal boundary of the Z1 region in the MAT locus. The 3' 4-b overhang created after HO endonuclease activity is underlined.

[b] Base insertions are indicated by boldface type.

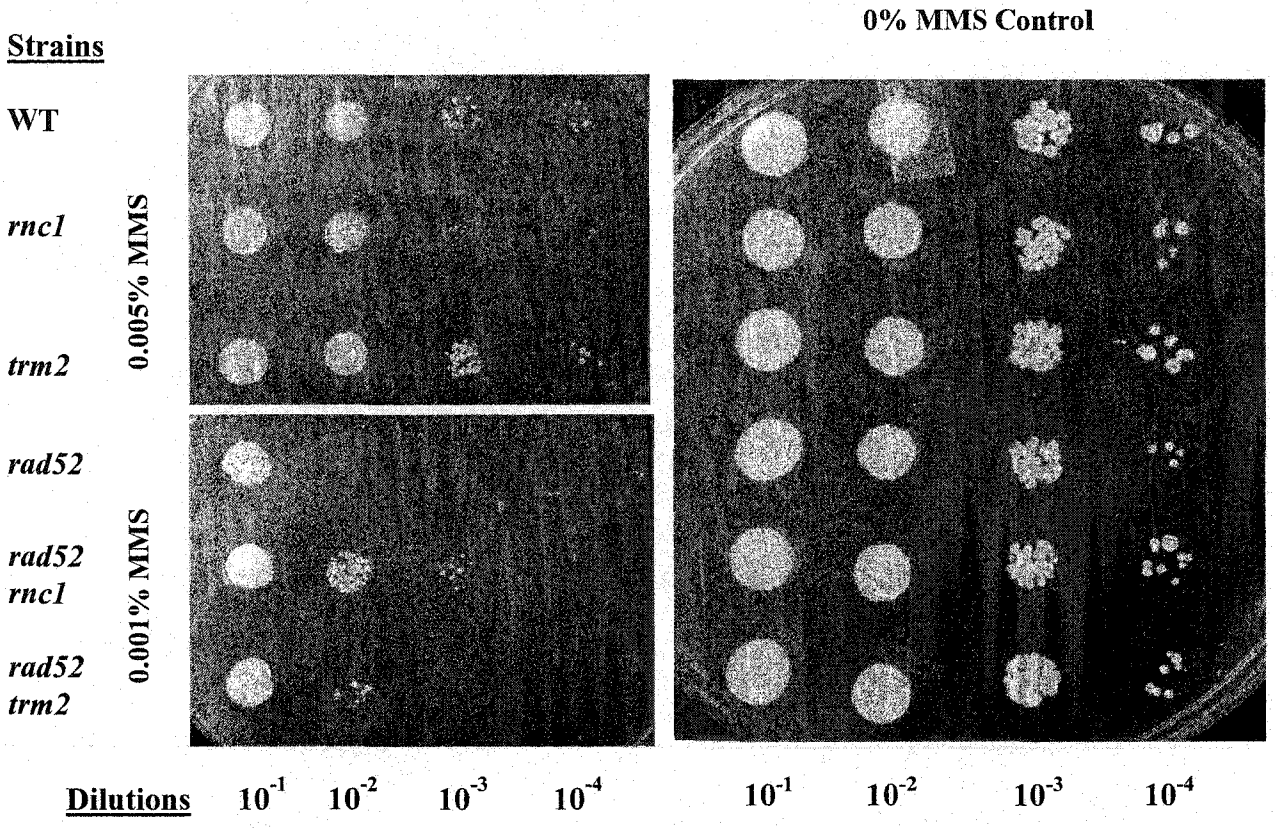
[C] Base substitutions are indicated by bold italics.

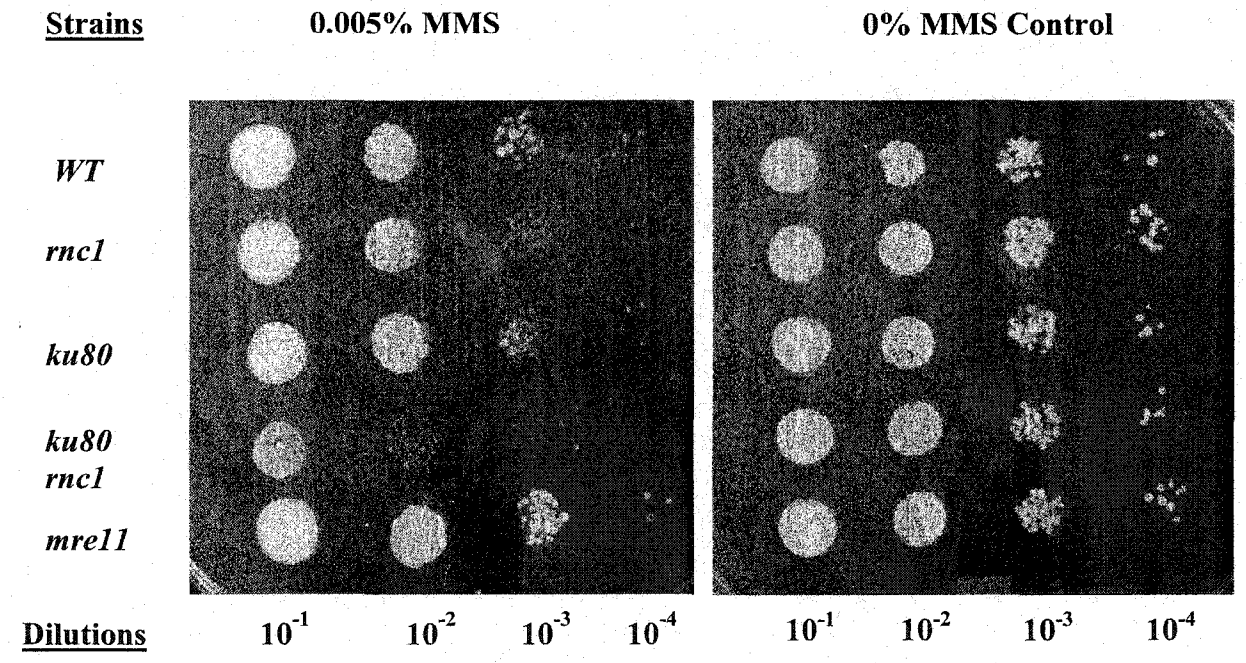
3.3 MMS survival assay.

Methyl methane sulfonate is an alkylating agent which effects a DNA damage pattern similar to that seen with ionizing radiation. Previously, we found that an *rnc1* mutation confers a slight sensitivity to ionizing radiation (IR) but not as significant as that observed in the HO endonuclease induction experiments [26]. Since that time, studies conducted by Nordlund *et al.* identified the *RNC1* gene as the structural gene for the tRNA(m^5U^{54}) methyltransferase [23] and consequently named the gene *TRM2*. Concomitantly, they demonstrated that the *trm2* mutant, which contained a disruption of the *TRM2/RNC1* gene at a unique *BamH*I site by a *HIS3* marker gene, failed to exhibit any sensitivity to specific DSB induction in the SK1 background. In addition, they showed that the phenotypic behavior of this particular construct of *trm2* is the same as a strain that is completely deleted of the *TRM2* gene. Therefore, we attempted to compare the *trm2* and *rnc1* knockouts in isogenic strains in relation to their sensitivity to the radiomimetic drug MMS. Consistent with earlier studies with ionizing radiation, the *rnc1* null mutant exhibited moderate sensitivity in 0.005% MMS as compared to wild-type (Fig. 2a). Surprisingly, however, the *trm2* mutant did not show any sensitivity in comparison to the wild-type strain. Furthermore, as with the specific DSB induction assays, we found that a *rad52* null mutation, normally highly sensitive to DNA damage, is more resistant to MMS in an *rnc1* null background (Fig. 2a). In contrast, the double mutant *rad52 trm2* is almost as sensitive as the *rad52* single mutant. Given that in this particular construct of *trm2*, the disruption insertion is situated at the 3' end of the gene, these results raise the possibility that this construct may retain the functional domain of the RNC1/TRM2 gene involved in DSB repair. In our strain background, we were not

able to obtain a strain that is completely deleted of the *TRM2* gene using the construct provided by Nordlund *et al.* [23].

Figure 2. MMS sensitivity in *rad52* mutants is partially suppressed in an *rnc1* background, but not a *trm2* background. Serial dilutions (10^{-1} to 10^{-4}) of YPD culture were spotted (8 µl) onto YPD plates containing 0 or 0.005% MMS. The plates were incubated at 30°C for 2 days. The *nud1* mutant displays slight sensitivity to MMS while the *trm2* mutant does not **(a).** The *rad52* single mutant shows much higher sensitivity to 0.001% MMS than does the *rad52 rnc1* double mutant. At 0.005% MMS, both *rad52* and *rad52 rnc1* display such high sensitivity to DNA damage that differences between the two strains is not very evident (data not shown). Suppression of the *rad52* lethality is not observed in the *rad52 trm2* double mutant, which is comparatively as sensitive as the *rad52* single mutant. A *ku80* null mutation has a additive effect on *rnc1* **(b)**; while the *rnc1* single mutant displays slight sensitivity to 0.005% MMS and a *ku80* mutation has no discernible effect on cell survival, the *ku80 rnc1* double mutant is hypersensitized to MMS. An *mre11* mutant disrupted in its nuclease domain does not display any sensitivity to MMS.


0% MMS Control
Strains
WT
rnc1
trm2
rad52
rad52 rnc1
rad52 trm2
0.005% MMS
0.001% MMS
Dilutions
10-1 10-2 10-3 10-4
10-1 10-2 10-3 10-4


Strains
0.005% MMS
0% MMS Control
WT
rnc1
ku80
ku80 rnc1
mre11
Dilutions
10-1 10-2 10-3 10-4
10-1 10-2 10-3 10-4

The results obtained appear to indicate that *RNC1* is epistatic to *RAD52* and that they act in the same repair pathway. We then attempted to clarify the role of the *RNC1* gene with respect to *Ku80*, a gene that encodes a critical DNA-binding subunit of the NHEJ pathway. The *ku80* single mutant displays minimal sensitivity to MMS as compared to wild-type (Fig. 2b). However, the *ku80 rnc1* double mutant displays sensitivity to MMS that is much higher than that of either mutant alone. This is a strong indication that *Ku80* and *RNC1* take part in two alternate but complementary DSB repair pathways, such that null mutations in both genes hypersensitizes the cell to DSB damage. An *mre11* mutant disrupted for its nuclease domain does not exhibit any sensitivity to MMS. This indicates that the nuclease activity of the Mre11 protein may not be critical for DSB processing. In fact, previous studies showed that the nuclease activity of Mre11 is essential for meiosis, but not required in mitotic homologous recombination events [37]. Also, it was shown that the Mre11 protein possesses two distinct functional domains, a nuclease domain and a DNA-binding domain. While disrupting the DNA-binding domain renders the cell extremely sensitive to MMS toxicity, a mutant disrupted for the nuclease domain exhibited almost wild-type phenotype in DSB repair [38].

3.4. Cell survival following UV irradiation.

The wild-type and *rnc1* strains were analyzed for their sensitivity to UVB (312 nm) irradiation over a dose range of 0.25 to 0.75 KJ/m^2. No appreciable difference was observed between wild-type and *rnc1* mutants in their response to UV irradiation (Fig. 3). Therefore, a null mutation in *RNC1* does not confer any sensitivity to UV irradiation, and

thus does not implicate RNC1 in the nucleotide excision repair and/or the base excision repair pathways.

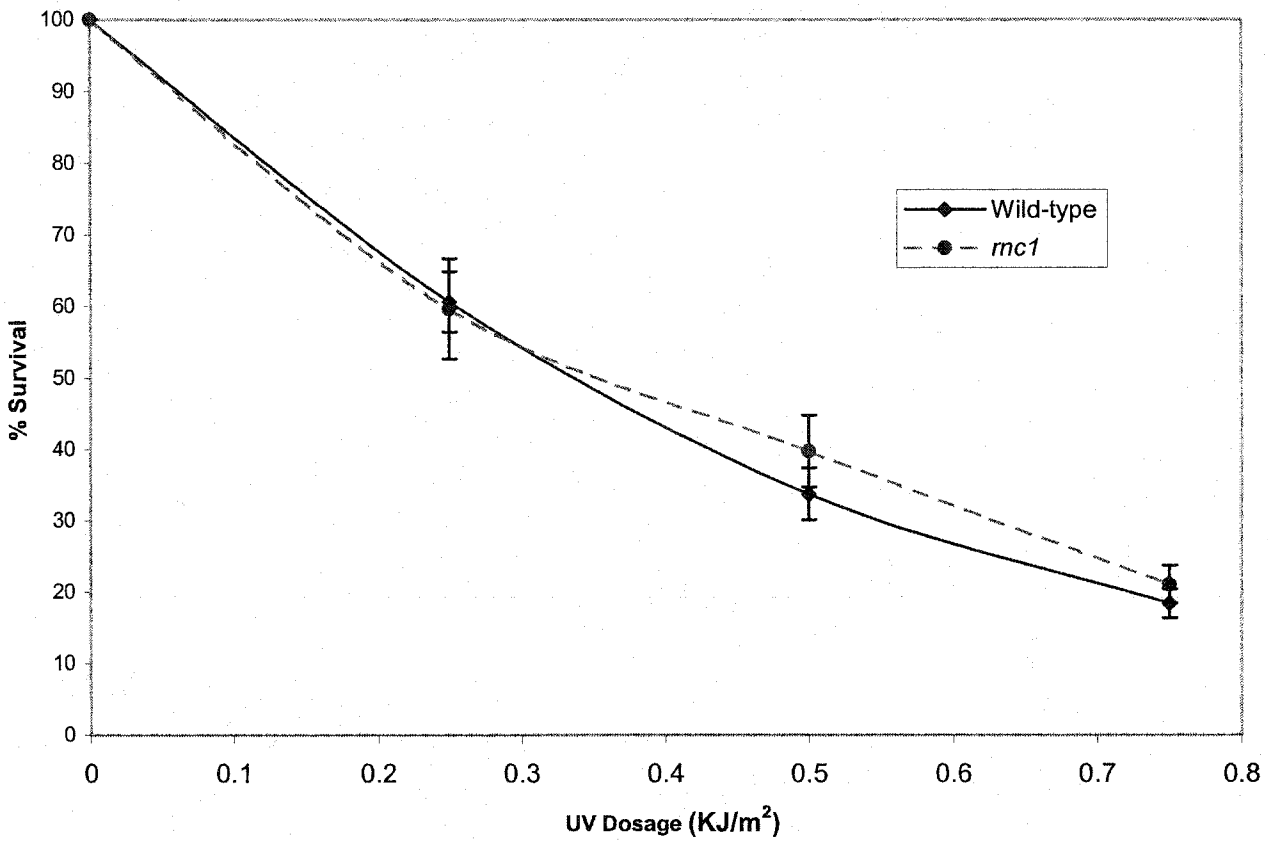


Fig. 3. UV survival curve. Symbols are: Wild-type —◆ and *rnc1* ---● . Survival of the *rnc1* strain was assayed over a UV dosage range of 0.25-0.75 KJ/m^2 at 312 nm (UVB). Approximately 1 x 10^3 cells/plate were plated onto selective medium, then after drying 10-20 minutes, were UV-irradiated. Following irradiation, plates were incubated under non-photoreactivating conditions over a period of 2-3 days at 30°C then counted. Survival was calculated as a function of the colony count of unirradiated cells plated under the same conditions. Data represented are the means of 3 independent experiments.

3.5 Expression of GFP-RNC1 fusion protein.

The pGFP-RNC1 construct was transformed into the strain DRY214, and grown in minimal medium lacking uracil and containing 2% raffinose. Expression of GFP-RNC1 was induced by addition of 0.5% galactose to exponentially growing cells OD600 ~ 1.0. Cells expressing GFP-RNC1 were viewed directly under a light microscope with a GFP filter. GFP-RNC1 expression was observed to be highly localized to the nucleus (Fig. 4). The nuclear localization of the GFP-RNC1 fusion protein suggests that the area of activity of RNC1 is tightly restricted to the nucleus.

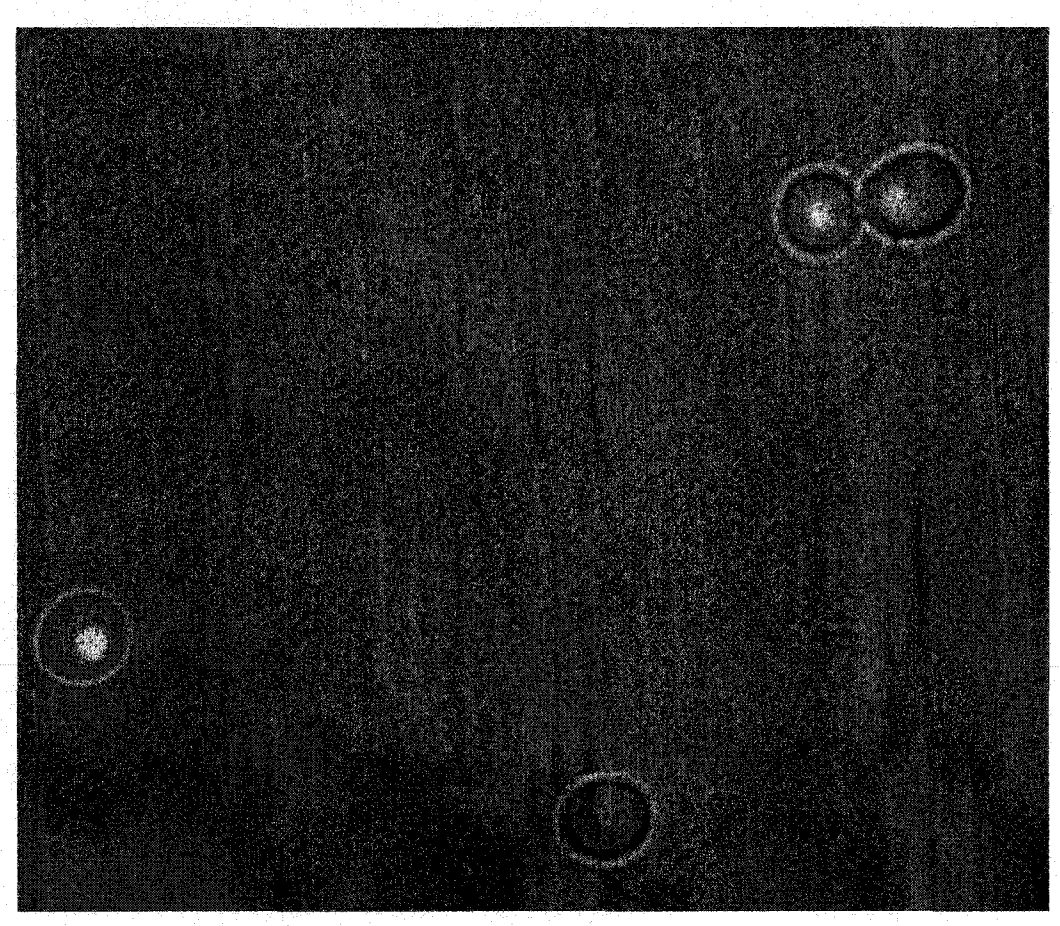


Figure 4. Localization of GFP-RNC1 fusion protein to the nucleus. GFP-RNC1 expression was induced by addition of 0.5% galactose to exponentially growing cells in 2% raffinose. GFP expression was visualized using a light microscope with a GFP filter.

3.6 Nuclease activity of the purified endo-exonuclease.

The single-strand endonuclease activity of the *RNC1* gene product was assayed on ssDNA substrate. The *in-vitro* nuclease activity was assayed by the release of fragments of α^{32}P-labelled DNA that is not co-precipitable with salmon-sperm carrier DNA in 10% trichloroacetic acid. Purified CBP-RNC1 protein was assayed for nuclease activity, along with CBP-ß-galactosidase expressed and purified under the same conditions from the *E. coli* expression strain BL21-Gold (Stratagene). Three independent experiments were carried out; one representative experiment is shown in Fig. 5. The immunoprecipitated GFP-RNC1 fusion protein was shown to have nuclease activity on ssDNA substrate while the CBP-ß-gal control displayed no appreciable DNase activity, which could have arisen from contaminating proteins from *E. coli* following the purification process. Specific nuclease activity ranged from 12 units/mg to 30 units/mg total protein (one unit is defined as the amount of deoxyribonuclease required to render 1 μg DNA acid-soluble in 30 min at 37°C). GFP-RNC1 fusion protein expressed in yeast and purified by immunoprecipitation with GFP monoclonal antibody was also shown to have nuclease activity on ssDNA substrate. In studies performed by Nordlund *et al.* [23], RNC1/Trm2 was expressed as a fusion protein with glutathione-S-transferase and affinity-purified on a GST-sepharose column. The purified protein did not degrade plasmid DNA, or degraded it with inconsistent efficiency [23]. However, the GST moiety was not cleaved, and may have interfered with nuclease activity. The purification process we applied for RNC1 uses the CBP tag which is only 4 kDa, as opposed to the GST tag, which is 30 kDa in size.

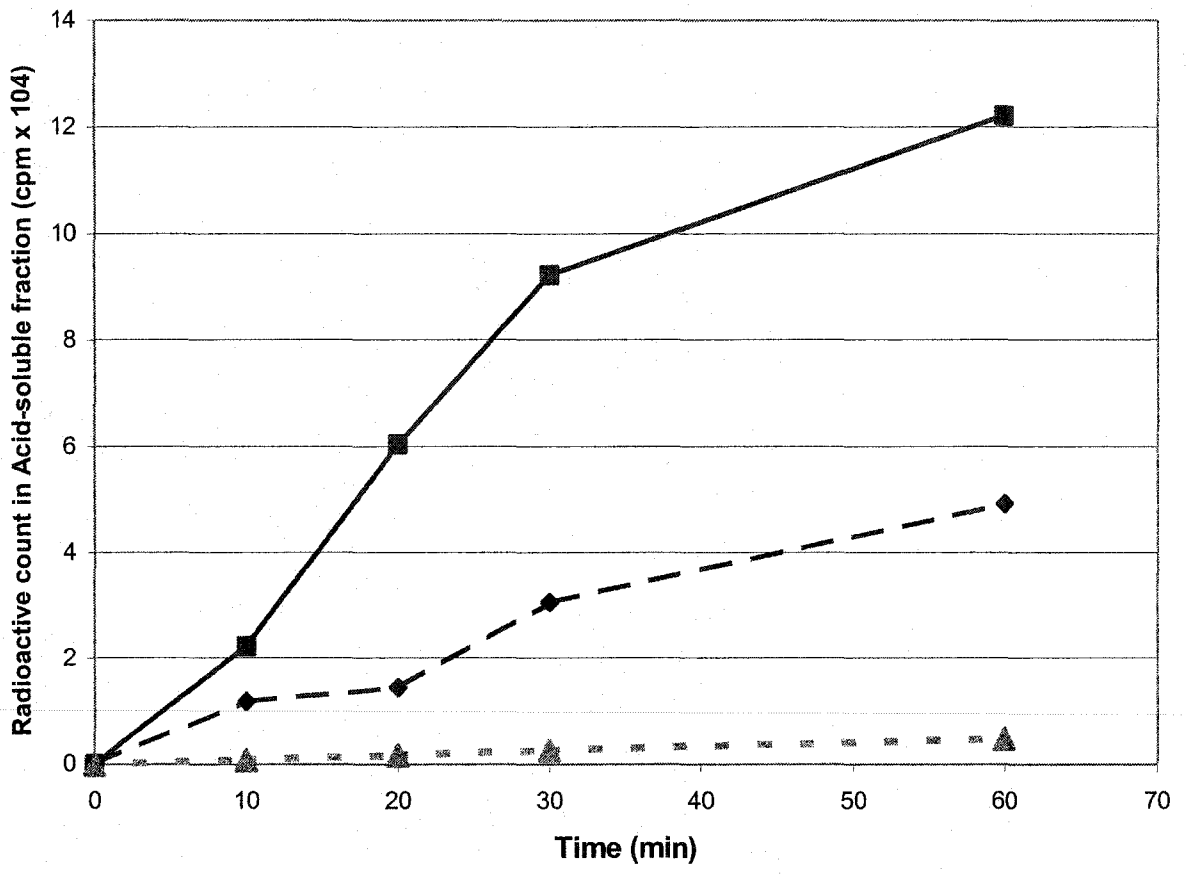


Figure 5. Deoxyribonuclease activity of RNC1 protein. Symbols are: CBP-RNC1 ——— GFP-RNC1 – – and CBP- ß -gal – – . GFP-RNC1 was overexpressed in the *rnc1* yeast strain and immunoprecipitated with the GFP monoclonal antibody. CBP-RNC1 was expressed in *E.coli* strain BL21-Gold and purified on calmudulin affinity resin (Stratagene). 50 µg purified CBP-RNC1 was incubated with 2 µg pUC19 DNA which was labeled with α^{32}P-dCTP using the Klenow Fragment, then denatured 5 min at 100°C. 50 µg CBP-ß-galactosidase expressed and purified in the same manner was used as a control.

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Directionality of the RNC1 DNase activity was also assayed with linear double-stranded pUC19 DNA substrate. Plasmid DNA was linearized with either *Sma*I or *Eco*RI restriction endonucleases to yield DNA ends with either blunt or 5' staggered ends, respectively, and incubated with 10μg CBP-RNC1. Purified CBP-RNC1 protein incubated with 5 μg pUC19 linearized with either restriction enzyme resulted in degradation of the linear substrate (Fig. 6). In addition, the RNC1 protein exhibits DNA-binding activity as band retardation is seen to occur consistently at 0 minutes, immediately after the substrate is added to the enzyme. However, it is interesting to note that the degradation of the linearized plasmid with the 5' staggered ends (a) lagged appreciably behind that of the blunt-end substrate (b). This indicates that the exonuclease activity of RNC1 proceeds in a 5' to 3' directionality; the 5' ends must be resected before processing of the DSB can occur.

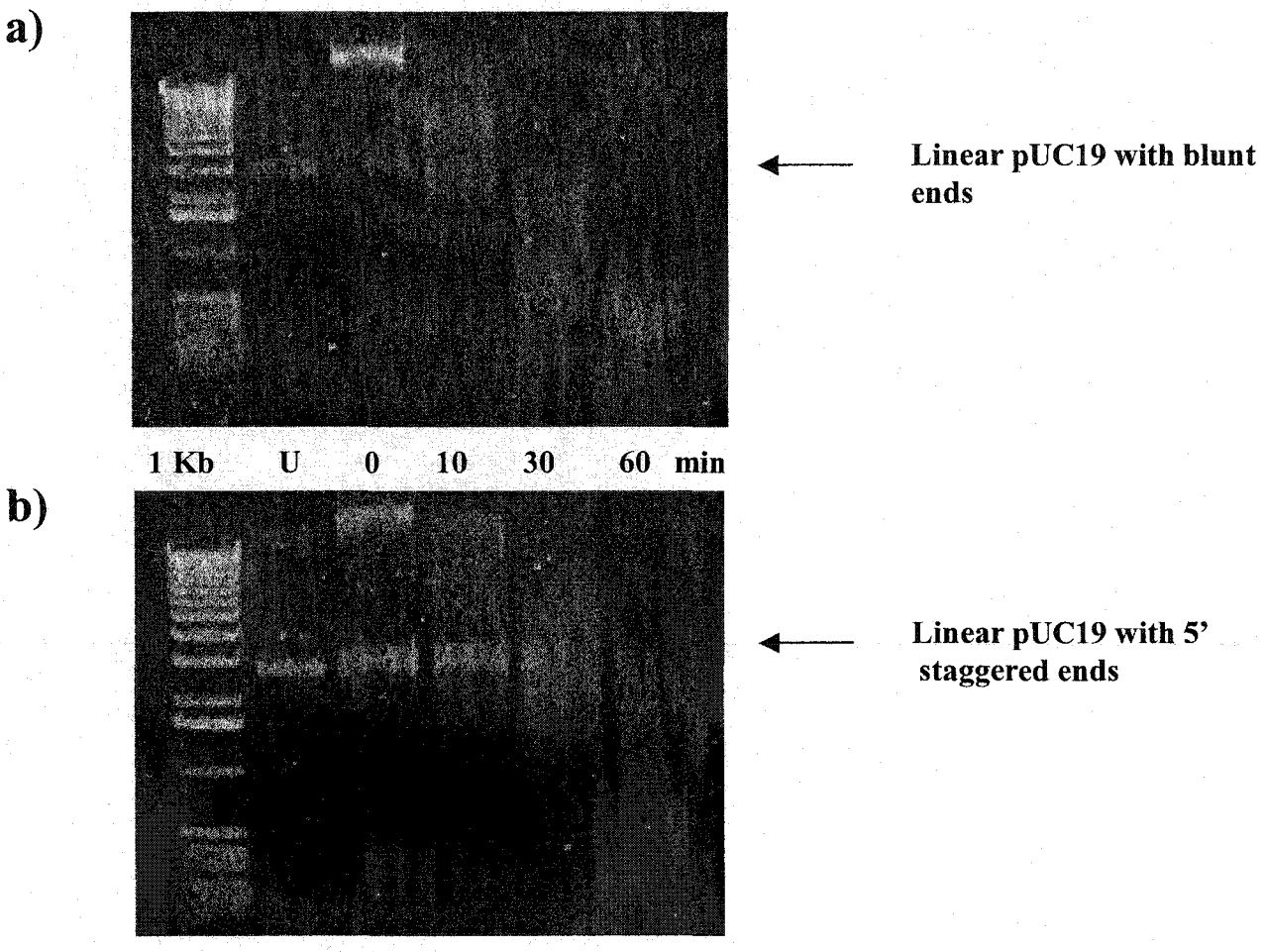

Figure 6. RNC1 nuclease activity on linear dsDNA. pUC19 DNA was linearized by either *Sma*I or *Eco*RI restriction endonucleases to yield either a blunt-end **(a)** or 5' staggered end **(b)** linear substrate, respectively. Cut and undigested linear DNA (U) was also run. At zero time, we immediately observed band retardation which is likely due to the binding of the substrate by RNC1. Exonucleolytic processing progressed at a slower pace from 10 – 60 min with the 5' staggered-end substrate possibly due to the necessity to resect the 5'single-strand ends before 5' to 3' exonuclease processing can occur.

This is corroborated by the manner in which 5' end-labeled linear plasmid DNA is degraded over time (Fig. 7). The intensity of the signal of the 5' end-labeled dsDNA substrate decreases over time, indicating that the 5' label is rapidly removed, with little indication of processing occurring via the 3'end. Labeled substrate incubated with CBP-β-gal purified in the same manner as CBP-RNC1 failed to display any nucleolytic processing of DNA ends, thus ruling out contaminating factors that may persist following the purification process.

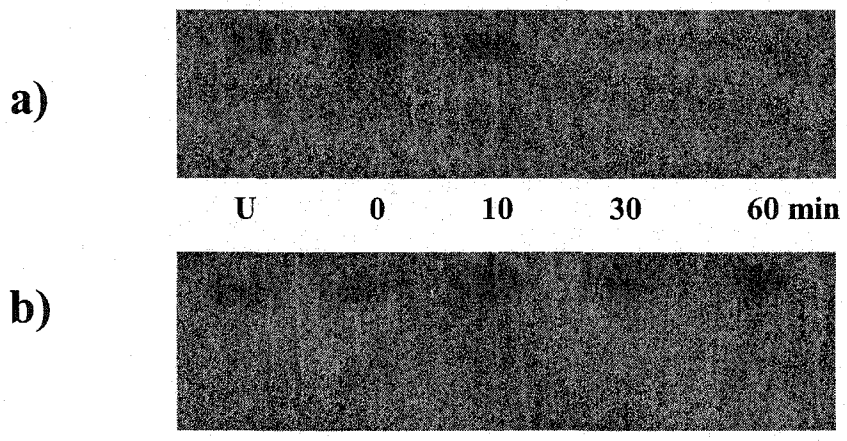

Figure 7. Degradation of 5'end-labelled linear DNA by RNC1. **(a)** 10μg of RNC1 was incubated with 5μg of pUC19 linearized with *Sma*I and 5'end-labelled with ^{32}P. Along with labeled substrate untreated with RNC1 (U), aliquots were taken at timepoints from 0 to 60 min and run on a denaturing gel. **(b)** A control reaction of 10 μg of CBP-β-gal purified under the same conditions as CBP-RNC1 was also incubated with linear pUC19 labelled DNA.

In order to monitor the *in vivo* nuclease activity of the RNC1 protein, we utilized an assay that measures the ssDNA formation directly at the HO cut site following the galactose-induced expression of HO endonuclease (see Materials and Methods). Figure 8 demonstrates that ssDNA forms to a greater extent in a *rad52* background as compared to *WT*, and persists after HO expression is repressed. This is consistent with a block in homologous recombination processes in the absence of Rad52 that results in the accumulation of extensively degraded DSB ends and a concomitantly low rate of recovery from DSB induction. In contrast, DNA degradation appears to occur to a lesser extent in the wild-type strain, which may be due to the presence of an active DNA repair process. This is also indicated by the manner in which signal hybridization diminishes after the 1 hr timepoint, suggesting that ssDNA is processed rapidly in the absence of recurring DSB formation. In the *rnc1* mutant, ssDNA formation appears to be dramatically reduced. This lack of processing is also apparent in the *rad52 rnc1* double mutant, though not to the same extent as in the *rnc1* single mutant. Possibly, the increase in signal hybridization in *rad52 rnc1* over time may be due to the absence of Rad52, which may otherwise protect the exposed DSB ends from general nucleolytic degradation.

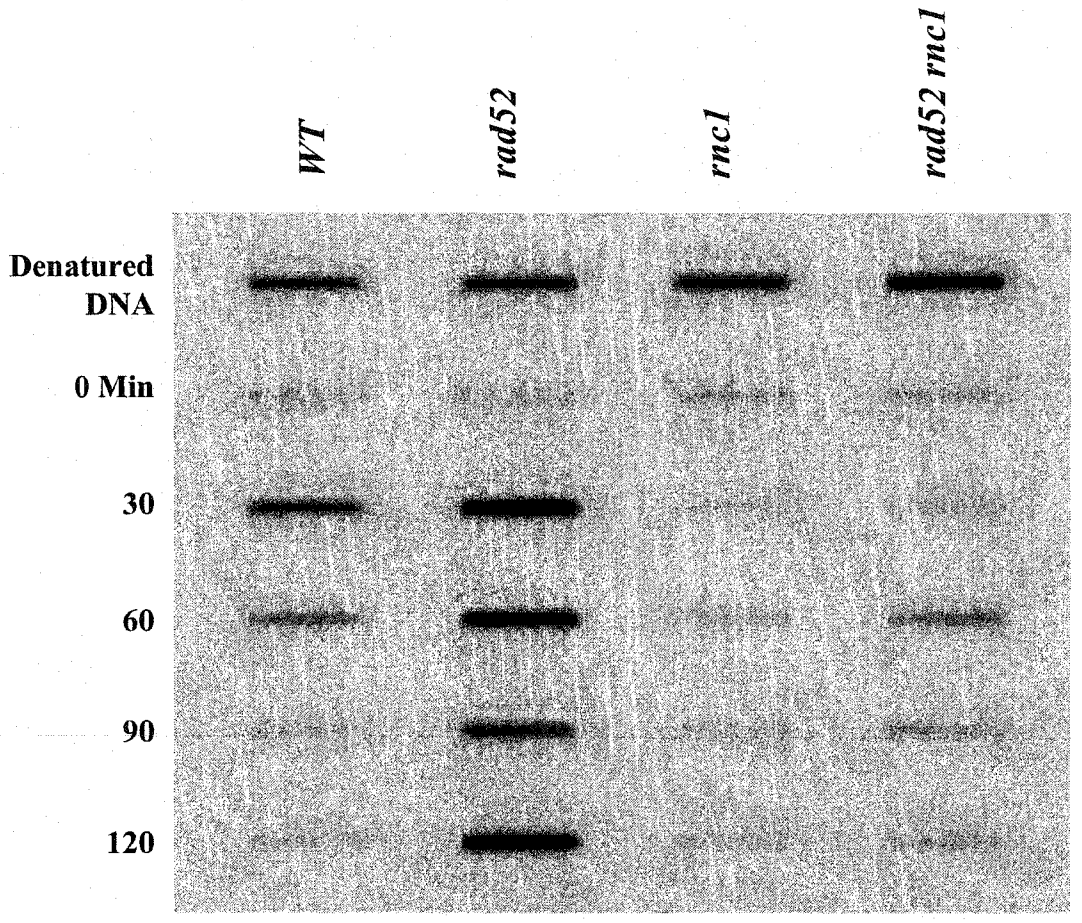

Figure 8. ssDNA formation at HO cut site. The 3' single-strand region directly at the HO cut site was probed in the above 4 strains. The probe used was the ^{32}P-end-labelled ssDNA oligonucleotide: 5' GAA GTA GTC CCA TAT TCC GTG CTG 3' which is specific for the 3' sequence of the right arm of the *MAT Ya/Z* junction. HO endonuclease expression was induced in liquid galactose medium from times 0 – 1 hr. After 1 hour, samples were spun down and resuspended in glucose medium. Time course samples were gently isolated and slot-blotted onto the membrane. Denatured samples were the same as native samples except they were alkali denatured and diluted 1/5 prior to blotting.

4. Discussion

In contrast to higher eukaryotes, homologous recombination is the dominant pathway through which double-strand breaks are repaired in yeast. The role that most of the proteins in the *RAD52* epistasis group play in this pathway has been elucidated. However, the steps involved in the initial processing of the DSB have yet to be clarified. In the most widely accepted model for DSB repair by homologous recombination, the strand invasion model [34,39-41], the DSB ends are processed by 5' to 3' exonuclease activity immediately following DSB induction. This enables the 3' single-stand ends to invade and hybridize to a complementary sequence located either on a sister chromatid or a homologous chromosome. The processing of the DSB is thought to involve a recBCD-like endo-exonuclease, but at present, no recBCD-like equivalent has conclusively been identified in eukaryotic cells responsible for processing DSB ends. In this study, we have shown that the *RNC1* gene product displays endonuclease activity on ssDNA substrate and 5' to 3' exonuclease activity on dsDNA that is the hallmark of endo-exonucleases that have been isolated and characterized in *Neurospora crassa* [1,14,42].

In the study by Nordlund and colleagues [23], the purified RNC1 protein displayed inconsistent nuclease activity on single-strand DNA. This was based on their analysis of the RNC1 protein purified as a GST fusion construct. When we repeated the experiments based on the same construct, we also obtained conflicting results when using the protein with the uncleaved GST moiety. While it was possible to cleave the tag with overnight thrombin treatment, we also realized that the RNC1 protein was sensitive to proteolytic degradation based secondary bands, which result following purification and treatment with thrombin. This, and the considerable difficulty we had in purifying the

GST-RNC1 fusion protein, led us to investigate an alternate purification method with a much smaller tag, in the calmodulin binding peptide. With this construct, we had much better success demonstrating that the purified RNC1 protein possesses DNase activity which is unlikely to be an artifact of the purification process, as the control CBP-β-galactosidase fusion protein purified under the same conditions displayed no appreciable nuclease activity. We were able to characterize the nuclease activity of RNC1 as being dual in functionality. Furthermore, we have localized the activity of the RNC1 protein via expression of the GFP-RNC1 fusion protein to the nucleus, thus corroborating that RNC1 is a nuclear protein consistent with its capacity as a component of the homologous recombination- repair pathway.

A single *rnc1* null mutation leads to slight sensitivity to DSB damage arising from MMS and ionizing radiation, and a 10-fold reduction in cell viability following persistent DSB induction. As demonstrated in the *rnc1/trm2* construct, insertional disruption of the C-terminal region of RNC1 has no effect on cell survival (Fig. 3a), so presumably this region may not be critical for DSB repair processes. Interestingly, the same region was implicated in the methyltransferase activity of RNC1, and it was shown that the sequence encoding it has considerable homology with the *trmA* gene of *E. coli* [23].

There is a high sterility rate associated with *rnc1* null strains emerging from continuous DSB induction. This suggests to us that the DSB events are alternatively being repaired through a more error-prone pathway in the *rnc1* mutants. Subsequent DNA sequence analysis revealed a proliferation of insertion or deletion events; in particular +CA insertions in *rnc1*, +AA insertions in *rad52 rnc1* and –G deletions in *ku80 rnc1* (Table 3). The spectra of mutations leading to sterility in *ku80* survivors shifts from

almost exclusive base substitutions to a predominance of minor deletions in *ku80 rnc1* survivors. Likewise, base substitutions make up all apparent mutation events in *rad52* mutants, as opposed to the more common insertion mutations seen in *rad52 rnc1*. A previous study by Moore and Haber [29], found that these insertion events; in particular the +CA insertion (35/45), were characteristic of the NHEJ pathway in the absence of the HR pathway in repairing DSBs induced through HO expression throughout the cell cycle. The high frequency of the +CA insertion events in *rnc1* suggests that a similar defect in the HR pathway may exist in this mutant.

Results from *in vivo* analysis of repair events are consistent with the hypothesis that DSBs are not primarily processed via the HR pathway in the *rnc1* mutant. In the wild-type strain we observed that DSB ends are degraded in an apparent 5' → 3' direction, a process that has been shown to be necessary for the priming of DSB ends for entry into the homologous recombination repair pathway [36,43], and that in the *rad52* mutant the ssDNA intermediate tends to accumulate without being processed. DSB processing in the *rnc1* mutant is not detectable, implicating the RNC1 protein in the 5' → 3' processing of DSB ends prior to homologous recombination. There is significantly less exonuclease degradation in the *rad52 rnc1* double mutant than in the *rad52* single mutant. This confirms the epistatic relationship between *RNC1* and *RAD52* in that an *rnc1* null mutation abrogates the recombinational block that otherwise occurs in the absence of a functional *RAD52* gene.

We hypothesize that the *RNC1* gene functions at an earlier step in the same pathway as *RAD52*, and in a separate but complementary pathway to *KU80* in the non-homologous end-joining pathway. As described in the DSB repair model (Fig. 9), in the

presence of *RNC1* the DSB ends may normally be resected to yield 3' ssDNA intermediate, which in the absence of RAD52 will accumulate [34,44], and eventually lead to cell death [45].

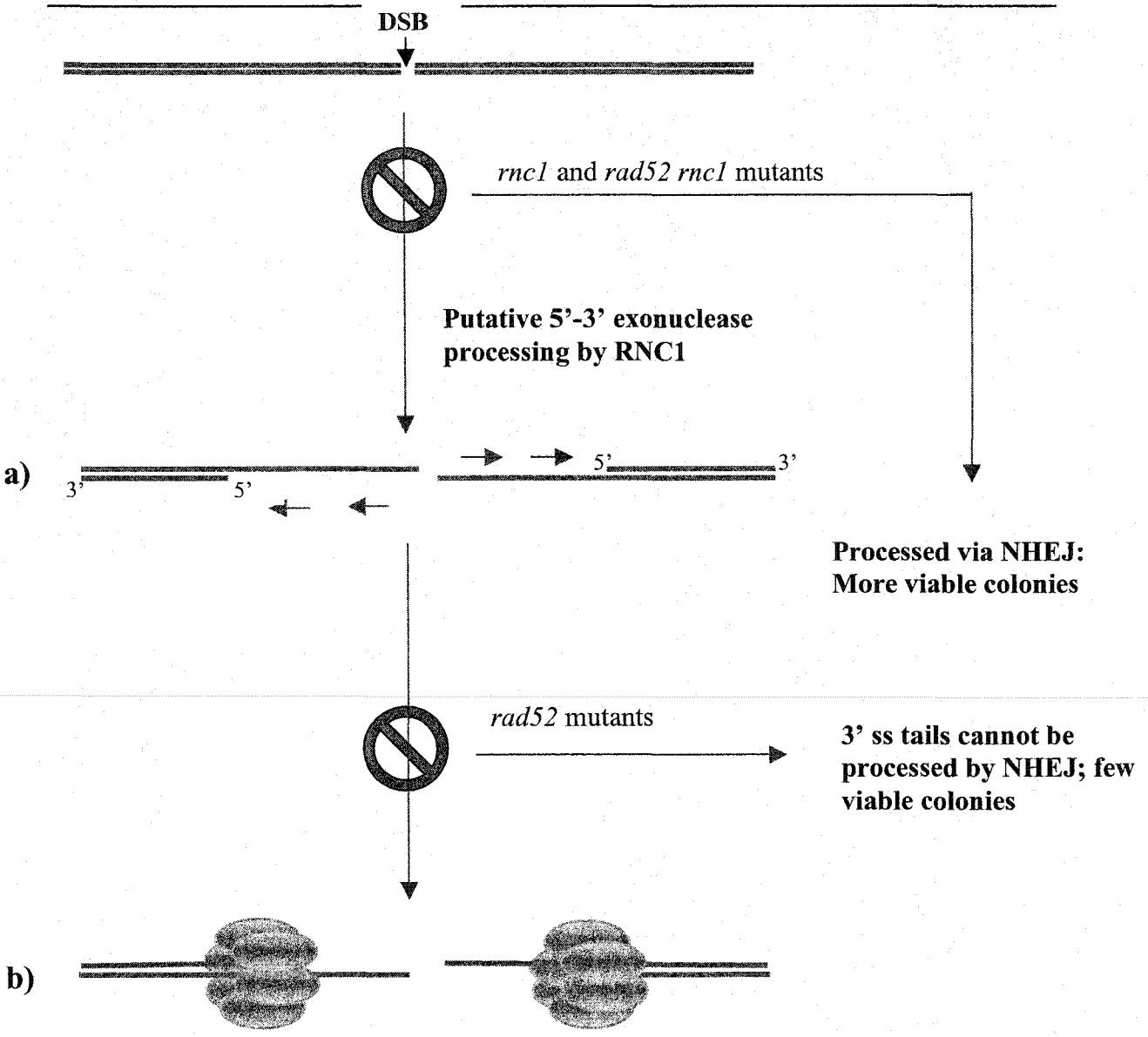

Figure 9. Model for phenotypic suppression of *rad52* lethality. 5' → 3' exonuclease processing by RNC1 **(a)**, is believed to be responsible for the producing the 3' ssDNA intermediate that is the substrate for RAD52 activity **(b)**. In the absence of RNC1, the NHEJ pathway can compete more effectively to repair the DSB. However, if the DSB is processed by RNC1, a *rad52* block is highly lethal since the NHEJ cannot process the extensive 3' overhang.

However, in the absence of RNC1, the DSB ends can effectively be acted on by the alternate repair pathway: the NHEJ pathway. As a result, a null *rnc1* mutation in a *rad52* background results in elevated levels of survival following DSB induction. In an interesting corollary, it was previously discovered that cell extracts from the *rnc1* mutant are defective in the recombination of plasmids with double-strand breaks but not of intact plasmids, as are cell extracts prepared from the *rad52* mutant [46]. However, it was found that elimination of RNC1 restores recombination in *rad52* extracts to levels comparable to those in wild-type extracts.

Mre11 is an exonuclease that, along with Rad50 and Xrs2, forms part of a heterotrimeric enzyme complex that is postulated to be necessary for both the introduction and processing of DSB ends during meiotic recombination [47,48]. *S. cerevisiae mre11* null mutants were found to be profoundly defective in DSB repair [49]. Along with its role in homologous recombination, a possible role has been indicated for the Mre11/Rad50/Xrs2 complex in NHEJ based on its ability to stimulate sister chromatid interactions that stabilize broken DNA ends [49]. The Mre11 protein was previously identified as the putative nuclease responsible for 5'→ 3' processing of DSB ends during mitotic recombination. However, its 3'→ 5' nuclease activity does not lend itself easily to this role [50-52]. Moreover, the nuclease activity of Mre11 was found to be required for meiosis, but not for mitotic recombination events or NHEJ [37,49]. In the course of our study, we utilized a knockout strain for *Mre11* specifically disrupted in its nuclease domain [38] and asked whether the *mre11* mutation confers a similar inability to *rnc1* to repair DSBs in an isogenic yeast strain. We found that the Mre11 nuclease-

deficient mutant does not display sensitivity to MMS or an impaired ability to undergo mating type switching.

In assaying for UVB sensitivity in wild-type, *ku80*, *rnc1* and *ku80 rnc1* strains, we did not find that the presence of an *rnc1* mutation conferred any additional UV sensitivity (Fig. 3). This underpins the hypothesis that the gene product of *RNC1* does not play a detectable role in repairing UV-damage, which consists primarily of pyrimidine dimers and DNA adducts and does not include DNA double-strand breaks [53,54]. It follows that *RNC1,* unlike *RAD27*, the yeast homolog of the *FEN1* 5' to 3' exonuclease [55], does not participate in excision repair. Taken together, the results presented here are consistent with the idea that the *RNC1* gene product plays an important role in homologous recombination repair pathway.

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Chapter IV.

General Discussion

4.1 Genetic interactions between *RNC1* and other DSB repair genes

The primary objective of this thesis was to elucidate the role that the yeast endo-exonuclease plays in DSB repair. Investigations in *S. cerevisiae* have revealed the most persuasive genetic evidence thus far that the eukaryotic EE is a major factor in recombination processes, which are essential for DSB repair in yeast. In this dissertation, I sought to provide a more detailed analysis of the involvement of *RNC1* in repair processes, and to present proof that it may be responsible for the commitment of double-strand breaks for repair via the HR pathway. This evidence is derived primarily from genetic studies examining what effect an *rnc1* null mutation has on the repair of induced double-strand breaks, as well as its influence on other factors regulating the major DSB repair pathways of *S. cerevisiae*.

4.1.1 Examining the epistatic relationship between *RNC1* and *RAD52*

Our present understanding of the HR pathway in yeast is mainly mapped around the *RAD52* epistasis group, relative to the importance of this family of genes in the homologous recombinational repair pathway. It is therefore important to draw on the relevance of *RNC1* to DSB repair processes as a function of its genetic interaction with members of the *RAD52* epistasis group. Previously, it had been shown that the expression of RNC1 is dependent on the presence of the *RAD52* gene product, as the *rad52* mutant possesses only 10% of the levels of RNC1 protein that is found in a wild-type strain (Chow and Resnick, 1988). Also, the immunoprecipitable nuclease activity in cell extracts of wild-type cells increases as they proceed through meiosis, but this increase in activity is not apparent in cell extracts prepared from *rad52*-deficient cells. Removal of

the RNC1 endo-exonuclease from cell extracts by treatment with EE-specific antibody abolishes recombination between homologous DNA duplexes, but not between single and double-stranded DNA substrates (Moore *et al.*, 1993). This indicates that RNC1 is functionally required prior to the strand invasion/annealing step of yeast DNA recombination processes.

A direct link between *RNC1* and *RAD52* was realized when we discovered an epistatic relationship between the two genes. In the presence of a functional *RNC1* gene, DSB induction is a lethal event in a *rad52* mutant. This is evident from the accumulation of ssDNA at the site of DSB induction, which indicates a recombinational block in the *rad52* mutant immediately following the 5'→ 3' processing of DSB ends. Other studies have indicated a similar buildup of unprocessed 3'-ended ssDNA products following DSB induction in the *rad52* mutant (Sugawara and Haber, 1992; White and Haber, 1990). Our studies using the *rad52 rnc1* double mutant demonstrated that its sensitivity to DSB damage is markedly reduced. This was consistently demonstrated by results from the IR and MMS toxicity assays as well as the HO endonuclease induction assay. Also, the buildup of unprocessed 3' ssDNA was not observed in the double mutant following DSB induction; in contrast to the *rad52* single mutant. Rather, the double mutant shows reduced exonuclease processing similar to the *rnc1* single mutant.

In an environment of persistent DSB induction, it is apparent that the *RNC1* gene product plays a major role in DNA repair processes. This is most notably seen in specific DSB induction assays using HO endonuclease, where the *rnc1* mutant shows as much as a 10-fold increase in sensitivity to persistent DSB induction in comparison to wild-type cells. This sensitivity is partially suppressed with the over-expression of *RNC1* on a

multicopy plasmid. It is likely that over-expression of *RNC1* may have a deleterious effect on cell survival such that wild-type levels of survival are not achieved. However, the sensitivity of the *rnc1* strain to DNA damage induced by ionizing radiation and the alkylating agent methyl methane sulfonate is minimal. This differs markedly from the response of the *rnc1* mutant to persistent specific DSB induction. A possible explanation may be that ionizing radiation and MMS induce a lesser quantity of DSB "hits" per cell as opposed to extended HO endonuclease induction. Increased doses of either mutagenic agent simply have the effect of elevating the background of general DNA damage, repair of which is not influenced by RNC1. Hence, the contribution of *RNC1* in the repair of DSBs might be masked. Notably, the wild-type strain and the *rnc1* mutant do not display appreciably different sensitivities to DSBs at shorter HO induction times (2-6 hrs), suggesting that a certain level of DSBs may be repaired in an *rnc1* background by being shunted through alternative repair pathways. Alternatively, there may be a functional overlap between RNC1 and another nuclease such that DSBs may still be processed to a certain degree in the absence of RNC1, without significantly hindering the HR pathway unless DSBs are persistently induced. Precedents for functional overlap have already been observed in DSB repair: helicase activities of recBCD and recQ of *E. coli* (Nakayama and Nakayama, 1984; Umezu and Nakayama, 1993), and the yeast DNA recombinases Rad51 and DMC1 (Bishop *et al.*, 1992; Masson and West, 2001; Shinohara *et al.*, 1997).

4.1.2 Contributions of the NHEJ pathway to DSB repair in the absence of RNC1

Once we established that that *RAD52* is epistatic to *RNC1*, thus implicating *RNC1* in the same recombination repair pathway as *RAD52*, we wanted to determine whether an *rnc1* mutation would have any meaningful impact on NHEJ events following DSB induction. The NHEJ pathway, of which *Ku80* is an integral component, is an essential DSB repair mechanism only in the absence of the HR pathway (Clikeman *et al.*, 2001). Since a *ku80* mutation has a deleterious effect on the yeast cells' ability to repair DSB only in the absence of a functional *RNC1* gene, this argues for a role for *RNC1* in the homologous recombination pathway. In-depth analyses of repair events following the introduction of a DSB at the mating type locus of haploid yeast cells support this hypothesis. Firstly, the fidelity of DSB repair in *rnc1* mutants, including *rnc1* and *ku80 rnc1*, is drastically reduced from wild-type levels, and is consistent with the level of error-prone repair in *rad52* mutants. The fidelity of DSB repair in the *ku80* mutant does not differ significantly from that of the wild-type strain.

Furthermore, sequence analysis of the DSB site of sterile survivors of HO induction has shed new light on the mutation events that lead to loss of mating proficiency. We found that there was a predominance of base insertions or deletions directly at the site of DSB induction in *rnc1* sterile survivors. This naturally suggests a repair process which tends to misalign free DSB ends. The HR pathway has been shown to be a highly precise repair pathway in yeast, and mechanistically is unlikely to yield small insertions and/or deletions at repair loci (Rudin and Haber, 1988; Sung *et al.*, 2000). In addition, the spectra of mutations, particularly the +CA insertion, has been

shown in other studies to occur with high frequency when HO-induced DSBs are directed for repair via the NHEJ pathway (Moore and Haber, 1996). Therefore, the presence of such mutations indicates that an alternate DSB repair pathway is at work in *rnc1* mutants. In contrast, *rad52* sterile survivors with base insertions/deletions at the HO cut site are rare. The divergent mutation spectra arising from *rnc1* and *rad52* backgrounds have engendered the following scenario: *rnc1* mutants undergoing multiple rounds of DNA breakage and repair with an error-prone DSB repair mechanism undergo a gradual selection process, whereby cells containing mutations at the DSB locus emerge as survivors due to their resistance to subsequent DSB induction. Hence, a high number of *rnc1* survivors possess mutations suggestive of misaligned repair. *rad52* survivors, on the other hand, may represent a small population of cells which already had mutations at the *MAT* locus, preventing DSB induction from occurring in the first place.

4.1.3 Does Mre11 play a functional role in DSB processing?

Mre11 forms a part of a heterotrimeric enzyme complex also consisting of the proteins Rad50 and Xrs2. Together, they have been implicated in meiotic double-strand break formation and processing (Furuse *et al.*, 1998; Ogawa *et al.*, 1995), non-homologous end-joining (Manolis *et al.*, 2001; Tsukamoto *et al.*, 1996) and telomere length maintenance (Le *et al.*, 1999; Ritchie and Petes, 2000). Based on the involvement of Mre11 in the initiation and processing of meiotic DSBs, it was originally postulated as the nuclease responsible for the 5' 3' processing of DSBs during mitotic homologous recombination. However, further examination has shown that Mre11 possesses two distinct functional domains: a nuclease domain and a DNA-binding domain (Furuse *et*

al., 1998). Of these, the DNA-binding domain has been identified as being critical in DSB repair. In the process of my investigations, I have shown that an *mre11* mutant disrupted for its nuclease domain does not display any sensitivity to DNA damage, while the *rnc1* mutant was demonstrated to be defective in DSB repair.

4.2 Functional analysis of the enzymatic properties of the RNC1 protein

Endo-exonucleases from the prokaryotic recBCD enzyme to mammalian endo-exonucleases display a diverse range of sizes and are highly diverged in sequence. They are, however, functionally inter-related in their ability to process DNA substrates via a true ssDNA-dependent endonuclease activity or a dsDNA 5' → 3' exonuclease activity. In addition, they have been shown to be immunochemically related based on the ability of the *N. crassa* polyclonal antibody to specifically bind to endo-exonucleases ranging from the *recC* subunit of the *E. coli* (Fraser *et al.*, 1990) to EEs isolated from *S. cerevisiae* (Chow and Resnick, 1987), *Aspergillus nidulans* (Koa *et al.*, 1990), monkey CV-1 cells and human leukemic cells (Fraser, 1996).

To date, only the EEs of *N. crassa* and *A. nidulans* have been localized in detail. Active endo-exonuclease in *N. crassa* (Chow and Fraser, 1983) and *A. nidulans* (Koa *et al.*, 1990), that was inhibited and immune-precipitable with a low concentration of antibody raised to the *N. crassa* EE, was found in three different types of organelles: nuclei, mitochondria and vacuoles. It is expected that different organelle localizations may reflect different biological roles of the enzymes. The distribution of EE in human leukemic cells is apparently restricted only to the nucleus; 98% being chromatin-bound and 2% nuclear matrix bound (Fraser *et al.*, 1996). Similarly, the RNC1 protein is highly

localized to the nucleus as demonstrated in *in situ* hybridization assays and by visualization of an RNC1 fusion protein with a GFP tag.

One of the earliest and still the best-studied example of EEs is the recBCD enzyme of *E. coli*. It has been shown to have single-strand specific endonuclease activity which is ATP-dependent, and exonuclease activity on both linear ss- and ds-DNA with no strand polarity (Karu *et al.*, 1973). Its nuclease activity has been implicated in recombination at Chi sites, octanucleotide sequences in *E. coli* which recBCD specifically recognizes and cleaves endonucleolytically (Smith and Stahl, 1985). Recombination was demonstrated to occur at the highest frequency at Chi sites of a Chi-marked bacteriophage as compared to an unmarked control (Fraser, 1994; Smith, 1988). The recBCD enzyme also possesses a highly processive 5' → 3' exonuclease activity on dsDNA that was implicated in the production of 3' ssDNA tails which are characteristic of the early stages of homologous recombination (Fraser, 1994).

In contrast, eukaryotic EEs are simpler in action. They are typically homodimeric enzymes with no ATP requirement and broader specificity, acting on both DNA and RNA. They have exonuclease activity which lacks strand specificity, but has strict 5' → 3' polarity (Fraser and Low, 1993). The results presented in this dissertation provide the first evidence that directly links the processive nuclease activity of an endo-exonuclease to recombination processes. The RNC1 protein has been shown to possess nuclease activity on single-strand DNA as well as exonuclease activity of a 5' → 3' polarity on double-strand DNA. *In vivo*, the RNC1 gene product is active in DSB repair processes in degrading DNA ends (Fig. 9, Chapter 3). This activity may be responsible for directing broken chromosome ends through the HR repair pathway in *S. cerevisiae*.

4.3 Addressing possible alternate functions of the RNC1 protein *in vivo*

While we have conclusively shown that the RNC1 gene product displays properties characteristic of other endo-exonucleases, there are intriguing questions to be addressed concerning the recent discovery that *RNC1* possesses a domain which is highly homologous to the *trmA* gene of *E. coli* (Nordlund *et al.*, 2000). This domain has been implicated in the modification of a specific base, uracil, at position 54 (m^5U_{54}) of elongator tRNA (Nordlund *et al.*, 2000). There is no conclusive proof that this serves a functional purpose in translational processes, though it was reported that m^5U_{54}-deficient $tRNA^{lys}$ was more efficient in an *in vitro* translation system as compared to modified tRNA (Kersten *et al.*, 1981). In the study by Nordlund *et al.* (2000), disrupting this domain had the effect of abolishing the m^5U_{54} modification of elongator tRNA but has no discernible effect on cell survival. Similarly, we found that disruption of the C-terminal domain of RNC1 does not confer any lethality to MMS mutagenicity. Therefore, it appears that the domain which confers methyltransferase activity in RNC1 is distinct from the regions in the protein implicated in DSB repair processes.

4.4 Conclusion and Future direction

Endo-exonucleases are a unique class of nucleases which have been implicated early on in recombinational repair processes, beginning with the recBCD EE, which is a major factor in recA-mediated DSB repair in *E. coli* (Sargentini and Smith, 1986). It was anticipated that more recently identified eukaryotic EEs would make similar contributions to recombination mechanisms. This dissertation offers the first direct evidence of the role that eukaryotic EEs play in DSB repair. We have demonstrated that

RNC1 exhibits nucleolytic properties characteristic of other identified EEs, and shown that these properties are implicated in the processing of DSBs, preparing them for entry into the HR pathway. Based on the studies illustrated in Chapters II and III, it appears that Rad52 acts directly on the end-product of RNC1 processing, as indicated by the epistatic relationship between *RNC1* and *RAD52*, and the accumulation of extensively degraded DSB ends in the absence of Rad52. Furthermore, we have shown that the NHEJ pathway appears to play a more dominant role in the absence of a functional *RNC1* gene, suggesting that RNC1 and the DNA-binding components of the NHEJ pathway compete to act on a ruptured chromosome immediately following DSB induction.

However, while these results are compelling, we have yet to identify distinct domains on the RNC1 protein which are responsible for its multiple enzymatic activities. While RNC1 was shown to possess methyltransferase activity which localizes to the C-terminal portion of the protein (Nordlund *et al.*, 2000), the nuclease domain has yet to be identified. Also, RNC1 consistently displays DNA-binding activity, but as yet no DNA-binding motif has been characterized in the *RNC1* gene. In addition, we have recently identified a mouse homologue which shares extensive amino acid sequence homology to RNC1 (Fig. 1). We are in the process of cloning cDNA encoding this homologue into a yeast shuttle vector with the purpose of performing complementation analysis in the *rnc1* mutant to observe if *mRNC1* displays conservation of function in yeast. Generating a mouse knock-out cell line as well as thorough biochemical analysis of the mammalian RNC1 counterpart would be the next step towards addressing what possible role endo-exonucleases play in mammalian DSB repair processes.

```
Sc-RNC1:  441 QIVTEYVDGYTFNFSAGEFFQNNNSILPIVTKYVRDNLQAPAKGDDNKTKFLVDAYCGSG 500
              Q + E + G TF  S   FFQ N     ++   +++  Q     D   T  ++D  CG+G
Mus-RNC1: 445 QCIQEDLLGLTFRISPHAFFQVNTPAAEVLYTVIQEWAQL----DGGST--VLDVCCGTG 498

          501 LFSICSSKGVDKVIGVEISADSVSFAEKNAKANGVENCRFIVGKAEKLFESIDT--PSEN 558
                +  +  V +V+G+E+  ++V  A  NA  N + N  F  G+AE L   + +   S
          499 TIGLALAPKVKRVVGIELCQEAVEDARMNALTNELSNVEFHCGRAEDLVPGLVSRLSSHQ 558

          559 TSVILDPPRKGC-DELFLKQLAAYNPAKIIYISCNVHSQARDVEYFLKETEN----GSAH 613
                +LDPPR G   ++ L    A N  +++Y+SCN  +   +     +   N       H
          559 LVAVLDPPRAGLHSKVILAIRKAENIKRLLYVSCNPRAAMGNFVDLCRAPSNRVKGTPFH 618

          614 QIESIRGFDFFPQTHHVESVCIMKRI 639
               ++++   D FPQT H E + + +R+
          619 PVKAV-AVDLFPQTPHCEMLILFERM 643
```

Figure 1: Alignment of the *S. cerevisiae* RNC1 and its mouse homologue protein sequence

Once the role of the RNC1 EE has been clarified with respect to DSB repair, it may be utilized as a promising tool for gene therapy and anticancer treatments. Already, it has been shown that transient expression of *RNC1* increases levels of extrachromosomal recombination frequencies in mouse Ltk-fibroblasts (Semionov *et al.*, 1999b), and that expression of *RNC1* in HeLa cells elevates their resistance to ionizing radiation (Semionov *et al.*, 1999a). This complements other studies of recombination proteins such as human Rad51 and Rad52: Over-expression of *RAD51* has been found to stimulate extra-chromosomal recombination and confer resistance to IR in CHO cells (Vispe *et al.*, 1998). Similarly, over-expression of *RAD52* results in enhanced recombination between *lacZ* direct repeats in monkey FSH2 cells (Park, 1995) as well as increased resistance to IR in human HT1080 cells (Johnson *et al.*, 1996a). Potentially, *RNC1* may be utilized to surmount the major obstacle to current gene targeting strategies: the extremely low rates of homologous recombination in mitotic mammalian cells. In combination with other recombination factors, it may be used to direct *in situ* repair of defunct somatic genes by targeted homologous recombination.

In summary, this dissertation describes a yeast endo-exonuclease which is strongly implicated in the degradation of DSB ends prior to their commitment to the homologous recombination repair pathway, and presents the first direct evidence of the role of endo-exonucleases in recombination processes. Hopefully, these findings will provide impetus towards further investigations into endo-exonucleases of higher eukaryotes with respect to their roles in DNA recombination and repair.

Chapter V

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