A Study of DNA Mismatch Correction

in *Saccharomyces cerevisiae*

By

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ABSTRACT

The potential roles of the yeast *PMS1*, *MSH2* and *RAD3* genes in the correction of mismatches formed during intracellular DNA replication in the yeast *Saccharomyces cerevisiae* were examined. *PMS1* and *MSH2* are required for mismatch correction whereas *RAD3* functions in nucleotide excision repair and transcription. Inactivation of any of these three genes confers a mutator phenotype. To characterize the specificities of these mutator effects, *SUP4*-o mutations arising spontaneously in strains having the *PMS1* or *MSH2* genes deleted (*pms1Δ* or *msh2Δ*, respectively), or *RAD3* replaced with the *rad3-1* allele, were sequenced. To assess the role of mismatch correction in the mutator effects, heteroduplex plasmid DNAs containing defined mismatches in *SUP4*-o were constructed *in vitro* and transformed into the *pms1Δ*, *msh2Δ*, *rad3-1* and wildtype strains, respectively. Comparisons of the resulting mutational spectra for the three mutator strains with the corresponding spectrum for the isogenic wildtype strain, together with the examination of heteroduplex repair in these strains, generated a number of interesting observations. Both the *pms1Δ* and *msh2Δ* mutators increased the rates of single base-pair deletions/insertions to a much greater extent than the rate of single base-pair substitutions. However, the magnitude of the increase in the rate of single base-pair insertion was larger than that of single base-pair deletion in both mutators. These findings were consistent with the analysis of heteroduplex repair which demonstrated that: 1) single nucleotide loop mismatches were among the most efficiently corrected substrates assessed in the wildtype strain; and 2) a single nucleotide loop due to an extra nucleotide in one DNA strand was repaired twice as

efficiently as a single nucleotide loop due to a missing nucleotide in the same DNA strand. Thus, the data suggest that when mismatches form naturally during DNA replication in yeast, single nucleotide loops are repaired much more efficiently than base mismatches. Furthermore, these results argue that loops which can give rise to a single base-pair insertion are repaired more efficiently than the loops which can result in a single base-pair deletion in yeast. In contrast to the situation in *E. coli*, the mutational specificities of the *pms1Δ* and *msh2Δ* mutators indicated that, overall, the transversion rates were increased more than the transition rates. This suggests that, in general, naturally-occurring transition mismatches are not corrected more efficiently than transversion mismatches in yeast. Analysis of the specificity of spontaneous mutagenesis in the *rad3-1* strain did not provide evidence for the involvement of the Rad3 protein in mismatch correction. Unexpectedly, however, repair of heteroduplex plasmids was more efficient in the *rad3-1* background than in the wildtype strain. Thus, the *RAD3* gene product might somehow function to limit the need for mismatch correction in yeast. Finally, to determine whether DNA strand breaks or gaps can direct intracellular mismatch repair in yeast, heteroduplex plasmids with a break or gap 3′ or 5′ to the mismatched base, on the transcribed or nontranscribed strand, with respect to the *SUP4*-o gene, were constructed. The results indicated that strand gaps can modulate the direction and efficiency of mismatch correction in yeast cells.

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LIST OF ABBREVIATIONS

A	adenine
AP	alkaline phosphatase
2-AP	2-aminopurine
amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base-pair
BPS	base-pair substitution
C	cytosine
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGMP	2'-deoxyguanosine 5'-monophosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTMP	2'-deoxythymidine 5'-monophosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dNTP	2'-deoxyribonucleoside 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
ddNTP	2',3'-dideoxyribonucleoside 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FUMP	5-fluoro-uridine-5'-monophosphate
FdUMP	5-fluoro-2'-deoxy-uridine-5'-monophosphate
G	guanosine

GO	8-oxo-7,8-dihydrodeoxyguanine
8-oxo-dGTP	8-oxo-7,8-dihydrodGTP
8-oxo-dGMP	8-oxo-7,8-dihydrodGMP
FOA	5-fluoro-orotic acid
g, mg, mg, μg	gram, milligram, nanogram, microgram
h(rs)	hour(s)
HNPCC	hereditary non-polyposis colon cancer
kb	kilobase-pair
kDa	kilodalton
l, ml, μl	litre, millilitre, microlitre
M, mM, μM	molar, millimolar, micromolar
m, cm, nm	metre, centimetre, nanometre
5-meC	5-methylcytosine
min	minute
mt DNA	mitochondria DNA
Na_2EDTA	disodium ethylenediaminetetraacetic acid
NER	nucleotide excision repair
no.	number
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMS	post-meiotic segregation
pol	polymerase
pur	purine
pyr	pyrimidine
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SSB	single-stranded DNA binding protein
T	thymine
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA
UV	ultraviolet
v/v	volume/volume
VSP	very short patch
w/v	weight/volume
w/w	weight/weight

1 INTRODUCTION

DNA replication fidelity is necessary to ensure that genetic information is faithfully passed from one generation to the next. Errors which are not repaired by the proofreading mechanism of DNA polymerases during DNA replication must be corrected by other mechanisms or mutations will result. One process that contributes to the fidelity of DNA replication is mismatch correction. Postreplicative mismatch repair corrects DNA polymerase misinsertion errors, which are a major source of spontaneous mutations in dividing cells (Leong *et al.* 1986). Both prokaryotes and eukaryotes correct DNA mismatches, pairing errors in which the DNA bases occur in noncomplementary opposition within the DNA helix. Mismatches also arise as a result of spontaneous chemical modification of bases in duplex DNA, such as deamination of 5-methylcytosine (5-meC) to form thymine, and DNA recombination between homologous, but nonidentical DNA sequences. By recognizing and eliminating mismatches, mismatch correction plays an important role in ensuring the accuracy of DNA replication and suppressing spontaneous mutation rates in bacteria, fungi and higher eukaryotes (Modrich 1987; Grilley *et al.* 1990).

1.1 Mismatch Correction in *Escherichia coli*

The biochemical mechanisms of mismatch correction are best known for *E. coli* which possesses multiple mismatch repair systems (Grilley *et al.* 1990; Au *et al.* 1992). There are three major types of mismatch repair systems which have been identified in *E. coli*: (1) the methyl-directed, *mutHLS*-dependent mismatch repair system recognizes and corrects a variety of mismatches with different efficiencies, and processes mispairs in a strand-specific manner with discrimination of the two DNA strands being

based on the state of adenine methylation of d(GATC) sequences; (2) the very short patch (VSP) mismatch repair system specifically corrects G/T mismatches to G·C pairs, and this system requires the removal of less than 10 residues during the correction process; (3) the *mutY*-dependent mismatch repair system specifically corrects G/A or 8-oxoG-A mismatches to G·C pairs.

1.1.1 Methyl-Directed Mismatch Correction

The *mutHLS* pathway can repair mismatches including most base mispairs and insertion/deletion mismatches involving a small number of nucleotides (Kramer *et al*. 1984; Dohet *et al*. 1985, 1987; Jones *et al*. 1987b; Su *et al*. 1988; Lahue *et al*. 1989). The repair efficiency varies with the nature of the mismatch (Kramer *et al*. 1984), and also is influenced by DNA sequence context (Jones *et al*. 1987b). In general, transition mismatches (G/T and A/C) are better repaired than transversion mismatches (C/T, G/A, G/G, A/A, T/T and C/C) (Kramer *et al*. 1984; Wagner *et al*. 1984; Dohet *et al*. 1986). An *in vitro* DNA mismatch repair assay showed that 1-, 2- or 3- base insertion and deletion heteroduplexes also were repaired by the methyl-directed mismatch repair pathway as efficiently as the G/T mismatch (Learn and Grafstrom 1989). These results are consistent with *in vivo* studies that demonstrate the involvement of methyl-directed mismatch repair in the efficient prevention of frame shift mutations (Dohet *et al*. 1986; Fishel *et al*. 1986). In addition, for a given mismatch, the repair efficiency increases with increasing G·C content in the neighbouring nucleotide sequence (Jones *et al*. 1987b), and helical structure at the site of the mismatch also is associated with the efficiency of mismatch correction (Fishel and Kolodner 1983; Fishel *et al*. 1986; Radman and

Wagner 1986; Jones *et al.* 1987b).

This system processes mismatches in a strand-specific manner based on the state of adenine methylation of d(GATC) sequences (Wagner and Meselson 1976; Pukkila *et al.* 1983; Kramer *et al.* 1984; Wagner *et al.* 1984; Dohet *et al.* 1986; Lahue *et al.* 1987; Raposa and Fox 1987). Methylation at the N^6 position of the adenine residues in the d(GATC) sequences is carried out by the product of the *dam* gene with a delay relative to DNA synthesis (Marinus 1976; Lyons and Schendel 1984), and newly synthesized DNA strands are transitorily undermethylated (Glickman *et al.* 1978). Repair of a hemimethylated heteroduplex is restricted to the unmodified strand. When both DNA strands are modified, the heteroduplex is refractory to repair. A mismatch within an unmethylated DNA duplex is corrected with little strand bias (Pukkila *et al.* 1983; Wagner *et al.* 1984; Dohet *et al.* 1986). This system excises and resynthesizes large regions of DNA, up to several kilobase-pairs (kb) in length (Au *et al.* 1992), and requires the MutS, MutH, MutL and MutU proteins as well as other enzymes and cofactors (Nevers and Spatz 1975; Rydberg 1978; Bauer *et al.* 1981; Pukkila *et al.* 1983; Lahue *et al.* 1989). Mutations in any of the *mut* genes result in a mutator phenotype with a bias for transitions over transversions (Nevers and Spatz 1975; Rydberg 1978; Bauer *et al.* 1981; Pukkila *et al.* 1983). These genes have been cloned and sequenced (Grafstrom and Hoess 1983; Lu *et al.* 1984; Su and Modrich 1986). In addition, the MutH, MutL and MutS proteins have been isolated in near-homogeneous and biologically active forms (Su and Modrich 1986; Welsh *et al.* 1987; Grilley *et al.* 1989).

MutS is a 97 kilodalton (kDa) protein involved in mismatch correction (Su and Modrich 1986; Su *et al.* 1988). It specifically binds to DNA regions containing a single base-pair mismatch. It binds to the 8 possible

mismatches with different affinities (Su and Modrich 1986; Su *et al.* 1988). MutS has the highest affinity for a G/T mispair and the lowest affinity for a T/C mispair. There is some correlation between MutS affinity and the mismatch repair efficiency, but it is not exact, suggesting that aspects of the repair reaction other than mismatch recognition contribute to repair efficiency (Grilley *et al.* 1990). The physical basis of mismatch recognition by MutS has not been established yet. However, MutS has a weak ATPase activity and it mediates formation of DNA loops stabilized at the DNA junction by bound protein (Grilley *et al.* 1990). This loop formation requires the presence of a mispair within the DNA and is also dependent upon the presence of ATP (Grilley *et al.* 1990).

MutH, a 25 kDa protein, possesses a d(GATC) endonuclease activity that makes a nick in the unmethylated strand at a hemimethylated d(GATC) site, and so is required for strand discrimination (Modrich 1987; Welsh *et al.* 1987). MutH is a latent endonuclease that is activated in a reaction which requires MutS, MutL, ATP and Mg^{2+} and depends upon the presence of a mismatch within the DNA (Au *et al.* 1992). Incision of an unmethylated strand of hemimethylated DNA occurs immediately 5′ to a d(GATC) sequence, leaving 5′ phosphate and 3′ hydroxy termini (Modrich 1989). This process has no obligate directionality as d(GATC) sequence can be situated either 3′ or 5′ to the mismatch on an unmethylated strand (Au *at al.* 1992). In addition, the activation of MutH is sensitive to DNA topology. Circular heteroduplex plasmids are better substrates than linear molecules (Au *et al.* 1992). An *in vitro* assay showed that symmetrically methylated DNAs are resistant to cleavage by MutH (Welsh *et al.* 1987). Both strands of unmethylated d(GATC) sites can be cleaved by activated MutH endonuclease, which may account for the killing of *dam⁻* mutants by 2-aminopurine (2-AP)

(Au *et al.* 1992). *dam⁻* mutants are deficient in adenine methylation of d(GATC) sequences. Thus, both strands are unmethylated and can be cleaved by activated MutH endonuclease in the mutants (Marinus and Morris 1973, 1975). 2-AP is a base analogue which may cause mispairs in DNA. Such mispairs may occur with a sufficiently high frequency that a lethal double-strand break may ensue when nearby mismatches cause excision on opposite DNA strand in *dam⁻* mutants (Glickman 1982).

MutL is a 70 kDa protein that binds to the MutS-mismatch DNA complex (Grilley *et al.* 1989). The purified MutL protein can bind to double- or single-stranded DNA (Bende and Grafstrom 1991). The affinity constant of MutL for unmethylated single-stranded DNA is twice that of its affinity constant for methylated single-stranded DNA and methylated or unmethylated double-stranded DNA. The binding of MutL to double-stranded DNA is not affected by the pattern of DNA methylation or even the presence of d(GATC) sequences (Bende and Grafstrom 1991). An *in vitro* experiment indicated that purified MutL protein interacts with the MutS-heteroduplex DNA complex in the presence of ATP (Grilley *et al.* 1989). Therefore, MutL may act to interface mismatch recognition by MutS with activation of the latent MutH d(GATC) endonuclease (Grilley *et al.* 1990). However, the biochemical function of MutL protein in this DNA repair pathway still remains largely unknown.

The *mutU* (*uvrD*) gene product, DNA helicase II/DNA-RNA helicase, unwinds a DNA duplex in an ATP-dependent manner (Längle-Rouault *et al.* 1987; Runyon and Lohman 1989). It is possible that helicase unwinding initiates in the vicinity of the strand break or at the mismatch (Längle-Rouault *et al.* 1987; Runyon and Lohman 1989). Therefore, it may facilitate removal of the incorrect base in the mismatch (Maples and Kushner 1982; Kumura and

Sekiguchi 1984; Längle-Rouault *et al.* 1987; Runyon and Lohman 1989). The product of the *mutU* gene is also known to be involved in nucleotide excision repair (NER) (Caron *et al.* 1985; Husain *et al.* 1985).

In addition to the *mut* gene products, an *in vitro* repair reaction also requires single-stranded DNA binding protein (SSB), DNA ligase, exonuclease I or exonuclease VII, DNA polymerase III holoenzyme, ATP, and the four deoxyribonucleoside triphosphates (dNTPs) and DNA ligase (Modrich 1987; Grilley *et al.* 1990, 1993). SSB polymerizes processively along single-stranded DNA following the action of DNA helicase. SSB stabilizes the single strands and offers protection against some nucleases, but it enhances the sensitivity of single strands to hydrolysis by exonuclease I, an enzyme with which it forms a molecular complex (Molineuz and Gefter 1975). SSB-bound DNA is also a better template for replication by DNA polymerase III holoenzyme than is naked single-stranded DNA (Kornberg 1980; Chase and Williams 1986).

The strand specificity of mismatch repair can be directed by single-strand breaks in DNA, instead of DNA methylation (Längle-Rouault *et al.* 1987; Lahue *et al.* 1989). The requirement for the d(GATC) sequence in the substrate DNA, and for MutH function in this mismatch repair system, can be complemented by the presence of a persistent nick in the DNA provided that DNA ligase is absent (Längle-Rouault *et al.* 1987; Lahue *et al.* 1989). The absence of the ligase is to prevent the closure of the strand break prior to the initiation of mismatch repair. This repair reaction requires MutS, MutL, helicase II, SSB, polymerase III holoenzyme, and exonuclease I, along with ATP and the four dNTPs (Lahue *et al.* 1989).

The possible molecular mechanisms for methyl-directed mismatch repair have been discussed (Au *et al.* 1992; Grilley *et al.* 1990, 1993; Modrich

1989, 1994). Analysis of the MutH, MutL and MutS proteins suggests that these three proteins are sufficient to start a methyl-directed mismatch correction event (Modrich 1989). It is believed that the repair is initiated by the recognition of a mismatch by MutS, followed by the addition of MutL and MutH (Grilley et al. 1989). Formation of this complex leads to activation of a latent d(GATC) endonuclease associated with the MutH protein, which scans along the helix and incises the unmodified strand at a hemimethylated d(GATC) sequence (Au at al. 1992). The process is ATP-dependent (Au at al. 1992). The resulting strand break can occur on either side of the mismatch. When the unmethylated d(GATC) sequence that directs repair is located 5′ to the mismatch on the unmethylated strand, mismatch repair requires the 5′ → 3′ hydrolytic activity of exonuclease VII or RecJ exonuclease. Repair directed by an unmethylated d(GATC) sequence situated 3′ to the mismatch depends on the 3′ → 5′ activity of exonuclease I (Cooper et al. 1993; Grilley et al. 1993). The excision reaction removes that portion of the unmodified strand spanning the d(GATC) site and the mismatch (Au at al. 1992). The repair event is completed by resynthesis of incised DNA by a DNA polymerase III (Kornberg 1980) and ligation by a NAD^+-dependent DNA ligase to seal the remaining nick and restore the covalently continuous form of DNA (Lahue et al. 1989).

MutS, MutL and MutH not only play a role in correction of DNA biosynthetic errors, but also ensure the fidelity of genetic recombination by blocking crossovers between sequences that have diverged genetically (Rayssiguier et al. 1989). That is why mutS or mutL defects lead to recombination errors. In E. coli, recombination between DNA sequences that diverged by 10% is 1000-fold lower than between homologous sequences.

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However, in *mutS*, *mutL* and *mutH* mutants, the frequency of these homeologous recombination events is increased to nearly the frequency observed for homologous sequences (Rayssiguier *et al.* 1989; Shen and Huang 1989). The finding that homeologous recombination is increased in mismatch repair mutants is consistent with the idea that mismatch repair plays a central role in regulating recombination by preventing the formation of heteroduplex DNA containing a large number of base pair mismatches (Rayssiguier *et al.* 1989; Shen and Huang 1989). This would prevent crossovers between related sequences which are present in multiple copies in the bacterial genome, and hence the duplication and deletion mutations that result from such events (Petit *et al.* 1991). The molecular mechanism underlying these effects of MutS, MutL and MutH is less well understood. However, these proteins interact with mispairs that occur in the heteroduplex joint, a key recombination intermediate generated by the transfer of a strand from one helix into a region of homology within a second helix and pairing according to Watson-Crick base-pairing rules (Worth *et al.* 1994).

1.1.2 Very Short Patch (VSP) Mismatch Correction

VSP repair is characterized by short excision tracts that rarely exceed 10 nucleotides in length. It depends on the *vsr* and *polA* genes and is strongly stimulated by MutL and MutS (Jones *et al.* 1987a; Lieb 1987; Zell and Fritz 1987; Sohail *et al.* 1990). It specifically restores G/T mismatches to G·C pairs, and is mainly responsible for repairing deaminated 5-meC (Raposa and Fox 1987; Lieb 1987, 1991). In *E. coli* K-12, 5-meC is formed by DNA cytosine methyltransferase (*dcm*) at the second position within the sequence d(CCATGG) (Marinus 1984). 5-meC can deaminate

spontaneously to thymine. This event produces a G/T mismatch which if left unrepaired, will result in a G·C → A·T transition.

The *vsr* gene product, which is an 18 kDa protein, has been purified and characterized as a DNA mismatch endonuclease (Hennecke *et al.* 1991). The Vsr endonuclease nicks double-stranded DNA within the sequence d(CTATGN) or d(NTATGG) 5′ to the underlined thymine residue, which is mispaired with deoxyguanine. The incision is mismatch-dependent and strand-specific (Hennecke *et al.* 1991). Once the cut is made, DNA polymerase I might remove the dTMP residue with its 5′ → 3′ exonuclease activity and commence repair synthesis in the usual template-directed fashion (Bambara *et al.* 1978).

VSP repair is also known to repair G/T mismatches that do not arise from 5-methylcytosine deamination (Lieb *et al.* 1986). In this case, if the original base pair was an A·T, VSP repair would cause a T → C transition. This result is consistent with an analysis of an *E. coli* sequence database, which suggests that VSP repair might tend to deplete the genome of the "T" containing sequences (e.g., CTAG), while enriching it for the corresponding "C" containing sequences (CCAG) (Bhagwat and McClelland 1992). This provides an explanation for the known scarcity of CTAG-containing restriction enzymes sites among the genomes of bacteria and identifies VSP repair as a force in shaping the sequence composition of bacterial genomes (Bhagwat and McClelland 1992). Additionally, it might also explain why in the *E. coli* genome the ratio of (G+C)/(A+T) is 1.09 (Belozersky and Spirin 1958).

The Vsr and Dcm proteins appear to be made from a single RNA transcript but there is a six-codon overlap between *vsr* and *dcm*, and translation of *dcm* is required for the efficient synthesis of Vsr (Sohail *et al.* 1990;

Dar and Bhagwat 1993). Since the translation of *dcm* also leads to the translation of *vsr*, VSP repair is likely to be active whenever Dcm is present in the cell though VSP repair does not require the *dcm* product (Sohail *et al.* 1990; Dar and Bhagwat 1993). Since Dcm is, indirectly, the major cause of the creation of postreplicative G/T mismatches in *E. coli* DNA, this strategy for Vsr synthesis may minimize the mutagenic effects of cytosine methylation, which causes C → T transition mutations (Sohail *et al.* 1990; Dar and Bhagwat 1993).

In addition to the *vsr* gene, another gene, named *mutG*, also functions in the prevention of C → T mutations in yeast. Mutations in this gene also lead to a high frequency of C → T transition mutations at the second G of the sequence d(CCATGG) (Ruiz *et al.* 1993). Although *mutG* is distinct from *vsr*, the exact function of the *mutG* gene product is not known yet.

1.1.3 Dependence of G/A → G·C Correction on *mutY*

The *mutY* system of *E. coli* specially corrects G/A mismatches, where the adenine resides on the nascent DNA strand, to G·C pairs. Mutations in the *mutY* gene confer hypermutability, specifically the induction of G·C → T·A transversions and result in a deficiency in methylation-independent G/A → G·C mismatch correction (Au *et al.* 1988; Nghiem *et al.* 1988). Because this system is independent of the state of d(GATC) methylation, it does not require the *mutH*, *mutL*, or *mutS* gene products (Nghiem *et al.* 1988). In addition, *in vivo* experiments with heteroduplex λ genomes showed that the repair tract is shorter than 27 nucleotides and longer than 9 nucleotides, and extends 3' from the corrected adenine (Radicella *et al.* 1993). Similarly, *in vitro* experiments with an A/G mismatch-containing M13mp18 heteroduplex incubated with *E. coli* cell extracts indicated that the

repair tract was shorter than 12 nucleotides and longer than 5 nucleotides, and was localized to the 3' side of the mismatched adenine (Tsai-Wu and Lu 1994). In the latter study, repair synthesis was catalyzed by DNA polymerase I (Tsai-Wu and Lu 1994).

The *mutY* gene has been cloned and found to encode a 39 kDa protein of 350 amino acids. The clone genetically complemented a *mutY* defective strain (Michaels *et al.* 1990a). The protein showed significant sequence homology to *E. coli* endonuclease III, an enzyme that has previously been shown to have glycosylase activity on damaged base pairs. Sequence analysis also indicated that, like endonuclease III, MutY is an iron-sulfur protein with a $[4Fe-4S]^{2+}$ cluster (Michaels *et al.* 1990a). Au *et al.* (1989) have shown that the MutY protein renders the strand containing the mispaired adenine sensitive to cleavage by several apurinic/apyrimidinic-site endonucleases. These findings suggest that MutY is a DNA glycosylase that hydrolyses the glycosyl bond linking the mispaired adenine to deoxyribose. MutY, DNA polymerase I, and DNA ligase are sufficient to repair G/A mismatches to G·C pairs *in vitro* (Au *et al.* 1988, 1989; Michaels *et al.* 1990b). However, *in vivo* experiments with heteroduplex λ genomes showed that DNA polymerase I played an important, but not essential role in the *in vivo* repair of apurinic sites generated by this system. In the absence of PolA, the repair tracts were modestly longer than in the *polA+* strain, and extended in the 5' direction from the corrected adenine, indicating a role for another DNA polymerase (Radicella *et al.* 1993).

Recently, it has been found that the primary function of MutY *in vivo* is to remove adenine that is misinserted opposite 8-oxo-7,8-dihydrodeoxyguanine (GO) (Michaels *et al.* 1992a, 1992b), an oxidatively

damaged form of guanine, during DNA synthesis. MutY, MutM (see section 1.1.4) and MutT (see section 1.1.4) combine to prevent mutation by this oxidatively damaged form of guanine in DNA (Figure 1). Therefore, MutY, MutM and MutT are parts of a multiple line of defense against oxidative damage to DNA (Michaels *et al*. 1992a, 1992b).

1.1.4 Related Pathways

In addition to the above mismatch repair systems, there may be other system(s) or other genes which are also involved in mismatch correction in *E. coli*.

mutA and *mutC* are two transversion-specific mutator loci (Michaels *et al*. 1990a). The mutant strains have higher frequencies of A·T → T·A and G·C → T·A transversions, and to a lesser degree, A·T → C·G transversions. It is likely that MutA and MutC are components of the same error-avoidance system.

The *mutM* gene is also another locus which is involved in the avoidance of G·C → T·A transversion (Cabrera *et al*. 1988). MutM protein is a glycosylase which removes GO lesions from DNA (Figure 1) (Tchou *et al*. 1991; Michaels *et al*. 1992a, 1992b.). Inactivation of the *mutM* gene leads specifically to G·C → T·A transversions (Cabrera *et al*. 1988; Michaels *et al*. 1991) because DNA synthesis past GO lesions leads to the misincorporation of dAMP opposite the damaged guanines (Wood *et al*. 1990; Moriya *et al*. 1991; Cheng *et al*. 1992).

mutT mutants are strong mutators, increasing by about 100-to 10,000-fold the frequency of A·T → C·G transversion both *in vivo* and *in vitro* (Yanofsky *et al*. 1966; Cox 1973, 1976; Schaaper and Dunn 1987). The MutT protein, which has a molecular mass of 15 kDa, was overproduced and

Figure 1. GO repair system. (A) The structure of the predominant tautomeric form of the GO lesion. (B) Oxidative stress can lead to GO lesions in DNA. The GO lesions can be removed by MutM protein and subsequent repair can restore the original G•C base pair. If the GO lesion is not removed before replication, translesion synthesis can be accurate, leading to a C•GO pair, the substrate for MutM protein. However, translesion synthesis by replicative DNA polymerases is frequently inaccurate, leading to the misincorporation of dAMP opposite the GO lesion. MutY glycosylase removes the misincorporated dA from the A/GO mispairs that result from error-prone replication past the GO lesion. If accurate, repair synthesis can lead to a C•GO pair again, a substrate for MutM. (C) Oxidative stress can also lead to damaged deoxynucleotides, such as 8-oxo-dGTP. MutT is active on 8-oxo-dGTP and hydrolyzes it to 8-oxo-dGMP, effectively removing it from the dNTP pool. If MutT were not active and replication occurred with 8-oxo-dGTP in the dNTP pool, replication could be accurate or inaccurate, with 8-oxo-dGTP being misincorporated opposite template adenines to form A/GO mispairs. MutY could be involved in fixing these mutations because it is active on the A/GO substrate and would remove the template A, leading to the A•T → C•G transversions that are characteristic of a *mutT* strain. This figure is modified from Michaels *et al.* (1992b).
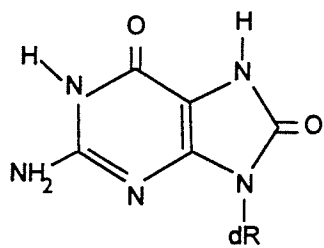
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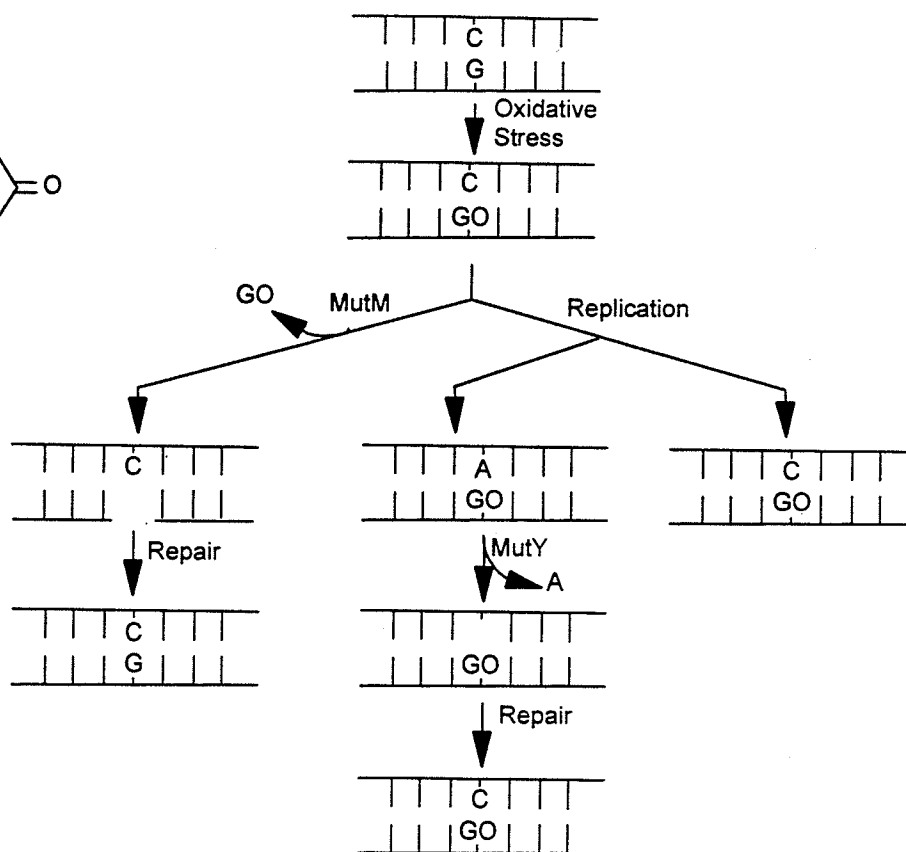
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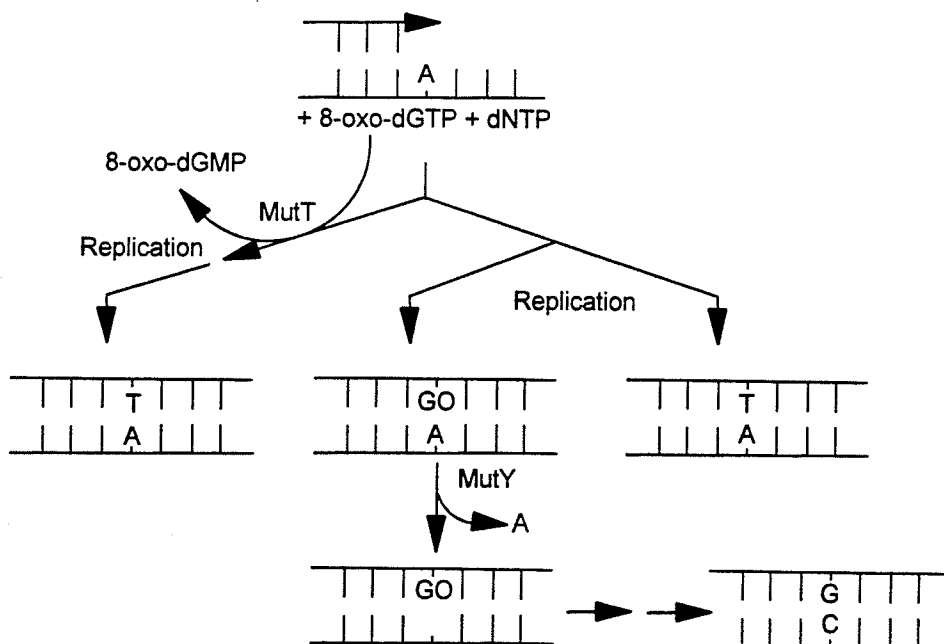
C

purified to near homogeneity (Bhatnagar and Bessman 1988). MutT possesses a distinct dGTPase activity and also an activity that prevents the misincorporation of dGMP into poly(dA) templates (Akiyama *et al.* 1989). Au *et al.* (1988) suggested that MutY and MutT provide complementary functions that serve to exclude G/A biosynthetic errors. Loss of mutT function results in the accumulation of A·T → C·G transversions mutation (Cox 1976) due to the failure to exclude G/A mispairs in which the guanine base of the mismatch resides on the newly synthesized DNA strand (Schaaper and Dunn 1987). Furthermore, it was found by Schaaper *et al.* (1989) that *mutT*-induced A/G replication errors are not recognized by the *mutHLS* system, which suggests that A/G mispairs may exist in several different conformations, some of which are recognized by the *mutHLS* system, others by *mutT*. However, it has been shown *in vitro* that MutT protein can hydrolyze 8-oxo-7,8-dihydrodGTP (8-oxo-dGTP) (Maki and Sekiguchi 1992), a mutagenic substrate which readily mispairs with adenine (Shibutani *et al.* 1991). This result suggested that one function for MutT may be to reduce the presence of 8-oxo-dGTP in the dNTP pool during DNA replication (Figure 1), therefore decreasing A/G mispairing. In the absence of MutT protein, 8-oxo-dGTP is frequently misincorporated opposite a template A (Maki and Sekiguchi 1992). This activity provides a potential explanation for the specific increase in A·T → C·G transversions in a *mutT* strain (Yanofsky *et al.* 1966). Therefore, MutT also defends against oxidative damage to DNA (Michaels *et al.* 1992a, 1992b) (Figure 1). Additionally, both *mutT* and proofreading (MutD) by DNA polymerase III make major contributions to the avoidance of A·T → C·G transversions. The double mutator strains produce more of these events than would be expected from simple additivity of the single mutator strains (Fowler *et al.* 1992). This suggests that the *mutT*

pathway interacts with the proofreading pathway.

1.2 Mismatch Correction in *Saccharomyces cerevisiae*

It has been found that mismatch repair also occurs in the yeast *Saccharomyces cerevisiae* (Bishop and Kolodner 1986; Bishop *et al*. 1987, 1989; Kramer *et al*. 1989a). *In vivo* evidence for mismatch correction was first obtained from studies of meiotic recombination (Fogel *et al*. 1979; Hastings 1984, 1987, 1988). For a pair of allelic gene markers, the expected Mendelian segregation ratio is 4:4. Non-Mendelian segregation ratios due to gene conversion (6:2) or post-meiotic segregation (PMS) (5:3 or 3:5) are associated with meiotic recombination (White *et al*. 1985; Hastings 1988). Gene conversion, the phenomenon of non-reciprocal transfer of genetic information, has been suggested to reflect mismatch repair of a heteroduplex DNA recombination intermediate which contains at least one mismatch (Holliday 1974; Meselson and Radding 1975; Radding 1978). PMS events are thought to reflect the failure to correct a mismatch resulting in the persistence of a heteroduplex region containing both parental genotypes during the process of meiotic recombination (White *et al*. 1985; Hastings 1988). The frequency of PMS per aberrant segregation was found to depend to a large extent on the type of mismatch that could be formed. C/C mismatches were inefficiently repaired in meiotic yeast cells as evidenced by their high PMS frequencies compared with other single base-pair mismatches (White *et al*. 1985; Detloff *et al*. 1991). C/C mismatches were also poorly repaired in mitotic yeast cells, in comparison with other single base-pair mismatches (Kramer *et al*. 1989a).

Mismatch correction in mitotic cells has been studied by transforming heteroduplexes into intact cells. The results demonstrated that defined

mismatches can be recognized and corrected *in vivo* and the repair tracts are shorter than 1 kb (Bishop and Kolodner 1986; Bishop *et al*. 1987, 1989; Kramer *et al*. 1989a). By using covalently closed heteroduplex plasmid substrates, it has been demonstrated that mitotic yeast cells are able to repair single base-pair substitution mismatches and single nucleotide loop mispairs, with the hierarchy of correction efficiencies depending on the nature of the mispair (Bishop and Kolodner 1986; Bishop *et al*. 1987, 1989; Kramer *et al*. 1989a). In general, most single base-pair substitution (transition and transversion) mismatches were corrected efficiently (Bishop and Kolodner 1986; Bishop *et al*. 1987, 1989; Kramer *et al*. 1989a), while the C/C mispair (Bishop *et al*. 1989; Kramer *et al*. 1989a) and the T/T mismatch (Bishop *et al*. 1989) were found to be poor substrates for correction. However, it has been demonstrated that single nucleotide loop mispairs, which can generate single base-pair deletions or additions, were corrected more efficiently than single base-pair substitution mismatches (Bishop *et al*. 1989; Kramer *et al*. 1989a). Thus, these studies with artificial substrates suggest that the specificity of repairing naturally-occurring mismatches might differ considerably in eukaryotes and prokaryotes.

In vitro experiments showed that yeast extracts can nick DNA fragments containing defined single base-pair mismatches. Furthermore, the nicking is strand-specific as the same DNA strands of the DNA fragments with mismatches were subject to mismatch-specific nicking. This activity may be involved in mismatch repair during genetic recombination (Chang and Lu 1991). Furthermore, a band shift assay showed mismatch-binding activity is present in nuclear extracts of wildtype strains (Miret *at al*. 1993). In wildtype strains, single nucleotide loops (T/-) and single base-pair

mismatches (T/G, G/G, G/A, A/C and T/C) are recognized by this activity, while recognition of a C/C mispair is very weak. The binding is primarily determined by the presence of a mispair rather than by DNA context (Miret *et al.* 1993).

Elements of the *E. coli mutHLS* system appear to have been evolutionarily conserved in prokaryotes and eukaryotes. *Salmonella typhimurium* possesses homologs of all of the *E. coli mut* genes and they are capable of complementing the corresponding mutations of *E. coli* (Pang *et al.* 1984). The gram-positive bacterium *Streptococcus pneumoniae* has the hex repair system which repairs heteroduplex DNA containing mismatches (Claverys and Lacks 1986). Genes encoding structural homologs of MutL and MutS have been identified in *S. cerevisiae* (Kramer *et al.* 1989b; Reenan and Kolodner 1992a; New *et al.* 1993; Prolla *et al.* 1994a). The first molecular evidence for the conservation of DNA mismatch repair came from the characterization of *PMS1* gene, the protein product of which exhibits homology to MutL (Williamson *et al.* 1985; Kramer *et al.* 1989b). Mutations in the *PMS1* gene result in elevated rates of spontaneous mutation and increased levels of PMS. By using the polymerase chain reaction (PCR) with degenerate oligonucleotide primers based on conserved regions of *E. coli* MutS and its homologs from *S. typhimurium* and *S. pneumoniae*, two DNA mismatch repair genes, *MSH1* and *MSH2*, were identified and found to encode proteins displaying homology to prokaryotic MutS (Reenan and Kolodner 1992a, b). Disruption of the *MSH2* gene affects nuclear mutation rates and PMS levels in a manner similar to mutation of the *PMS1* gene. The *MSH1*-encoded protein is involved in mitochondrial DNA (mt DNA) maintenance. A third *mutS* homolog, *MSH3*, was identified by PCR with degenerate oligonucleotides (New *et al.* 1993). Disruption of the *MSH3* gene slightly

increases PMS for some gene markers but does not induce a strong mitotic mutator phenotype (New *et al.* 1993). By using the same PCR approach, a new yeast gene, *MLH1*, that encodes a predicted protein with sequence similarity to the MutL and HexB proteins of bacteria and the yeast Pms1 protein, was identified (Prolla *et al.* 1994a). The mutator and meiotic phenotypes of the *MLH1* deleted strain is indistinguishable from those of the *PMS1* deleted strain (Prolla *et al.* 1994a).

1.2.1 *MSH* Genes

The *MSH1* (*mutS* homolog) and *MSH2* genes of *S. cerevisiae* were isolated, characterized and predicted to encode proteins that are homologous to the *E. coli* MutS and *S. pneumoniae* HexA proteins. The deduced amino acid sequences predict polypeptides of 109.3 kDa and 109.1 kDa for Msh1 and Msh2, respectively (Reenan and Kolodner 1992a). The overall amino acid sequence identity with the *E. coli* MutS protein is 28.6% for the Msh1 protein and 25.2% for the Msh2 protein. A comparison between Msh1 and Msh2 revealed that they are 17.2% identical and 31.5% similar when conservative amino acids substitutions are considered (Reenan and Kolodner 1992a). Disruption of the *MSH1* gene causes mitochondrial mutagenesis and large scale rearrangement of mt DNA. These findings suggest that the *MSH1* gene is either involved in repair of mt DNA damage or in the maintenance of mt DNA (Reenan and Kolodner 1992b), although mismatch repair has not been demonstrated in mitochondria yet. The mt DNA polymerase lacks a $3' \rightarrow 5'$ proofreading exonuclease and could therefore have a high in *vivo* misincorporation rate (Dujon 1981). This suggests that a mismatch repair system may be required in mitochondria to increase the fidelity of DNA replication. The phenotype conferred by defects in the *MSH1* gene supports

this idea.

msh2 mutants display an increase of 85-fold in the rate of spontaneous mutation to canavanine resistance and a high level of lethality during sporulation of homozygous *msh2/msh2* diploids (Reenan and Kolodner 1992b). *msh2* mutations also decrease meiotic gene conversion and increase PMS events of two *HIS4* alleles, two 4-base insertion mutations, suggesting the *MSH2* gene product appears to play a role in repair of 4-bp insertion/deletion mispairs in the nucleus (Reenan and Kolodner 1992b). It was shown *in vitro* that mismatch-binding activity is present in nuclear extracts of wildtype strains but is completely absent from *msh2* strains (Miret *at al.* 1993). This is evidence for a general mismatch-binding activity in nuclear extracts of yeast which is dependent on the product of *MSH2*.

A third yeast *mutS* homolog (*MSH3*) has been identified and is more closely related to mammalian *mutS* homologs than are *MSH1* and MSH2 (New *et al.* 1993). Additionally, no loss of mismatch-binding activity was observed in extracts missing *MSH3* function (Miret *at al.* 1993). The effect of *MSH3* disruption on reversion rates and postmeiotic segregation appears to be much less than that of other yeast DNA mismatch repair genes (New *et al.* 1993). Moreover, the *MSH3* disruption apparently has no effect on the canavanine spontaneous mutations (Alani *et al.* 1994). However, *MSH3* may play a role in DNA mismatch repair since the reversion rates of the frame shift mutations *hom3-10* and *lys2ΔBgl* are increased 10-fold and 7-fold, respectively, in *msh3* disruption strains relative to their isogenic wildtype parent strains (New *et al.* 1993). Allignment of the *MSH3* sequence with all of the known MutS homologs indicates that *MSH3* may be more closely related to the mouse *Rep-3* gene and other similar eukaryotic *mutS*

homologs, than to the yeast gene *MSH2* and other *mutS* homologs that are involved in replication repair (New *et al.* 1993). Thus, it has been suggested that the primary function of *MSH3* may be analogous to one of the other known functions of *mutS*, such as its role in preventing recombination between non-identical sequences (New *at al.* 1993).

The existence of more than one yeast *mutS* homolog suggests that there may be multiple mismatch repair systems in yeast, similar to the situation in *E. coli* (Reenan and Kolodner 1992a).

1.2.2 *PMS* Genes

PMS1, *PMS2*, *PMS3* and *PMS6* also are required for mismatch repair in yeast (Kramer *et al.* 1989a; Jeyaprakash *et al.* 1994). Mutations in the *PMS1*, *PMS2* and *PMS3* loci result in a mitotic mutator phenotype, with homozygous mutant diploids exhibiting elevated PMS frequencies and reduced spore viability (Williamson *et al.* 1985; Kramer *et al.* 1989a). The reduced spore viability has been attributed to accumulation of recessive lethal mutations in the diploid backgrounds (Kramer *et al.* 1989b). Transformation of wildtype and *pms* cells with heteroduplex plasmid substrates has provided direct evidence for the recognition and processing of mismatches in yeast (Bishop and Kolodner 1986; Bishop *et al.* 1987, 1989; Kramer *et al.* 1989a). In contrast to results obtained upon transformation of the wildtype strains, repair of all mismatches including single nucleotide loop mispairs was dramatically reduced in *pms1* and *pms2* strains (Bishop and Kolodner 1986; Bishop *et al.* 1987, 1989; Kramer *et al.* 1989a). Low-efficiency repair also was characteristic of *pms3* mutant strains, except that correction of single nucleotide loop mispairs occurred with an efficiency close to that of *PMS* wildtype strains (Kramer

et al. 1989a). In addition, strains with the *pms1* mutation had a 10-fold elevated frequency of instability of a palindromic repeat inserted upstream of the ß-galactosidase gene (Henderson and Petes 1993).

The *PMS1* gene has been cloned and sequencecd (Kramer *et al.* 1989b). The nucleotide sequence showed a 2,712 bp open reading frame (ORF) which predicts a polypeptide of 103 kDa. The deduced amino acid sequence of the Pms1 protein contains regions conserved in the MutL protein of *S. typhimurium* and the HexB protein of *S. pneumoniae* (Kramer *et al.* 1989b). These findings provide strong support for the idea that *the PMS* genes encode components of a mismatch repair system in yeast. The yeast *PMS*-dependent pathway may be similar to the bacterial nick-dependent repair pathway. The *pms1* and *msh2* phenotypes are indistinguishable, suggesting that the wildtype gene products act in the same repair pathway (Alani *et al.* 1994). However, the exact natures of the protein products encoded by the yeast *PMS2*, *PMS3* and *PMS6* genes are not known yet (Jeyaprakash *et al.* 1994).

1.2.3 *MLH1* Gene

The *MLH1* (*mutL homolog*) gene was isolated using PCR to detect sequences homologous to *PMS1* (Prolla *et al.* 1994a). *MLH1* encodes a predicted 769 amino-acid protein, and the protein displays significant homology to bacterial mismatch repair proteins (MutL, HexB), and the yeast Pms1 protein. Overall, the *MLH1* gene product has 24% amino acid sequence identity to the Pms1 protein, although this homology is limited mostly to the N-terminal 300 amino acids (Prolla *et al.* 1994a). Thus, in contrast to *E. coli*, there are at least two MutL/HexB-like proteins (Mlh1 protein and Pms1 Protein) in *S. cerevisiae*.

Disruption of the *MLH1* gene in haploid cells resulted in elevated spontaneous mutation rates during vegetative growth, as measured by forward mutation to canavanine resistance and reversion of the *hom3-10* allele. *MLH1* inactivation in diploid strains increased PMS and spore lethality (Prolla *et al.* 1994a). The mutator and meiotic phenotypes of the *mlh1pms1* double mutant are indistinguishable from those of the *mlh1* and *pms1* single mutants (Prolla *et al.* 1994a). Thus, each of the MutL/hexB-like proteins likely is a component of the same DNA mismatch repair system in *S. cerevisiae*.

1.2.4 Tract Instability and Mismatch Correction Genes

The genomes of all eukaryotes contain "microsatellite" sequences, tracts of DNA in which a single base or a small number of bases is repeated. Among these tracts, poly(GT)$_{10-30}$ is the most common simple repeat (Hamada *et al.* 1982). Tracts of simple repetitive DNA change length at frequencies that are considerably higher than expected for "standard" point mutations (Strand *et al.* 1993). Expansions and contraction of these microsatellite sequences or similar repeats have been associated with cancer and several heritable diseases (Kuhl and Caskey 1993). Transformation of yeast strains with plasmids containing 29-bp or 33-bp poly (GT) repeats, showed that mutations in the yeast DNA mismatch repair genes *MLH1*, *MSH2* and *PMS1* lead to 100- to 700-fold increases in tract instability, while mutations that eliminate the proofreading function of DNA polymerases have little effect (Strand *et al.* 1993). These results suggest that DNA polymerases have a very high rate of slippage *in vivo* on templates containing simple repeats. However, most of these errors are corrected by cellular mismatch repair systems. Therefore, the instability

of simple repeats observed for some human cancers and genetic diseases may be a consequence of decreased efficiency of mismatch repair (see section 1.3.2) (Strand *et al*. 1993).

1.2.5 Possible Mechanism of Mismatch Correction in Yeast

The fact that yeast contains the *mutL* homologs *MLH1* and *PMS1*, as well as the mutS-like *MSH2* gene, suggests that yeast possesses a mismatch repair system similar to the *mutHLS* system of *E. coli*. However, as yeast DNA is not detectably methylated (Proffitt *et al*. 1984), the yeast system is unlikely to be methyl-directed like the *E. coli* system. How does the yeast mismatch repair system distinguish the template from the nascent strand following replication so as to eliminate the incorrect base in a mismatched base pair? It has been proposed that strand breaks may be involved. One possible way to generate strand breaks in the newly replicated DNA is to incorporate dUTP into the DNA followed by the sequential action of uracil-DNA-glycosylase (*UNG1*) and an apurinic/apyrimidinic endonuclease to form a small gap (Aprelikova *et al*. 1989; Tomilin and Aprelikova 1989). These strand breaks might be the ultimate signal for strand discrimination in mismatch correction of yeast. However, the research done by Impellizzeri *et al*. (1991) did not support involvement of the *UNG1* gene in strand-specific mismatch repair in *S. cerevisiae*. Instead, *UNG1* was suggested to function in the repair of spontaneous cytosine deamination events. These results are consistent with the data of Verri *et al*. (1992), which showed that the uracil-DNA glycosylases encoded by the human, Herpes simplex 1 and *E. coli* genomes, excise uracil from U/G mispair more efficiently than from U/A mispair. In addition, a study done by Neddermann and Jiricny (1994) showed that a

uracil DNA glycosylase is required for efficient removal of uracil from U/G mispairs in HeLa cells. However, the enzyme did not recognize uracil in either U/A pairs or single-stranded substrates. So, in yeast, factors controlling the strand specificity of mismatch repair have not yet been established.

The yeast Mlh1 and Pms1 proteins physically associate, possibly forming heterodimer, and Mlh1 and Pms1 act in concert to bind a Msh2-heteroduplex complex containing a G/T mismatch (Prolla et al. 1994b). Thus, it appears that the Msh2, Mlh1 and Pms1 proteins likely form a complex during the initiation of DNA mismatch repair. On the basis of above experiments, a model of early events in eukaryotic DNA mismatch repair has been proposed (Marx 1994; Prolla et al. 1994b) as follows. Mismatch correction is initiated by Msh2 protein recognition of DNA single base-pair mismatches or small heterologies, the latter presumed to occur as a result of strand misalignment during DNA replication of microsatellites and other repeated sequences, or base-pair runs. After recognition of a DNA mispair, Msh2 protein may undergo a conformational change that increases its DNA binding affinity or alters the local DNA conformation. This is followed by binding of an Mlh1-Pms1 heterodimer to the Msh2-DNA complex, which in turn is likely to recruit additional proteins involved in actual repair. An excision reaction removes a portion of the single-stranded DNA which contains the mismatch or other defect, and this is followed by resynthesis by a DNA polymerase and ligation by a ligase to finish the repair (Marx 1994; Prolla et al. 1994b).

1.2.6 Interaction between Mismatch Repair and Genetic Recombination

Unlike *E. coli*, the functions of mismatch repair genes in yeast DNA

recombination are not clear. In yeast, recombination between homeologous sequences is not under as tight control as has been observed in *E. coli* and homeologous sequences containing 27% sequence divergence recombine at about 5-10% of frequency observed for homologous sequences (Bailis and Rothstein 1990; Mezard *et al*. 1992). *pms1* mutants showed no change in the frequency of homeologous recombination, suggesting that the yeast mismatch repair system involving *PMS1* does not act to prevent recombination between divergent DNA sequences (Bailis and Bothstein 1990). It is not known whether any other putative mismatch correction genes play a role in regulating recombination between divergent DNA sequences. However, a number of studies in yeast have shown a strong correlation between meiotic gene conversion and reciprocal recombination events; 20-50% of gene conversion events at a particular locus are accompanied by crossing over at an adjacent locus (reviewed in Petes *et al*. 1991). In addition, since heteroduplex DNA represents an intermediate step in the formation of a gene conversion event, analysis of gene conversion events can be used to examine the efficiency of mismatch repair (White *et al*. 1985; Bishop *et al*. 1987, 1989; Kramer *et al*. 1989a; Lichten *et al*. 1990; Detloff *et al*. 1991). By comparing meiotic gene conversion and PMS events between a wildtype strain and *msh2* mutants, it was found that gene conversion was greatly decreased while PMS events were increased in the *msh2* mutator, indicating that mismatch repair plays an important role in genetic recombination (Alani *et al*. 1994). As the *msh2*, *pms1* and *mlh1* mutant phenotypes are indistinguishable (Kramer *et al*. 1989a; Reenan and Kolodner 1992b; Prolla *et al*. 1994a), the wildtype gene products likely act in the same repair pathway. Thus, it has been suggested that mismatch repair proteins specifically interact with recombination enzymes to regulate the

length of symmetric heteroduplex DNA (Alani *et al.* 1994).

1.2.7 A Potential Role for *RAD3* in Mismatch Correction

Besides the *MLH1*, *MSH* and *PMS* genes, the *RAD3* gene might also be involved in postreplicative mismatch correction in yeast. *RAD3* is required for the incision step of nucleotide excision repair (NER) in *S. cerevisiae* (Reynolds and Friedberg 1981; Wilcox and Prakash 1981). The demonstration that *rad3* deletion mutants are inviable (Higgins *et al.* 1983; Naumovski and Friedberg 1983) indicated that the Rad3 protein also has an essential role and thus, is multifunctional. This essential role is now known to be in transcription. *RAD3* encodes the 85 kDa subunit of yeast RNA polymerase II initiation factor b, a homolog of human TFIIH (Feaver *et al.* 1993). Accordingly, a temperature-sensitive, conditional lethal *rad3* allele renders mRNA synthesis thermolabile, *in vitro* the temperature-sensitivity of transcription is offset by Rad3 protein, and factor b restores NER to extracts from a *rad3* mutant (Guzder *et al.* 1994; Wang *et al.* 1994). Analysis of the Rad3 protein revealed that it has DNA-dependent ATPase/helicase activity and unwinds DNA and DNA·RNA duplexes (Sung *et al.* 1987a,b; Harosh *et al.* 1989; Bailly *et al.* 1991; Naegeli *et al.* 1992). The Rad3 ATPase/DNA helicase function likely is necessary for NER (Sung *et al.* 1988), but may not be required for transcription (Feaver *et al.* 1993).

Defects in *RAD3* not only can sensitize yeast cells to a variety of DNA-damaging agents, but also can increase spontaneous and induced mutation and mitotic recombination (Haynes and Kunz 1981; Friedberg 1988; Song *et al.* 1990). These effects are not all produced to the same extent by all *rad3* mutations (Montelone *et al.* 1988; Song *et al.* 1990). With some exceptions, potent *rad3* mutator alleles were found to have mutations in

consensus motifs for DNA helicases (Song *et al.* 1990). This correlation led to the suggestion that the Rad3 helicase also is needed for the fidelity of DNA replication or postreplicative mismatch repair (Song *et al.* 1990; Montelone *et al.* 1992). Subsequently, the specificity of the *rad3-102* mutator was determined and interpreted as support for the involvement of the Rad3 protein in mismatch correction (Montelone *et al.* 1992).

In contrast, an earlier report indicated that post-meiotic segregation, was not increased in a *rad3-2* strain (DiCaprio and Hastings 1976). Furthermore, deletion of *RAD1*, which also is required for NER, did not influence correction of mismatches on heteroduplex plasmids (Kang and Kunz 1992). These findings appear to downplay a role for the Rad3 protein in mismatch correction. However, *rad3-2* confers a dominant negative phenotype when overexpressed (Naumovski *et al.* 1985), unlike other highly UV-sensitive *rad3* mutants. This suggests that *rad3-2* might influence cellular sensitivity to UV, and so other properties, through a mechanism(s) different from that of most *rad3* alleles. Consequently, whether the mutator phenotype conferred by the majority of *rad3* mutations reflects participation of the Rad3 protein in mismatch repair remains to be determined.

1.3 Mismatch Repair in Higher Eukaryotic Cells

Compared to bacteria and yeast, mismatch correction in higher eukaryotic cells is not well understood. However, there is evidence that mismatch repair does occur in human, monkey, and mouse cells and cell-free extracts from human cells, *Xenopus* eggs, and *Drosophila melanogaster* cell lines.

1.3.1 Mismatch Correction in Higher Eukaryotic Cells

Studies of mismatch repair in higher eukaryotic cells have relied on transfection of artificially constructed heteroduplex-containing viral or plasmid vectors (Brown and Jiricny 1987, 1988; Brown et al. 1989), or the use of cell-free extracts (Holmes et al. 1990; Thomas et al. 1991). Both types of experiment revealed that there appear to be three enzyme systems for mismatch correction in higher organisms, similar to the situation in E. coli: (1) a general mismatch repair system corrects all eight single base-pair mismatches with different efficiencies (Brown and Jiricny 1988; Holmes et al. 1990; Thomas et al. 1991; Yeh et al. 1991); (2) a G/T mismatch-specific repair system corrects G/T mismatches to G·C pairs (Brown and Jiricny 1987, 1988; Wiebauer and Jiricny 1989, 1990); (3) a G/A specific system corrects only DNA containing a G/A mismatch to G·C pairs (Yeh et al. 1991; McGoldrick et al. 1995).

The existence of a general mismatch repair system is supported by experiments using simian and human cells, cell-free extracts from human cells, D. melanogaster cell lines and Xenopus eggs (Brown and Jiricny 1988; Brown et al. 1989; Holmes et al. 1990; Varlet et al. 1990; Thomas et al. 1991; Yeh et al. 1991). This system appears able to process a wide variety of mismatches with different efficiencies. By using extracts of human HeLa cells, it has been found that the specificities of repairing M13mp2 DNA substrates containing single base-pair mismatches are similar for human cells and E. coli (Thomas et al. 1991). HeLa nuclear extracts nicked eight single base-pair mismatches at a single position in synthetic double-stranded DNA fragments with different efficiencies (Yeh et al. 1991). Nuclear extract from D. melanogaster Kc cell lines can correct single base-pair mispairs within open circular DNA heteroduplexes with

different efficiencies (G/T > G/G ≈ A/C > C/C) (Holmes *et al*. 1990). Mismatch repair was studied using cultured monkey kidney cells by transfection with SV40 DNA molecules harbouring a single mispair in a defined orientation within the intron of the large T antigen. All possible single base-pair mismatches were corrected and the repair efficiencies were 96% (G/T), 92% (G/G), 78% (A/C), 72% (C/T), 66% (C/C), 58% (A/A), and 39% (T/T and A/G) (Brown and Jiricny 1988). A protein which specifically binds to A/C, T/C and T/T mispairs has been detected in human cells extracts and it was suggested that this protein might be involved in a broad specificity DNA repair pathway for the correction of single base-pair mismatches in human cells (Stephenson and Karran 1989).

The above findings suggest that higher eukaryotic cells possess a general, strand-specific mismatch repair system, analogous to the *E. coli mutHLS* system. In *E. coli*, the strand specificity of mismatch correction in the methyl-directed pathway is determined by the methylation state of d(GATC) sequences. It has also been shown that a persistent strand break can bypass the requirements for both the d(GATC) sequences and MutH protein, which recognizes these sequences (Längle-Rouault *et al*. 1987; Welsh *et al*. 1987; Lauhue *et al*. 1989). In African green monkey cells (CV-1), hemimethylation at cytosine or adenine residues was found to direct strand selection during mismatch repair so as to correct the base on the unmethylated strand (Hare and Taylor 1985). However, the presence of a strand break may be a stronger determinant of strand bias than methylation, with the strand containing the nick farthest from the mismatch serving as the template strand for repair (Hare and Taylor 1985, 1988). Experiments using HeLa cell extracts (Thomas *et al*. 1991) or nuclear extracts from *D. melanogaster* cell lines (Holmes *et al*. 1990)

confirmed that strand discrimination could be directed by strand breaks. Curiously, in *Xenopus* egg extracts, mismatch repair of twelve single base-pair mismatch heteroduplexes was not strand-specific (Varlet *et al*. 1990). This suggests either that there are different repair systems or different modes of mismatch recognition in *Xenopus* or that components required for strand selectivity were missing from the *Xenopus* extracts.

It has been found that a mismatch-binding protein in HeLa nuclear extracts has associated ATPase and helicase activities (Hughes and Jiricny 1992), similar to the *mutU* (*uvrD*) gene product in the *mutHLS*-mismatch repair pathway in *E. coli* (Längle-Rouault *et al*. 1987; Runyon and Lohman 1989; Grilley *et al*. 1990). Moreover, the human pathway of mismatch correction may possess a bidirectional excision capability (Fang and Modrich 1993) similar to that of the *E. coli* methyl-directed system (Au *at al*. 1992). By using nuclear extracts from a HeLa cell line and analysis of the excision tracts, it was shown that the strand break can occur either 3' or 5' to the mismatch, and mismatch-provoked gaps rarely span the longer distance separating the mismatch and the strand break (Fang and Modrich 1993). However, mismatch repair capabilities of bacterial and mammalian cells may not be identical, since bacterial mismatch repair can't handle unpaired loops containing more than three nucleotides (Learn and Grafstrom 1989), while human cells have the ability to repair loops containing five or more nucleotides (Umar *et al*. 1994a) (see section 1.3.2.1). It is possible that this is a new activity acquired by the hMsh2 protein (see section 1.3.2.1). Conceivably, MutS has evolved to recognize and correct the relatively simple replication errors of the bacterial genome, while hMsh2 has evolved to recognize and repair more complex replication errors associated with the repetitive sequences found in the

human genome (Fishel *et al*. 1994b).

Among the different types of mismatches, the G/T mismatch generally is repaired the most efficiently, which may suggest the existence of a G/T specific mismatch repair activity (Hare and Taylor 1985; Brown and Jiricny 1988; Wiebauer and Jiricny 1989). G/T mispairs, incorporated into the SV40 genome and transfected into monkey (Brown and Jiricny 1987, 1988) or human cells (Brown *et al*. 1989), were found to be corrected with high efficiency and produce mainly G·C pairs. Subsequently, a G/T mismatch repair activity was confirmed using human cell extracts (Wiebauer and Jiricny 1990). This system involved a DNA glycosylase activity which removes the mispaired thymine to generate an apyrimidinic site opposite the guanine (Wiebauer and Jiricny 1990). This DNA glycosylase was purified and the enzymatic activity attributed to a 55 kDa polypeptide (Neddermann and Jiricny 1993). Additionally, by using HeLa and other mammalian cell extracts, Griffin and Karran (1993) found that incision of the T-containing strand of a G/T mismatch exhibited a strong sequence specificity. Efficient strand cleavage was only observed when the mismatched G was in a CpG sequence. The most probable source of G/T mismatches is deamination of 5-meC, which is found almost exclusively in CpG dinucleotides in mammalian DNA (Bird 1986). Thus, the data suggest that incision was targeted to CpG sequences in which G/T mismatches are formed by the mutagenic deamination of DNA 5-meC (Griffin and Karran 1993). Therefore, this pathway resembles the VSP pathway in *E. coli* (see section 1.1.2), and it may protect the cells from the potentially mutagenic effects of spontaneous deamination of 5-methylcytosine.

A G/A mismatch specific nicking enzyme system has been detected in HeLa nuclear extracts. This system nicks the "A"-containing strand but not the

"G"-containing strand of synthetic double-stranded DNA fragments containing a G/A mismatch (Yeh *et al.* 1991). Incisions are made simultaneously at the first phosphodiester bond both 5′ and 3′ to the mispaired adenine (Yeh *et al.* 1991). Recently, evidence has been presented that the HeLa G/A specific nicking activity may be mediated by a DNA glycosylase-apurinic/apyrimidinic endonuclease, and that the human G/A specific enzyme is similar to the *E. coli* MutY DNA glycosylase and AP endonuclease system involved in G/A specific repair. A 65 kDa protein homologous to the *E. coli* MutY protein, MYH, has been identified in nuclear extracts of calf thymus and human HeLa cells by western blot analysis using polyclonal antibodies to the *E. coli* MutY protein (McGoldrick *et al.* 1995). Partial purification of MYH from calf thymus cells also revealed that the protein is a DNA glycosylase that specifically removes mispaired adenines from A/G and A/GO mismatches (McGoldrick *et al.* 1995). A nicking activity that is either associated with or copurified with MYH was also detected. The nicking occurred at the first phosphodiester bond 3′ to the apurinic/apyrimidinic site generated by the glycosylase activity. Although the nicking on A/GO-containing DNA was 2-fold weaker than that on A/G-containing substrates, binding of the MYH protein was 7-fold greater on the A/GO than A/G substrates.

The results indicate that the eukaryotic MYH protein may be involved in the repair of both replication errors and oxidative damage to DNA, the same functions as those of the *E. coli* MutY protein. Oxidative stress and metabolic processes produce active oxygen species, which react with DNA to produce GO, and have been implicated in cancer and aging (Ames and Gold 1991; Kasai and Nishimura 1991). Therefore, mechanisms to prevent the accumulation of GO in DNA are also necessary in mammalian cells.

Furthermore, it has been noted that G·C → T·A transversions occur frequently as mutations of the *p53* tumor suppressor gene in human lung, breast, and liver cancers (Hollstein *et al.* 1991). Thus, the MYH pathway may be linked to protection against certain cancers.

1.3.2 Genes Involved in Mismatch Correction in Human Cells

Several genes, which appear to be involved in DNA mismatch correction have been found in human cells. Defects in these genes are associated with some hereditary diseases, including a particular form of colon cancer.

1.3.2.1 *hMSH2* Gene

By using PCR technology, the *hMSH2* (human *MSH2* homolog) gene has been cloned, and predicted to encode a 909 amino-acid long protein that has 41% identity with the 966 amino-acid long *S. cerevisiae* Msh2 protein (Fishel *et al.* 1993). The evolutionary relationship among the known MutS-related proteins indicates that hMsh2 is a member of a group of MutS homologs that includes all of the bacterial homologs and the *S. cerevisiae* Msh1 and Msh2 proteins (Fishel *et al.* 1993). Expression of *hMSH2* in *E. coli* causes a dominant mutator phenotype, suggesting that hMsh2, like other divergent MutS homologs, interferes with the normal bacterial mismatch repair pathway. Possibly the heterologous protein interacts with mismatched nucleotides but cannot interact with other proteins in the repair pathway (Fishel *et al.* 1993). The above observation provides support for the idea that *hMSH2* functions in mismatch repair in humans.

hMSH2 has been mapped to human chromosome 2p22-21 at a locus implicated in hereditary non-polyposis colon cancer (HNPCC) (Fishel *et al.* 1993; Leach *et al.* 1993), which affects as many as 1 in 200 individuals in the western world (Lynch *et al.* 1993). It has been demonstrated that a

mutation in *hMSH2* is the basis of HNPCC (Fishel *et al.* 1993; Leach *et al.* 1993), tumors of which exhibit microsatellite instability (Parsons *et al.* 1993). Variations in microsatellites can be generated by errors in DNA replication, such as the slippage of one strand relative to the other (Kornberg and Baker 1992). These errors create regions of non-complementarity that are normally corrected by the mismatch repair system. Mutations in the genes that comprise this system result in defective proteins that fail to correct replication errors, including those resulting from slippage during DNA replication (Loeb 1994). Furthermore, it has been suggested that microsatellites might provide a sensitive indicator for genetic instability in tumors and some genetic diseases (Loeb 1994). This is because microsatellite instability is not only associated with some cancers, but also with a number of heritable genetic diseases, including fragile X syndrome, Kennedy's disease, myotonic dystrophy, Huntington's disease and spinocerebellar ataxia type 1 (Kuhl and Caskey 1993).

Purified hMsh2 protein efficiently and specifically binds DNA containing mismatched microsatellite DNA sequences, as well as single base-pair mismatches (Fishel *et al.* 1994a, b). Using human cell extracts and M13mp2 DNA substrates, it has been demonstrated that a sporadic colorectal cancer cell line with deletions in both *hMSH2* alleles is deficient in the repair of loops of one to five or more unpaired bases as well as single base-pair mismatches (Umar *et al.* 1994a, b). The repair is strand-specific and is directed by a nick located 5′ or 3′ to the loop (Umar *et al.* 1994a). These results support a direct and general role for *hMSH2* in mutation avoidance and microsatellite stability in human cells. As defects in this gene may account for up to 50% of the cases of HNPCC

(Fishel *et al.* 1994b; Service 1994), *hMSH2* is considered to be very important in limiting the occurrence of HNPCC.

1.3.2.2 *hPMS* Genes

Two human homologs of the bacterial *mutL* gene, called *hPMS1* (human *PMS1* homolog) and *hPMS2* have been sequenced. *hPMS1* is located at human chromosome 2q31-33 (Nicolaides *et al.* 1994), while *hPMS2* is a member of a subfamily which includes at least two related genes located on chromosome 7q (Bronner *et al.* 1994). The ORF of *hPMS1* is 2,795 bp long and has been predicted to encode a 932 amino-acid long protein that has 27% identity with the 904 amino-acid yeast Pms1 protein. *hPMS2* contains a 2,586 bp ORF and has been predicted to encode an 862 amino-acid long protein that has 32% identity with the yeast Pms1 protein (Nicolaides *et al.* 1994). Both *hPMS1* and *hPMS2* have been found to be mutated in the germline of HNPCC patients (Nicolaides *et al.* 1994). Furthermore, a somatic alteration of the remaining allele in a tumor from a patient with a germline *hPMS2* mutation has been identified (Nicolaides *et al.* 1994). HNPCC is inherited in an autosomal dominant fashion, with normal cells from affected individuals containing one functional and one defective copy of the repair gene (Bronner *et al.* 1994; Nicolaides *et al.* 1994; Papadopoulos *et al.* 1994). Normal cells from HNPCC patient typically display low mutability due to the presence of a wildtype gene and in one case have been shown to be proficient in mismatch repair (Parsons *et al.* 1993). Tumour cells, on the other hand, are defective in both copies of the affected gene, with inactivation of the wildtype allele as result of somatic mutation (Nicolaides *et al.* 1994). These observations support the idea that inactivation of both alleles of mismatch repair genes is required in tumor

formation (Nicolaides *et al.* 1994). *hPMS1* and *hPMS2* may each account for 5% of the HNPCC cases (Fishel *et al.* 1994b).

1.3.2.3 *hMLH1* Gene

The *hMLH1* (human *MLH1* homolog) gene, homologous to the bacterial DNA mismatch repair gene *mutL*, is located on human chromosome 3p21.3-23 (Bronner *et al.* 1994; Papadopoulos *et al.* 1994). hMlh1 protein has 34% amino acid identity with the yeast Mlh1 protein (Papadopoulos *et al.* 1994). *hMLH1* missense mutations were identified in nine of ten chromosome 3-linked HNPCC families (Papadopoulos *et al.* 1994), and *hMLH1* may be involved in 30% of the cases of HNPCC (Fishel *et al.* 1994b; Service 1994). Using sporadic colorectal cancer cell line extracts, it been shown that the *hMLH1* gene is involved in the repair of single base-pair mismatches (Parsons *et al.* 1993; Umar *et al.* 1994a, b), as well as heteroduplexes containing loops of one to four unpaired bases, but not heteroduplexes with loops of five or more unpaired bases (Umar *et al.* 1994a). This suggests that *hMLH1* plays a role in DNA mismatch repair in human cells, and that repair of heteroduplexes containing loops of five or more bases is distinct from repair of smaller loops. It appears that *hMLH1* and *hMSH2* may have different functions in the repair of heteroduplexes with loops, depending on the size of the loop.

The finding that human cells contain at least three *mutL* homologs (hMlh1, hPms1 and hPms2) suggests that there may be unique functions for each of these genes in human cells. This is similar to the situation in yeast in which inactivation of either yeast *mutL* homolog (*MLH1* or *PMS1*) results in a mutator phenotype (Prolla *et al.* 1994a), and the encoded proteins apparently interact in a complex (Prolla *et al.* 1994b).

Consistent with this idea, inactivation of any one of three human *mutL*-related genes or *hMSH2* can result in HNPCC, which is associated with mismatch repair deficiency. Mutations in the mismatch repair genes could be an early event in the carcinogenic process, since these mutations have been demonstrated in diploid colon cancer cells (Leach *et al.* 1993). Thus, the potential role for *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* in the development of cancer suggests that a defect in the mismatch repair system may destabilize the genome. Presumably, this would lead to other genetic alterations, such as the activation of oncogenes or the inactivation of tumour suppressor genes, that might cause cancer (Marx 1994; Modrich 1994).

1.4 Purpose of This Study

In this study, I have sought to characterize the mutator phenotypes conferred by defects in *S. cerevisiae* mismatch correction genes *PMS1* and *MSH2*, as well as the DNA repair gene *RAD3*, assess the potential role of mismatch correction in the *rad3* mutator phenotype, and begin to determine whether DNA strand breaks direct intracellular mismatch repair. To date, much less is known about mismatch correction in eukaryotes than prokaryotes but it occurs in yeast, simian and human cells and in extracts of *D. melanogaster*, human cells and *Xenopus* eggs (Bishop *et al.* 1989; Brooks *et al.* 1989; Brown *et al.* 1989; Holmes *et al.* 1990; Thomas *et al.* 1991). Currently, investigations using higher eukaryotic cells or their extracts have to rely on systems where mismatch correction, even when occurring intracellularly, only can be detected *in vitro*. Contrary to the situation for higher eukaryotes, it has been possible to assay mismatch correction in yeast cells. Several yeast genes have been found to be

involved in mismatch correction. However, the specificities of the mutator effects conferred by mismatch correction deficiencies, and the mechanism of mismatch correction in yeast remain unclear.

To obtain detailed spontaneous mutational specificity data and assay mismatch correction, I have used a system (Pierce *et al.* 1987) which facilitates: (1) rapid DNA sequence analysis of mutations occurring spontaneously in a yeast suppressor tRNA gene (*SUP4*-o); (2) construction of defined mismatches in this gene and analysis of their repair; and (3) construction of heteroduplex plasmids with a break or gap on the 5' or 3' of the mismatched base, on the transcribed strand or nontranscribed strand, with respect to the *SUP4*-o gene. Specifically, I have characterized spontaneous *SUP4*-o mutations arising in *pms1Δ*, *msh2Δ* or *rad3-1* backgrounds and have compared the resulting spectra to that obtained in an isogenic wildtype (MKP-o) strain. Additionally, different heteroduplex plasmids containing defined mismatches were constructed *in vitro* and transformed into *pms1Δ*, *msh2Δ*, *rad3-1*, *rad3-25* and wildtype strains. Finally, I have examined the influence of strand breaks or gaps on the direction of mismatch correction in yeast. A number of interesting findings have emerged: (1) the *pms1Δ* and *msh2Δ* mutator phenotypes were strongly associated with increased rates of single base-pair deletion/insertion; (2) mismatch correction of heteroduplex plasmids was more efficient in the *rad3-1* background than the wildtype strain; (3) strand breaks appeared to modulate the direction of mismatch correction in yeast cells.

2 MATERIALS AND METHODS

2.1 Chemicals and Media Components

All chemicals were analytical reagent grade and were obtained commercially from Fisher Scientific, Mallinckrodt, Sigma, or Bethesda Research Laboratories (BRL). Media components such as yeast extract, tryptone, peptone, yeast nitrogen base w/o amino acids, and agar were purchased from CanLab.

2.2 Bacterial and Yeast Strains

The complete genotypes of all strains used in this study are given in Table 1. The *Escherichia coli* strain JF1754 was used to recover plasmid DNA from yeast cells. *E. coli* NR3837 and JF1754$^+$ were used to produce single-stranded plasmid DNA. MKP-o, a haploid repair-proficient strain of the yeast *Saccharomyces cerevisiae* has been described (Pierce *et al.* 1987).

Isogenic derivatives of MKP-o having mutations in *RAD3*, a yeast gene required for nucleotide excision repair, were constructed by Dr. W. Siede (University of Texas) as follows. First, the 0.8 kb *Bgl*II-*Eco*RI YRp7 (Stinchcomb *et al.* 1979) fragment carrying the yeast *TRP1* gene was cloned into the *Xba*I site immediately downstream of the *rad3-1* or *rad3-25* alleles in plasmids pWS3001-1 or pWS3001-25 (Naumovski *et al.* 1985; Naumovski and Friedberg 1986), respectively, to generate pWS3001-T1 or pWE3001-T25. Next, pWE3001-T1 and pWS3001-T25 were digested with *Sal*I and *Mlu*I to liberate 5.5 kb fragments consisting of a *TRP1* plus *rad3-1* or *rad3-25* segment surrounded by chromosomal DNA that flanks the *RAD3* locus. These fragments were gel-purified and then transformed (Ito *et al.* 1983) into MKP-o. Trp$^+$ transformants that arose were tested for UV sensitivity and

Table 1. Yeast and Bacterial Strains

Strain	Genotype	Reference/Source
MKP-o	*MATα, can1-100, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901*	Pierce et al. (1987)
WS3-1	as for MKP-o but *rad3-1:TRP1*	W. Siede
WS3-25	as for MKP-o but *rad3-25:TRP1*	W. Siede
YY1	as for MKP-o but *pms1Δ*	This study
YY2	as for MKP-o but *msh2Δ*	This study
JF1754	*Δlac, gal, metB, leuB, hisB436, hsdR*	Pierce et al. (1987)
NR3837	*F'pro-lac/ara, Δpro-lac, thi, trpE9777, dam*	B.W. Glickman

replacement of the *RAD3* gene with a single copy of the 5.5 kb *Sal*I-*Mlu*I

fragments in the UV-sensitive isolates WS3-1 (*rad3-1*) and WE3-25 (*rad3-25*)

was confirmed by DNA hybridization analysis. MKP-o, WS3-1 and WS3-25 were

transformed (Gietz *et al*. 1992) in our laboratory with YCpMP2 (see section

2.3) to make MKP-op, WS3-1p and WS3-25p, respectively. Construction of YY1

and YY2 is described in sections 3.1.1 and 3.1.2 of the Results.

2.3 Plasmids

YCpMP2 (Pierce *et al*. 1987) is a hybrid yeast-bacterial shuttle vector

which can replicate autonomously in yeast and in *E. coli* (Figure 2). The

vector contains a replication origin (*ARS1*), a centromere sequence (*CEN4*)

and a selectable marker (*URA3*) from yeast and the replication origin (*REP*)

and the ampicillin resistance determinant (*AMP*^{R}), the ß-lactamase gene,

from the bacterial plasmid pBR322. The *CEN4* sequence allows YCpMP2 to be

maintained predominantly at a copy number of one in haploid yeast cells

(Newlon 1988). In addition, YCpMP2 carries *SUP4*-o, an ochre suppressor

allele of a yeast tyrosine tRNA gene. The replication origin from the

filamentous phage M13, which permits the generation of single-stranded

plasmid DNA (Dente *et al*. 1983; Levinson *et al*. 1984; Zagursky and Berman

1984), is also part of this vector.

YCpMP2 and its derivatives were used to construct heteroduplexes, which

were used for the study of mismatch correction in MKP-o, WS3-1 (*rad3-1*),

WS3-25 (*rad3-25*), YY1 (*pms1Δ*) and YY2 (*msh2Δ*) strains respectively. The

derivatives included the following plasmids. YCpMP2-18G and YCpMP2-51G

carry *SUP4*-o alleles which have a G·C → C·G transversion at site 18 or 51

of the coding sequence, respectively. Similarly, YCpMP2-27A and YCpMP2-32T

have a G·C → A·T transition at site 27 or 32, YCpMP2-35G has an A·T → G·C

Figure 2. Plasmid YCpMP2.

M13 ori

SUP4-o

Hind III

Sac II

EcoRI

BamHI

Hind III

BamHI

EcoRI

Sal I

PstI

TET

AMP^R

PstI

REP

URA 3

YCpMP2
8.5 kb

PstI

ARS1

CEN4

XhoI

HpaI
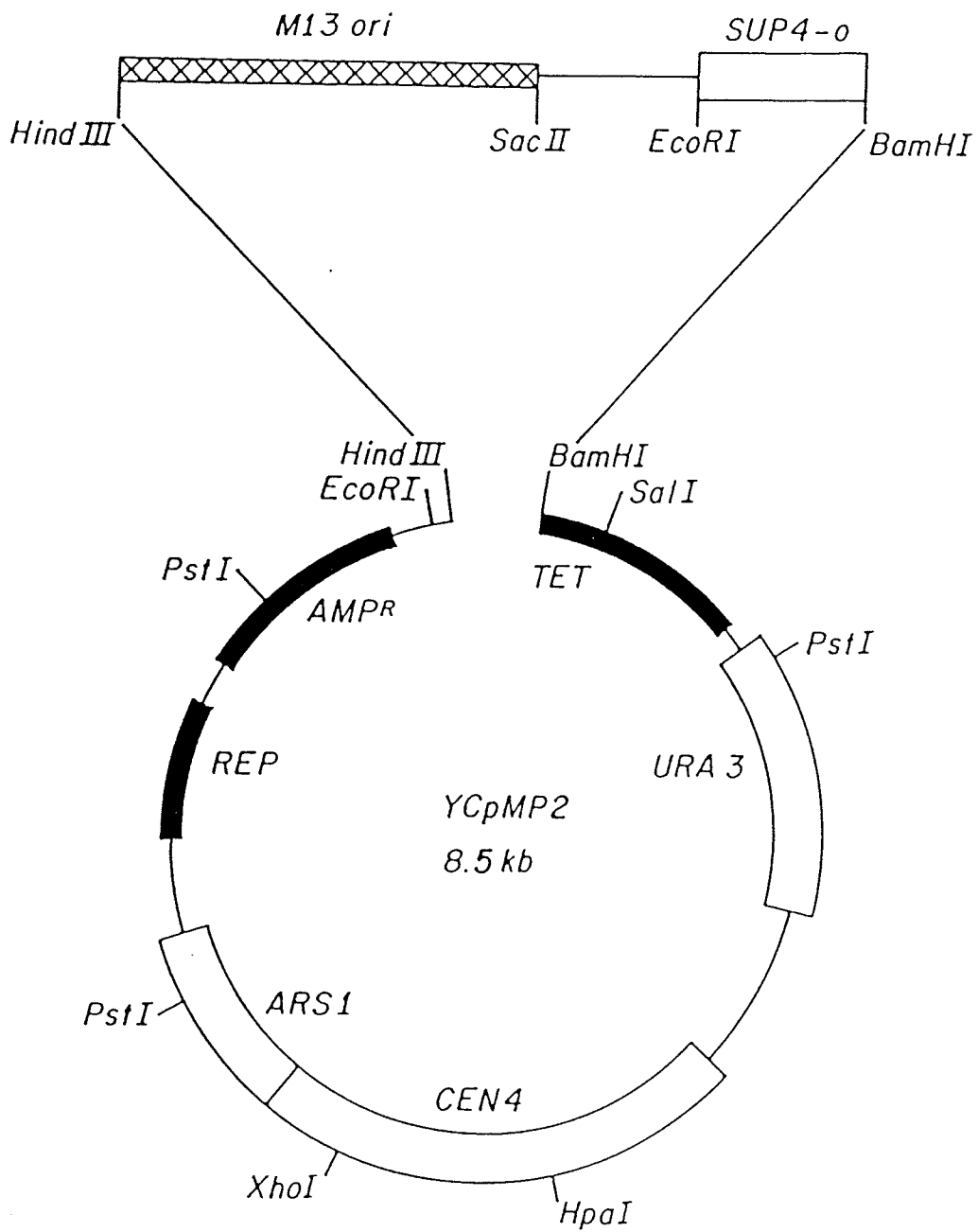
transition at site 35 and YCpMP2-55T has an A·T → T·A transversion at site 55. These six plasmids were obtained from our collection of *SUP4*-o mutants.

YCpLK1E and YCpLK3EB (Figure 3), or their derivatives carrying *SUP4*-o alleles with defined base-pair substitutions, were used to study the influence of strand breaks on mismatch correction. YCpLK1E and YCpLK3EB were modified from YCpMP2 and YCpJA1, respectively by L. Kohalmi using site-specific mutagenesis. YCpLK1E was made by removing the *Eco*RI site immediately adjacent to the *Hind*III site of YCpMP2. YCpJA1 (Armstrong and Kunz 1995) is similar to YCpMP2 except that *SUP4*-o is inverted relative to its orientation on YCpMP2. YCpLK3EB was constructed by eliminating unwanted *Bam*HI and *Eco*RI sites in linker sequences flanking the *SUP4*-o gene in YCpJA1, and removing the same *Eco*RI site as in the construction of YCpLK1E. YCpLK1E-32T and YCpLK3EB-32A, which each have a G·C → A·T transition mutation located at sites 32 within the *SUP4*-o gene, were made by X. Kang via site-specific mutagenesis.

Plasmid pHR307a was used to isolate a 24 bp *Bam*HI fragment and a 42 bp *Eco*RI fragment in order to construct gapped heteroduplex plasmids (see section 2.13).

Plasmids used for construction of yeast strains included pII-2 (Reenan and Kolodner, 1992a), pEN63 (E. Alani, personal communication) and pWBK4 (Kramer *et al*. 1989b).

Figure 3. Plasmids YCpLK1E and YCpLK3EB.

M13 ori   SUP4-o

Sac II   Eco RI

Hind III   Bam HI
Sal I

Pst I

AMP^R

TET^S   Pst I

REP   YCpLK1E   URA3
8.5 kb

ARS1

Pst I   CEN4

Xho I   Hpa I

M13 ori   SUP4-o

Sac II   Bam HI Eco RI

Hind III   Sal I

Pst I

AMP^R

TET^S   Pst I

REP   YCpLK3EB   URA3
8.3 kb

ARS1

Pst I   CEN4

Xho I   Hpa I

2.4 Media

A. SD (Synthetic Minimal Dextrose) (Sherman *et al*. 1983):

per litre: 40 g Dextrose
                      6.7 g Bacto yeast nitrogen base w/o amino acids
                      (Difco)

Required nutrients were added at the concentrations suggested by Sherman *et al*. (1983). To enhance colouring due to the *ade2-1* allele (see section 2.5), adenine was added at half the recommended concentration (Zimmermann 1973).

B. YPD (Sherman *et al*. 1983):

per litre: 20 g Dextrose
                      20 g Bacto peptone (Difco)
                      10 g Bacto yeast extract (Difco)

For yeast transformation, 10 mg adenine sulphate was added per litre of YPD and the medium was designated YPDA.

C. YT (Miller 1972):

per litre: 8 g Bacto tryptone
                      5 g Bacto yeast extract
                      5 g sodium chloride

When required, ampicillin (100 mg/1), tetracycline (5 mg/1) or kanamycin (70 mg/1) was added after autoclaving and cooling of the medium to 45°C. The medium was then designated YT + amp, YT + tet, or YT + kan, respectively. Medium containing twice the concentration of the YT components was designated 2x YT and was used for bacterial transformation. Medium containing both tetracycline and kanamycin was designated YT + tet + kan.

D. M9 + amp (Miller 1972):

per litre: 500 μg thiamine
                      500 mg sodium chloride
                      246 mg magnesium sulphate
                      200 mg glucose
                      100 mg L-tryptophan
                       10 mg calcium chloride
                        6 g disodium hydrogen phosphate
                        3 g potassium hydrogen phosphate monobasic
                        1 g ammonium chloride

Ampicillin (100 mg/1) was added after autoclaving and cooling of the medium to 45°C.

E. FOA (Boeke *et al*. 1984)

per litre:
```
16.7 g Bacto agar
 1.7 g Bacto yeast nitrogen base w/o amino acids
 5   g (NH₄)₂SO₄
 1   g 5-fluoro-orotic acid (FOA) (Sigma)
20   g glucose
```

Required amino acids were added at the concentrations suggested by Sherman *et al*. (1983). Bacto yeast nitrogen base w/o amino acids, $(NH_4)_2SO_4$, FOA, glucose and all required amino acids were added in a total volume of 500 ml, heated to 60°C to dissolve and filter-sterilized (Nalgene TM Disposable Filterware, 115 ml). Simultaneously, 16.7 g agar in 500 ml H_2O was autoclaved and then cooled to 60°C. The two solutions were mixed just prior to pouring the plates.

F. For solid media, 20 g/l Bacto agar was added, except for FOA medium.

2.5 Detection of *SUP4*-o Mutation and *lys2-1* Reversion/Suppression

Forward mutations in the *SUP4*-o gene are detected by scoring for reduced suppression of three ochre markers. The haploid yeast strains used in this study carry ochre alleles which confer either resistance to the arginine analog canavanine (*can1-100*), cause red pigmentation (*ade2-1*) or result in lysine auxotrophy (*lys2-1*). Since these mutations are suppressed by *SUP4*-o, cells harbouring YCpMP2 are canavanine-sensitive and form white, lysine-independent colonies. Mutants lacking suppressor activity are selected as canavanine-resistant, red, or pink colonies unable to grow when replicated to lysine omission medium. Selection for reduced suppression of all three ochre markers detects at least a 30% decrease in the production of functional suppressor tRNA (Wang and Hopper 1988), and is unlikely to bias mutant recovery significantly for several reasons. First, mutations that prevent suppression of only two of the ochre mutations are rare (<1% of mutations analyzed) at the chromosomal *SUP4*-o locus (Kurjan and Hall 1982). Second, sequence alterations have not been detected in the plasmid copy of *SUP4*-o from mutants isolated using less

stringent selection methods (Kunz *et al*. 1987). Third, 178 different substitutions can be recovered at 68 of the 75 exon sites and at 2 of the 14 intron positions in the gene, and a wide range of mutational classes has been identified, using the selection protocol employed here (Kunz *et al*. 1990; Kohalmi and Kunz 1992).

Reversion of *lys2-1* was detected by selecting for lysine prototrophs. Since all of the strains used in this study also carry the *ade2-1* allele, *lys2-1* intragenic reversion results in the emergence of red, Lys⁺ colonies whereas extragenic ochre suppression of *lys2-1* gives rise to white, Lys⁺ colonies.

2.6 Selection of Spontaneous Mutants and Determination of Plasmid Stability, Mutation Frequency and Mutation Rate

To isolate spontaneous *SUP4*-o mutants, yeast strains were grown from low titre inocula (33 cells/ml) to stationary phase (ca. 1-2 X 10^7 cells/ml, as determined by a Coulter counter) in uracil omission medium. Cell suspensions were diluted and plated on uracil to measure viability and on fully supplemented medium to determine plasmid retention. To select for red, canavanine-resistant colonies, the cell suspensions were plated on uracil omission medium containing 60 mg/ml canavanine sulphate. All plates were scored after 6 days incubation at 30°C. Red colonies that emerged on canavanine-containing medium were transferred to uracil omission medium, grown for 2-3 days at 30°C, and replicated to uracil-lysine omission medium which was then incubated at 30°C for 2-3 days. Lysine auxotrophs were scored as *SUP4*-o mutants.

To isolate Lys⁺ colonies, MKP-o, YY1 or YY2 were inoculated (33 cells/ml) in supplemented minimal medium and grown at 30°C with shaking to

stationary phase (ca. 1-2 X 10^7 cells/ml, as determined by a Coulter counter). Cell suspensions (25 ml) were centrifuged (3,020 x g, 5 min, 4°C) to pellet the cells which were then resuspended in 2.5 ml sterile water. These cell suspensions were diluted when necessary and plated on appropriately supplemented minimal medium, with or without lysine, to assess viability and select Lys⁺ colonies, respectively.

Plasmid retention, mutation frequency and mutation rate per round of DNA replication were calculated as follows:

A. Plasmid Retention:

$$P = \frac{N_-}{N_+} \ x \ 100\%$$

P =   plasmid retention
N_- =   number of cells able to grow on uracil omission medium
N_+ =   number of cells able to grow on medium containing uracil

B. Mutation Frequency:

$$f = \frac{N}{N_c}$$

f =   mutation frequency
N =   number of mutants
N_c =   number of viable cells plated to select mutants

C. Mutation Rate (Drake 1991):

$$r = \frac{(0.4343 \cdot C \cdot f_m)}{\log(N_m \cdot r)}$$

r =   mutations in the target per DNA replication (solved algorithmically)

C = reciprocal of efficiency of base-pair substitution (BPS) detection, calculated as {no. of non-BPS detected + [no. of BPS detected · (no. of possible BPS / no. of detectable BPS)]} / no. of mutations sequenced [of 267 possible BPS in *SUP4*-o, 178 are detectable with the genetic screen used in this study (Kohalmi and Kunz 1992). C = 1.25, 1.42, 1.34, 1.30 and 1.32 for MKP-op, WS3-1p, WS3-25p, YY1-p, YY2-p, respectively. C for WS3-25p was estimated as the mean of the corresponding values for MKP-op and WS3-1p. C could not be used for calculation of the *lys2-1* → Lys$^+$ rates since determination of C requires DNA sequence analysis of the mutations recovered. Thus, in this case the rates may be slightly underestimated, but the degree of underestimation should be similar for MKP-o, YY1 and YY2.

f_m = the median mutation frequency

N_m = the median population size at the time of sampling including residual growth on the plates (3 generations for all yeast strains on canavanine-containing medium), N_m = 3.75 x 10^8, 5.04 x 10^8, 5.03 x 10^8, 4.37 x 10^8 and 4.46 x 10^8 for MKP-op, WS3-1p, WS3-25p, YY1-p and YY2-p, respectively. For the *lys2-1* → Lys$^+$ reversion/suppression, the N_m = 3.60 x 10^8, 4.91 x 10^8 and 4.23 x 10^8 for MKP-o, YY1 and YY2 respectively.

2.7 DNA Isolation

2.7.1 Large Scale Yeast DNA Preparation

SCE buffer: 900 mM sorbitol
100 mM Na$_2$EDTA, pH 7.5

Zymolyase: Zymolyase 100,000 (Seikagaku Kogyo) was dissolved at a concentration of 10 mg/ml in SCE buffer plus 10% ß-mercaptoethanol.

TE buffer(H): 50 mM Tris, pH 7.4
20 mM Na$_2$EDTA, pH 7.4

TE buffer: 10 mM Tris, pH 7.4
1 mM Na$_2$EDTA, pH 7.4

RNase: 1 mg/ml RNase A was dissolved in 5 mM Tris (pH 8.0), 4,000 U/ml RNase T1 was added and the mixture was heated at 100°C for 10 min and cooled slowly to room temperature. Aliquots (100 μl) were stored at -20°C.

Potassium acetate: 5 M potassium acetate was dissolved in double-distilled H$_2$O.

Total yeast DNA used for hybridization was isolated by a modification of a procedure described by Sherman *et al.* (1983). Yeast was grown in 12 ml YPDA to stationary phase (ca. 1-2 x 10^8 cells/ml, as determined by a Coulter counter) and the cell suspension was centrifuged (3,020 x g, 5 min, 4°C) to pellet the cells. The supernatant was aspirated and the cells were transferred to a microfuge tube. The cells were pelleted by centrifugation (Brinkman 5415C, 1 min, room temperature), washed with 500 μl SCE buffer, resuspended in 300 μl zymolyase and then the cell suspension was incubated at 37°C for 1 h. The resulting spheroplasts were pelleted by centrifugation (Brinkman 5415C, 10 s, room temperature) and resuspended in 357 μl TE buffer(H). Sodium dodecyl sulphate (SDS, 36 μl, 10%, w/v) was added, and the contents were mixed gently by inversion and incubated for 30 min at 65°C. Potassium acetate (107 μl, 5 M) was added and the suspension was chilled for 15 min on ice. The precipitate was pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), the supernatant transferred to a fresh tube and 1 ml of ice-cold ethanol (95%) added. The precipitated nucleic acids were pelleted by centrifugation (Brinkman 5415C, 5 s, room temperature) and the pellet dried and dissolved in 400 μl TE buffer. RNase (6 μl) was added and the suspension was incubated for 30 min at 37°C. Then, 400 μl isopropanol (room temperature) was added, the solution mixed by inversion and the DNA pelleted by centrifugation (Brinkman 5415C, 1 min, room temperature). The DNA pellet was washed with 70% ice-cold ethanol, dried and dissolved in 80 μl TE buffer and stored at

-20°C.

2.7.2 Glass-Bead Prep for Yeast DNA Isolation

Glass beads: Glass beads (0.45-0.50 mm, Braun) were soaked in
                      nitric acid for 1-2 h and then rinsed carefully
                      in double-distilled H_2O for 2-3 h. The H_2O was
                      discarded and the glass beads were dried for 2 h
                      at 160°C.

STET solution: 8% (w/v) sucrose
                      50 mM Tris, pH 8.0
                      50 mM Na_2EDTA, pH 8.0
                      5% (v/v) Triton X-100

Ammonium acetate: .7.5 M ammonium acetate was dissolved in double-
                      distilled H_2O while being heated to 65°C.

TE buffer: see section 2.7.1

This glass-bead technique (modified from Robzyk and Kassir 1992) was

used for fast processing of a larger number of samples for bacterial

transformation. Putative *SUP4*-o mutants were grown to stationary phase at

30°C with shaking in 1.5 ml uracil omission medium. Next, the cell cultures

were transferred to a microfuge tube and the cells were collected by

centrifugation (Brinkman 5415C, 1 min, room temperature). The cells were

resuspended in 200 μl STET solution and 200 mg acid-washed glass beads was

added to the tube which was mixed vigorously for 5 min using a vortex

mixer. The tube was then incubated briefly on ice and the beads and

cellular debris pelleted by centrifugation for in a microfuge (Brinkman

5415C, 10 min, 4°C). Then, 100 μl of the supernatant was transferred to a

tube containing 50 μl ammonium acetate, mixed well, and the tube was

incubated on ice for 30 min. After the mixture was centrifuged (Brinkman

5415C, 10 min, 4°C), 100 μl of the supernatant was transferred to a fresh

tube and the nucleic acids were precipitated by adding 200 μl of 95% ice-

cold ethanol. The tube was then centrifuged (Brinkman 5415C, 10 min, 4°C)

to pellet the DNA, the supernatant aspirated, and the DNA pellet was

washed with 70% ethanol, dried by aspiration and dissolved in 20 μl TE

buffer. DNA samples were stored at -20°C.

2.7.3 Rapid Alkaline Procedure for Plasmid DNA Isolation

GTE Buffer	50 mM glucose 25 mM Tris, pH 8.0 10 mM Na_2EDTA, pH 8.0
NaOH/SDS:	200 mM NaOH 1% (w/v) SDS Prepared just prior to use.
Ammonium acetate:	6.17 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C to achieve a pH of 5.8.
TE buffer:	see section 2.7.1

To isolate a large number of plasmid DNA samples for DNA sequencing, a procedure modified from Morelle (1989) was used. Bacteria were grown overnight in 5 ml YT + amp at 37°C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 1 ml of GTE buffer and transferred to a microfuge tube. Cells were pelleted (Brinkman 5415C, 30 s, room temperature), the supernatant was removed by aspiration, and the cells were resuspended in 190 μl GTE buffer. Next, the tube was transferred to ice, 400 μl NaOH/SDS was added drop-wise and the mixture was incubated on ice for 5 min. Ammonium acetate (300 μl) was added drop-wise and the mixture was incubated on ice for 10 min. The precipitate was pelleted by centrifugation (Brinkman 5415C, 4°C, $\geq$ 15 min) and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated two more times. Then, 500 μl isopropanol (room temperature) was added, the contents were mixed by inversion and the tube was held at room temperature for 10 min. Following centrifugation (Brinkman 5415C, 2 min, room temperature), the nucleic acid pellet was washed with 70% ice-cold

ethanol, dried by aspiration and dissolved in 50 μl TE buffer. DNA samples were stored at -20°C.

2.7.4 Large Scale Preparation of Plasmid DNA

STE buffer:	100 mM NaCl 10 mM Tris HCl, pH 8.0 1 mM Na$_2$EDTA, pH 8.0
GTE buffer:	see section 2.7.3
Lysozyme:	Lysozyme (Boehringer Mannheim) (10 mg/ml) was dissolved in GTE buffer.
NaOH/SDS:	see section 2.7.3
Potassium acetate:	3 M potassium acetate was adjusted to pH 4.8 with glacial acetic acid.
Ammonium acetate	10 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C to achieve a pH of 7.8.
PEG:	13% (w/v) polyethylene glycol$_{8000}$ (Sigma) was dissolved in 1.6 M sodium chloride solution and filter-sterilized just prior to use.
TE buffer:	see section 2.7.1
RNase:	see section 2.7.1

Double-stranded plasmid DNA used for heteroduplex construction, probe isolation or strain construction was prepared using a scaled-up version of the alkaline procedure of Birnboim and Doly (1979). Bacterial cultures were grown overnight in 5 ml M9 + amp at 37°C with shaking. Then, 2 ml overnight culture was transferred to 500 ml M9 + amp or 500 ml YT + amp and grown overnight at 37°C with shaking. Cells were pelleted by centrifugation (2,000 x g, 15 min, 4°C), resuspended in 100 ml ice-cold STE buffer, pelleted again, and resuspended in 18 ml GTE buffer. Lysozyme (2 ml) was added and the cell suspension was incubated at room temperature for 10 min. NaOH/SDS (40 ml) was added, mixed by inverting several times

and the mixture was chilled on ice for 10 min. Potassium acetate (20 ml) was added and the contents were mixed by inversion and shaking the bottle several times to produce a flocculent white precipitate. The bottle was then chilled on ice for 10 min, the precipitate was pelleted by centrifugation (2,000 x g, 20 min, 4°C), and the supernatant was filtered through 5 layers of cheesecloth and transferred to a fresh centrifuge tube. The nucleic acids were precipitated by adding 50 ml of isopropanol (room temperature) and incubating the tube at room temperature for 15 min. Then, the precipitate was pelleted by centrifugation (4,080 x g, 15 min, room temperature) and the pellet was washed with 70% ethanol, dried and dissolved in 3 ml TE buffer. The large RNA molecules were removed by precipitation with 3 ml ice-cold 5 M lithium chloride followed by centrifugation (12,100 x g, 15 min, 4°C). The supernatant was transferred to a fresh tube, 6 ml isopropanol (room temperature) was added and the tube was incubated at room temperature for 10 min. The resulting precipitate was pelleted by centrifugation (12,100 x g, 10 min, room temperature), washed with 70% ethanol, dried by aspiration, dissolved in 500 μl TE buffer and transferred to a microfuge tube. Then, 4 μl RNase was added and the tube was incubated at 37°C for 30 min. The DNA was precipitated with 500 μl PEG, pelleted by centrifugation (Brinkman 5415C, 5 min, 4°C) and the supernatant was discarded and the pellet was dissolved in 570 μl TE buffer. The nucleic acids were then extracted once with 570 μl TE-saturated phenol, once with 540 μl TE-saturated phenol:chloroform (1:1) and once with 500 μl TE-saturated chloroform. After each extraction the samples were centrifuged (Brinkman 5415C, 2 min, 4°C) and the upper aqueous layer was transferred to a fresh microfuge tube. Following the last extraction, one-fourth the volume of ammonium acetate and twice the

volume of 95% ice-cold ethanol were added, the contents were mixed by inversion and the precipitate was pelleted by centrifugation (Brinkman 5415C, 5 min, 4°C). The pellet was washed with 1 ml 70% ice-cold ethanol, dried by aspiration and dissolved in 500 μl TE buffer. DNA samples were stored at -20°C.

2.7.5 Preparation of Bacterial Nucleic Acid

STE buffer: See section 2.7.4

NaOH/SDS: See section 2.7.3

Potassium acetate: See section 2.7.4

Bacterial nucleic acid (primarily RNA), which was used for yeast transformation as a plasmid carrier, was prepared by modifying the latter half of the previous protocol (2.7.4), beginning after the first isopropanol precipitation and wash with 70% ethanol. Following the wash, the pellet was dried, dissolved in 6 ml TE buffer and transferred by pipette to a 30 ml sterile Corex tube (Corex no. 8433). The nucleic acids were then extracted once with 6 ml TE-saturated phenol, once with 6 ml TE-saturated phenol:chloroform (1:1) and once with 6 ml TE-saturated chloroform. After each extraction, the samples were centrifuged (6,780 x g, 10 min, 4°C) and approximately 5 ml of the top aqueous layer was transferred to a sterile Corex tube. Next, 6 ml of isopropanol was added to precipitate the nucleic acids, the contents of the tube were mixed well by inversion and the tube was allowed to stand at room temperature for 10 min. After incubation, the nucleic acids were pelleted by centrifugation (12,100 x g, 15 min, 4°C), washed with 1 ml ice-cold 70% ethanol, dried by aspiration, dissolved in 200 μl TE buffer, and then stored at -20°C.

2.8 Preparation of Nicked Heteroduplex Plasmid DNA

2.8.1 Preparation of Single-Stranded Plasmid DNA

A. Preparation of phage M13KO7

The procedure described by Vieira and Messing (1987) was used. *E. coli* JF1754F$^+$ was grown overnight in 5 ml YT + tet at 37°C with shaking. Then, the overnight culture diluted 5-fold by adding 1 ml of overnight culture to 4 ml YT + tet. Diluted JF1754F$^+$ cell suspension (200 μl) was mixed with 100 μl of phage M13KO7 (10^7 - 10^8 pfu/ml), and incubated at room temperature for 20 min. An aliquot (50 μl) of the mixture was then plated onto a YT + tet + kan plate, and the plate was incubated at 37°C overnight. A single colony that emerged on the YT + tet + kan plate was picked, inoculated into 30 ml of 2 x YT + tet + kan and the culture was grown for 24 h at 37°C with shaking. The cells were removed by centrifuging twice (17,300 x g, 15 min, 4°C), and the phage in the supernatant was used for the production of single-stranded plasmid DNA.

B. Production of single-stranded plasmid DNA

Formation of single-stranded plasmid DNA was induced by infection of NR3837 carrying YCpMP2, or YCpMP2 derivatives, with M13KO7 helper phage as described previously (Vieira and Messing 1987). Plasmid-containing NR3837 cells carrying plasmid were grown overnight in M9 + amp at 37°C with shaking. Then, 1 ml of overnight culture was transferred into 100 ml M9 + amp and incubated at 37°C with shaking (New Brunswick Gyrotory Water Bath Shaker G76, 220 rpm) for 30 min. The cells were then infected with 600 μl of freshly-prepared phage (7 x 10^{10} pfu) and incubated at a lower shaking speed (60 rpm) for 1 h. Next, 1 ml of kanamycin (7 mg/ml) was added and the culture was incubated at 37°C with shaking (220 rpm) for 24 h. The cells were removed by centrifugation (9,150 x g, 30 min, 4°C) and the

supernatant was kept at 4°C for purification of single-stranded plasmid DNA.

C. Purification of single-stranded plasmid DNA

TES: 20   mM Tris, pH 7.5
                        10   mM NaCl
                        0.1 mM Na_2EDTA, pH 8.0

PEG: 20% (w/v) polyethylene $glycol_{8000}$ was dissolved
                        in 2.5 M NaCl solution and filter-sterilized
                        just prior to use.

Sodium acetate: 3 M sodium acetate was dissolved in glacial
                        acetic acid while being heated to 65°C to
                        achieve a pH of 4.8.

TE buffer: see section 2.7.1

Loening's buffer: 400 mM Tris
                        200 mM sodium acetate
                        10 mM Na_2EDTA
                        Adjusted to pH 8.0 with glacial acetic acid and
                        stored at room temperature.

An aliquot (24 ml) of the supernatant containing the single-stranded plasmid DNA was transferred to sterile Corex tube (Corex no. 8433), centrifuged (6,780 x g, 30 min, 4°C) and transferred to a fresh sterile Corex tube. PEG (4.2 ml) was added to the tube, the contents were mixed well and the tube was incubated at room temperature for 20 min before being centrifuged (6,780 x g, 30 min, 4°C). The supernatant was discarded, the pellet was dissolved in 250 μl TES buffer and transferred to a microfuge tube. Two lots of dissolved pellet (total = 500 μl) were transferred to each tube. The solution was extracted once with 500 μl TE-saturated phenol and once with 500 μl TE-saturated chloroform (1:24). For each extraction the contents of the tube were mixed vigorously for 30 s using a vortex mixer, the tube was centrifuged (Brinkman 5415C, 4 min, room temperature), the upper aqueous layer was transferred to a fresh

microfuge tube, and 36 μl sodium acetate and 1 ml ice-cold 95% ethanol were added. The contents were mixed well and the tube was held at -20°C for 15 min. The DNA was pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), dissolved in 40 μl TE buffer and two lots of dissolved pellet (total = 80 μl) originating from the same 100 ml culture were transferred to one microfuge tube. An aliquot (1 μl) of sample from these preparation was electrophoresed on a agarose gel (0.7% w/v in Loening's buffer) for 2 h at 2 volt/cm. The gel was stained with ethidium bromide (0.5 μg/ml) and destained for 20 min in double-distilled H_2O. The concentration of single-stranded plasmid DNA was estimated from the relative intensities of the ethidium bromide-stained bands corresponding to the single-stranded plasmid and phage DNAs and the quantity of total DNA in the preparation (determined by A_{260}). The DNA was stored at -20°C.

2.8.2 Linearization of Plasmid DNA for Heteroduplex Construction

TE buffer: see section 2.7.1

Digest mix: 500 μl plasmid DNA (100 μg DNA in TE buffer)
                        58 μl 10x reaction buffer 2 from BRL
                        20 μl XhoI (15 U/μl) from BRL or Pharmacia
                        Incubated at 37°C for 2.5 hours.

Plasmid DNA was linearized by digestion with XhoI. Following digestion, the reaction mixture was extracted once with TE-saturated phenol, once with TE-saturated phenol:chloroform (1:1) and once with TE-saturated chloroform. The DNA was precipitated with 95% ice-cold ethanol, pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), washed with 70% ice-cold ethanol, dried by aspiration, and dissolved in 60 μl TE buffer. The concentration of DNA was determined by A_{260} and the DNA was stored at -20°C.

2.8.3 Construction and Purification of Nicked Heteroduplex Plasmid DNA

TE buffer: see section 2.7.1

20x SSC
                  3   M NaCl
                  0.3 M sodium citrate
                  Adjusted to pH 7.0 with 10 N NaOH.

Glycine-NaOH buffer: 1 M glycine was adjusted to pH 9.4 with NaOH and filter-sterilized.

Annealing mix:
                  30 μg linearized DNA in 48 μl TE buffer
                  60 μg single-stranded DNA in 80 μl TE buffer
                  92 μl 2.5x SSC buffer (diluted from 20x SSC buffer)
                  736 μl sterile double-distilled H_2O

10x Endonuclease V reaction buffer:
                  66.7 mM glycine-NaOH buffer, pH 9.4
                  5   mM $MgCl_2$
                  8.3 mM 2-mercaptoethanol
                  0.5 mM ATP

Endonuclease V digest mix:
                  10 μl 10x endonuclease V reaction buffer
                  89 μl DNA in TE buffer (ca. 2.8 μg DNA)
                  1 μl 3.5 U/μl   *Micrococcus*   *luteus* endonuclease V (United States Biochemical) Incubated at 37°C for 30 min.

Loening's buffer: see section 2.8.1

Heteroduplex plasmid DNA was constructed from combinations of double-stranded linear and single-stranded circular DNA by using a modification of a thermal denaturation and renaturation procedure (Figure 4) (Kramer *et al.* 1989a). Linearized plasmid DNA (30 μg) was added to single-stranded circular DNA (ca. 60 μg of plasmid DNA plus M13K07 DNA) in a final volume of 956 μl SSC buffer. Aliquots (60 μl) of the mixture were dispensed into screw cap microfuge tubes. The tubes were incubated at 100°C for 4 min, transferred to a small 75°C bath, cooled slowly to 65°C, incubated at 65°C for 15 min, removed from the bath, and then incubated at room temperature for 30-45 min. The mixture was then separated by agarose gel (0.7% w/v in Loening's buffer) electrophoresis at 2 volt/cm overnight. Then the gel was

Figure 4. Heteroduplex construction.

HETERODUPLEX CONSTRUCTION

Plasmid with functional
SUP4-o allele

Plasmid with mutant
SUP4-o allele

Infect with helper phage,
isolate phagemids and
extract circular ss DNA

Linearize plasmid
and isolate ds DNA

Mix DNAs and heat to | denature linear ds DNA

Slow cool to anneal circular ss DNA | to the complementary linear strand.
Purify nicked heteroduplex by | agarose gel electrophoresis

Nicked heteroduplex plasmid
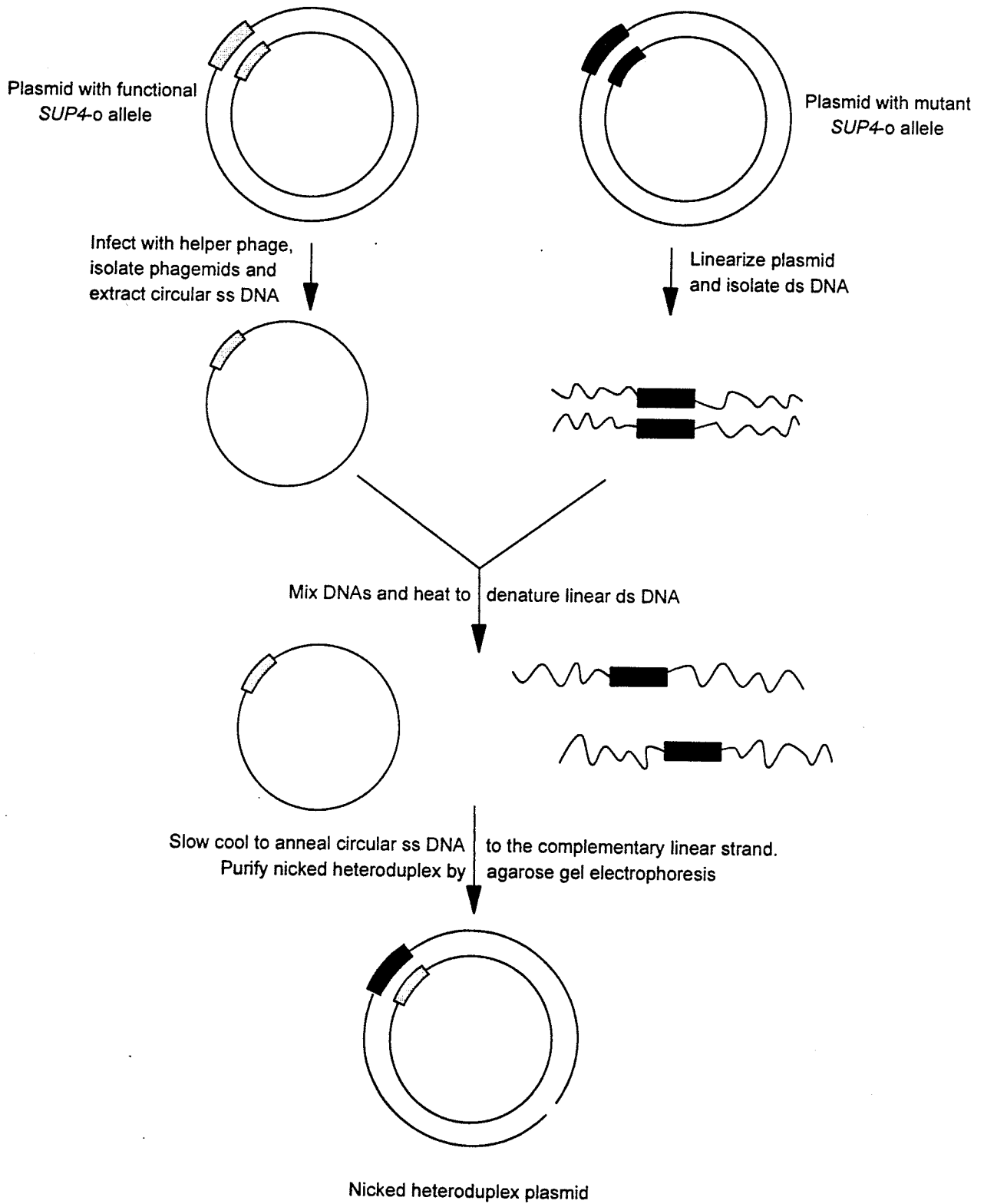
stained with ethidium bromide solution (0.5 μg/ml) for 20 min, followed by rinsing with tap water for 10 min and two quick rinses with double-distilled H_2O. The position of the heteroduplex-containing band on the gel was visualized on a UV transilluminator. Ten heteroduplex-containing gel segments were excised and each piece was put into a separate microfuge tube. The heteroduplex DNA was recovered from the agarose by modification of a freeze-extraction procedure (Thuring et al. 1975). Each gel slice was frozen in a microfuge tube at -20°C overnight and the tube was centrifuged at maximum speed in a microfuge (Brinkman 5415C, 1.5 h, 4°C). The supernatant was then transferred to a sterile 6" glass tubes (Kimax) so that each tube contained about 4 ml of supernatant. Then, 2 volumes of butanol were added to remove ca. 50% of the H_2O from each tube, the tubes were inverted several times, and the aqueous layers were transferred to microfuge tubes, each tube receiving 500 μl. Following extraction of the supernatant with TE-saturated chloroform, and ethanol precipitation, the DNA was dissolved in TE buffer. Then, linear duplex DNA was eliminated from the heteroduplex preparation by digestion with *Micrococcus luteus* endonuclease V at 37°C for 30 min to 1 h. The remaining DNA was precipitated with 95% ice-cold ethanol, washed with 70% ice-cold ethanol, and dissolved in 50 μl TE buffer. Agarose gel electrophoresis (see section 2.8.1) of samples from these preparations indicated that the heteroduplex DNA was not detectably contaminated with single-stranded circular DNA or linearized DNA. Yields were determined by comparing the intensities of ethidium bromide-stained heteroduplex DNA bands with the intensities of DNA bands of known quantity. The nick and the mismatch sites were at least 4 kb apart.

2.9 Transformation Procedures

2.9.1 Bacterial Transformation

Buffer A:　　　　　　　　　100 mM NaCl
　　　　　　　　　　　　　　5 mM Tris, pH 7.5
　　　　　　　　　　　　　　5 mM $MgCl_2$

Buffer B:　　　　　　　　　100 mM $CaCl_2$
　　　　　　　　　　　　　　5 mM Tris, pH 7.5
　　　　　　　　　　　　　　5 mM $MgCl_2$

Bacterial cells were transformed using a modification (Pierce *et al*. 1987) of the calcium chloride procedure of Mandel and Higa (1970). *E. coli* strains were grown overnight in 5 ml YT. The cells were then diluted 1:100 in YT (40 ml of YT for every 10 transformations), grown for 1 h 45 min at 37°C with shaking ($O.D._{600}$ = 0.6) and chilled on ice for 10 min. The culture was centrifuged (3,020 x g, 10 min, 4°C) to pellet the cells and for every 40 ml of culture, the pellet was washed and resuspended in 10 ml of buffer A and then chilled on ice for 20 min. The cells were next collected by centrifugation (3,020 x g, 10 min, 4°C) and, for every 40 ml of original culture, resuspended in 10 ml of buffer B, chilled on ice for 1 h, pelleted by centrifugation (3,020 x g, 10 min, 4°C) and resuspended in 2 ml of the same solution. For each transformation, a 200 μl aliquot of the cell suspension was transferred to a sterile microfuge tube containing yeast DNA (10 μl) that was isolated previously. The tube was chilled on ice for 1 h, heated at 42°C for 2 min and then chilled on ice for 2 min, and 2x YT (200 μl) was added to each tube which was then incubated at 37°C for 1 h. The cell suspensions were then plated on YT + amp medium and incubated overnight at 37°C.

2.9.2 Yeast Transformation

TE buffer:　　　　　　　　10 mM Tris, pH 7.0
　　　　　　　　　　　　　　1 mM Na_2EDTA, pH 8.0

Lithium acetate: 100 mM dissolved in double-distilled H_2O and
                        autoclaved.

PEG: 44% (w/v) polyethylene glycol$_{4000}$ (Sigma) was
                        dissolved in 100 mM lithium acetate and filter-
                        sterilized just prior to use.

Yeast cells were transformed using the lithium acetate procedure described
by Ito et al. (1983) as modified by Schiestl and Gietz (1989) and Gietz et
al. (1992). YPDA (100 ml) was inoculated with stationary phase yeast cells
and the culture was incubated with shaking at 30°C overnight until the cell
titre reached 1-3 x 10^7 cells/ml as determined by a Coulter counter. The
culture was centrifuged (4,080 x g, 5 min, room temperature) to pellet the
cells which were washed twice in 5 ml TE buffer and resuspended in 1 ml lithium
acetate. The suspension was then incubated at room temperature for 5 min
without shaking. For each transformation, a 100 μl aliquot of the cell
suspension was transferred to a sterile 13 mm tube (Sarstedt). Bacterial
RNA (20 μl, 3.4 μg/μl) (see section 2.7.5) was added as carrier and the DNA
to be transformed into the cells was added. (To disrupt PMS1 and MSH2, 1-2 μg
of the isolated DNA fragment described in the Results sections 3.1.1 and
3.1.2 was used. To introduce YCpMP2 into yeast cells, 0.1-0.3 μg plasmid
DNA was used. To assay mismatch correction, 0.01-0.05 μg heteroduplex DNA
was used.) PEG (440 μl) was then added and the tube was incubated for an
additional 30 min without shaking at room temperature. DMSO (final
concentration: 10% v/v) was added to the tube, followed by heating at 42°C
for 15 min. Cells were then pelleted by centrifugation (1,850 x g, 5 min,
room temperature), and washed twice with, and resuspended in, 1 ml sterile
double-distilled H_2O. Aliquots (0.2 ml) of the cell suspension, or 10-fold
dilutions of the cell suspension, were plated on appropriately supplemented
minimal medium to select transformants, and the plates were incubated at

30°C for 5 days. For transformation with heteroduplex DNA, the proportions of red, white and red/white sectored transformants that emerged were determined. For other transformations, selected transformants were purified by streaking onto the same type of medium used to select the transformants, and incubating the plates for 2-3 days at 30°C. The genotypes of the transformants were then characterized by testing for growth on appropriately supplemented media.

2.10 DNA Sequencing

10x Buffer 3:	Reaction buffer 3 from BRL
RNase:	see section 2.7.1
*Bam*HI:	50 U/μl *Bam*HI (BRL) was diluted to a concentration of 1 U/μl in 1x buffer 3 just prior to use.
RP primer:	0.1 A_{260} U/ml M13 reverse sequencing primer (Pharmacia). This 17 bp primer has the sequence 5'-d(CAGGAAACAGCTATGAC)-3' and binds at position +167 to +183 relative to the first base-pair of the *SUP4*-o gene on YCpMP2. Stored at -20°C.
[^{32}P] dATP:	3000 Ci/mmol α-[^{32}P] dATP (DuPont). Stored at -60°C.
Klenow:	1 U/μl DNA polymerase I Klenow fragment from *E. coli* (BRL). Stored at -20°C.
Hin buffer:	240 mM dithiothreitol 60 mM Tris, pH 7.5 60 mM NaCl 60 mM MgCl$_2$ 1 ml aliquots were stored at room temperature.
Termination mixes:	All dNTPs and ddNTPs were purchased from Pharmacia. All four mixtures were stored at -20°C.

ddA: 150 μM ddATP   ddG: 250 μM ddGTP
     2.5 μM dATP   2.5 μM dATP
     50 μM dGTP   12.5 μM dGTP
     50 μM dCTP   50 μM dCTP
     50 μM dTTP   50 μM dTTP
     25% (v/v) Hin buffer   25% (v/v) Hin buffer

```
ddC:  250   µM ddCTP              ddT:  400   µM ddTTP
      2.5  µM dATP                      2.5  µM dATP
      50   µM dGTP                      50   µM dGTP
      12.5 µM dCTP                      50   µM dCTP
      50   µM dTTP                      8    µM dTTP
      25% (v/v) Hin buffer             25% (v/v) Hin buffer
```

Deionized formamide: 2.5 g amberlite (Bio-Rad) was mixed with 50 ml formamide. The solution was covered with parafilm, stirred at 4°C for 2 h or overnight and filtered twice through Whatman filter paper #1. 1 ml aliquots were stored at -60°C.

Stop buffer:
- 10 mM Na_2EDTA, pH 8.0
- 95% (v/v) deionized formamide
- 0.1% (w/v) xylene cyanol FF
- 0.1% (w/v) bromophenol blue

10x TEB buffer:
- 20 mM Na_2EDTA, pH 8.0
- 1 M Tris
- 0.9 M boric acid

Stored at room temperature.

Insta-gel:
- 7.6% (w/v) acrylamide
- 0.4% (w/v) bis-acrylamide
- 7 M urea
- 1x TEB buffer

Acrylamide and bis-acrylamide were dissolved in 10x TEB buffer, and distilled water and urea were then added step-wise. The solution was filtered first through a double layer of Whatman filter paper #1 and then through a 0.2 µm Millipore filter. Insta-gel was stored in a brown bottle at 4°C.

Sequencing gel: The Insta-gel was polymerized by the addition of ammonium persulfate and TEMED (N,N,N',N'-tetramethylethylenediamine) to final concentrations of 0.25% (w/v) and 0.1% (v/v), respectively, and then stored at 4°C overnight.

A modification of the dideoxynucleotide chain termination sequencing procedure (Sanger *et al*. 1977) as described by Korneluk *et al*. (1985) was used. To prepare double-stranded plasmid DNA for sequencing, the DNA was first linearized by mixing 1.2 µl of 10x buffer 3, 1.0 µl RNase, 1.0 µl of RP primer, 8 µl plasmid DNA (0.5-1 µg) and 1.8 µl *Bam*HI in a screw cap microfuge tube and incubating the tube for 30 min at 37°C. The tube was heated at 100°C for 3 min and immediately transferred to ice-water and allowed to

cool for 2 min before proceeding. Successively, 1 μl dithiothreitol (100 mM), 1 μl Klenow and 1 μl [^{32}P] dATP were added and the mixture was stirred with the pipetman tip. An aliquot (2 μl) of this mixture was added to the side of each of 4 microfuge tubes containing 2 μl of either the ddA, ddG, ddC, or ddT termination mixture and the tubes were centrifuged (Brinkman 5415C, 2 s, room temperature) to mix both solutions. These microfuge tubes were then transferred to a 46°C waterbath and incubated for 20 min, and the reactions were terminated by adding 2.4 μl stop buffer. Finally, the microfuge tubes were heated for 3 min at 100°C, transferred immediately to ice, and 2.5 μl of each reaction mixture was electrophoresed on a sequencing gel (electrolyte: 1x TEB buffer) at constant power, to heat the gel to 50°C, for about 3.5 h. The gel was then vacuum-dried at 80°C for 1 h and exposed to Kodak XAR-5 film at room temperature (the exposure time varied according to the radioactive intensity of the dried gel).

2.11 Preparation of DNA Fragments for Strain Construction

2.11.1 Restriction Digests

Enzyme digest:	1x reaction buffer (10x buffer supplied by BRL) 1 μg DNA 5 units of enzyme (BRL) per μg of DNA
Stop buffer:	50 mM Na$_2$EDTA 50% (w/v) sucrose 0.1% (w/v) bromophenol blue Stored at 4°C.
λ DNA:	*Hind*III digested λ DNA, 500 mg/ml (BRL). The DNA was stored at 4°C and heated at 65°C for 5 min before use.
Loening's buffer:	see section 2.8.1

DNA fragments used in the strain constructions were obtained by agarose gel electrophoresis of restriction enzyme-digested DNA. Plasmid DNA was digested with the appropriate enzyme for 2.5 h at 37°C, stop buffer (1/4

of the reaction volume) was added to terminate the reaction, and the resulting DNA fragments were separated by agarose gel electrophoresis (0.7% w/v agarose dissolved in Loening's buffer), for 18 h at 1 volt/cm. A λ DNA size standard accompanied the DNA samples each time. The gel was stained with ethidium bromide (0.5 μg/ml) and destained for 20 min in double-distilled H_2O.

2.11.2 Isolation of DNA Fragments

TE buffer   See section 2.7.1

Silanized glass-wool: Glass-wool was silanized with dimethyldichlorosilane in a 500 ml glass beaker by shaking a few times and rinsing thoroughly with sterile double-distilled H_2O. The silanized glass-wool was stored at room temperature in sterile double-distilled H_2O.

Ammonium acetate: See section 2.7.4

DNA fragments were isolated from agarose gels by a silanized glass-wool method (Heery *et al.* 1990). The caps of two 1.5 ml microfuge tubes were removed. A small hole was made in the base of a 0.6 ml microfuge tube with a syringe needle. A small amount of silanized glass wool was cut and packed in the bottom of the 0.6 ml tube (3-4 mm in depth), which was then placed inside the 1.5 ml tube. The 1.5 ml tube was centrifuged (Brinkman 5415C, 20 min, room temperature) to remove any excess dimethyldichlorosilane from the glass wool. After centrifugation, the 1.5 ml outer tube was discarded and replaced with a fresh tube. The band containing the DNA fragment was excised from the agarose gel and trimmed. The trimmed agarose slice was placed in the 0.6 ml tube, which was then placed inside the 1.5 ml tube, and the tubes were centrifuged (Brinkman 5415C, 20 min, room temperature) until all the liquid had been forced out of the agarose gel slice. After centrifugation, the 0.6 ml tube was discarded and the supernatant in the 1.5 ml outer tube was then transferred to a fresh 1.5 ml tube without transferring any of the

pellet. One-third the volume of ammonium acetate and 2.5 times the volume of 95% ice-cold ethanol were added to the eluate. The contents of the tube were mixed well by inversion and the tube was chilled on ice for 10 min. After incubation, the nucleic acids were pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), washed with 0.5 ml ice-cold 70% ethanol, dried by aspiration, dissolved in 10-20 μl TE buffer, and then stored at -20°C.

2.12 Hybridization Analysis

2.12.1 Agarose Gel Electrophoresis

Enzyme digest:
  1x reaction buffer 3 or 1x reaction buffer 10 (10x buffer supplied by BRL)
  5 μg DNA
  50 units of *Bam*HI, *Eco*RI or *Sal*I (BRL) per μg of DNA

Stop buffer: See section 2.11.1

λ DNA: See section 2.11.1

Loening's buffer: See section 2.8.1

Total yeast DNA (5 μg) was digested with *Bam*HI, *Eco*RI or *Sal*I overnight, stop buffer (1/4 of the total reaction volume) was added to terminate the reaction and the resulting DNA fragments were separated by agarose (0.7% w/v, dissolved in Loening's buffer containing 0.5 μg/ml ethidium bromide) gel electrophoresis, for 18 h at 1 volt/cm (a λ DNA standard was included). A picture was taken for later sizing of the bands, and the gel was soaked for 45 min in 0.5 M NaOH and then for 45 min in 1 M Tris (pH 8.0) at room temperature with slow shaking. Next, the gel was sandwiched between 3MM Chr Whatman Chromatography paper and a single layer of Saran Wrap and vacuum-dried at 60°C for 1 h (Tsao *et al.* 1983). The dried gel was stored at room temperature.

2.12.2 Random Primers DNA Labelling System

The Random Primers Buffer Mixture, dNTPs, stop buffer, and DNA polymerase were supplied by BRL with the Random Primers Labelling Kit.

Random Primers Buffer Mixture:

$$
\begin{aligned}
&670 \quad \text{mM HEPES} \\
&170 \quad \text{mM Tris, pH 7.2} \\
&17 \quad \text{mM MgCl}_2 \\
&33 \quad \text{mM 2-mercaptoethanol} \\
&1.3 \text{ mg/ml bovine serum albumin} \\
&18 \text{ OD}_{260} \text{ units/ml oligodeoxyribonucleotide primers}
\end{aligned}
$$
(hexamer fraction), pH 6.8

[^{32}P]dATP: see section 2.10

dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na$_2$EDTA

dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na$_2$EDTA

dTTP solution: 0.5 mM dTTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na$_2$EDTA

Reaction mix:

$$
\begin{aligned}
&5 \ \mu\text{l Random Primers Buffer Mixture} \\
&20 \ \mu\text{M dCTP} \\
&20 \ \mu\text{M dGTP} \\
&20 \ \mu\text{M dTTP} \\
&50 \ \mu\text{Ci [}^{32}\text{P]dATP}
\end{aligned}
$$

Stop solution: 200 mM Na$_2$EDTA, pH 7.5

DNA polymerase: 6 U/μl *E. coli* DNA polymerase I Klenow fragment (BRL). Stored at -20°C.

Yeast tRNA: Yeast tRNA (Boehringer Mannheim) was dissolved in sterile double-distilled H$_2$O (20 mg/ml) and stored at -20°C.

Sodium acetate: See section 2.8.1

[^{32}P]-labelled probes for hybridization were prepared by using the BRL Random Primers DNA Labelling kit according to a modification of the protocol supplied by the manufacturer. DNA (25 ng), dissolved in 5-20 μl of distilled water in a sterile screw cap microfuge tube, was denatured by heating in a boiling water bath for 5 min and then immediately cooling the tube on ice. The reaction mixture was added to the tube on ice and the final volume of

49 μl was made by adding sterile double-distilled H_2O. Next, DNA polymerase (1 μl) was added and the tube was incubated at 25°C for 2 hours. The reaction was stopped by adding 5 μl stop buffer. Successively, 5 μl yeast tRNA, 7 μl $MgCl_2$ (100 mM), 7 μl sodium acetate and 200 μl ice-cold ethanol (95%) were added. The tube was then held at -60°C for 1 h and the precipitate was pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C). The supernatant was removed with a Pasteur pipette. Then, the pellet was washed with 1 ml ice-cold ethanol (70%), dried and dissolved in 100 μl sterile double-distilled H_2O. The tube was heated at 100°C for 5 min and then rapidly transferred to ice-water.

2.12.3 Hybridization Procedure

20x SSC buffer: see section 2.8.3

Salmom sperm DNA: Salmon sperm DNA (Sigma) was dissolved in sterile double-distilled H_2O (10 mg/ml) and then passed through a fine syringe several times to shear the DNA. Aliquots were stored at -20°C.

50x Denhardt's solution:
    10 mg/ml ficoll
    10 mg/ml polyvinylpyrrolidone
    10 mg/ml bovine serum albumin (BRL)
    Stored at -20°C.

Prehybridization solution:
    6x SSC
    5x Denhardt's solution
    0.5% (w/v) SDS
    0.2 mg/ml salmon sperm DNA
    Prepared just prior to use and heated to 65°C.

Hybridization solution:
    10 mM Na_2EDTA
    6x SSC
    5x Denhardt's solution
    0.5% (w/v) SDS
    Prepared just prior to use and heated to 65°C.

Solution I: 2x SSC
    0.5% (w/v) SDS

Solution II:	2x SSC
	0.1% (w/v) SDS
Solution III:	0.1x SSC
	0.5% (w/v) SDS

Direct DNA hybridization in agarose gels was carried out as described by Tsao *et al.* (1983) and Maniatis *et al.* (1989). The dried gel was floated paper-side down on 2x SSC for 30 s and gently peeled off from the paper backing. The gel was then rolled into a tight spiral, using a nylon mesh to prevent gel-gel overlap, and placed in a 35 mm x 150 mm glass hybridization bottle (BIO/CAN Scientific). Prehybridization solution (20 ml) was added and the bottle was sealed and incubated for 10 min at 65°C in a BIO/CAN Scientific Turbo Speed Rotary Hybridization Oven. After 10 min, the bottle top was opened to release the pressure build-up, refastened, and the bottle was further incubated for 2-4 h at 65°C in the oven. The prehybridization solution was removed and replaced with 20 ml of hybridization solution, the labelled DNA probe was added, and the bottle was sealed and incubated overnight at 65°C. The gel was then removed carefully from the bottle and the following washes were performed: 1. 50 ml of solution I for 5 min at room temperature (twice); 2. 50 ml of solution II for 15 min at room temperature (twice); 3. 50 ml of solution III for 2 h, 1 h and 0.5 h at 65°C. The gel was then placed on 3MM Chr Whatman Chromatography paper, air-dried, covered with a single layer of Saran Wrap and exposed to Kodak XAR-5 film with an intensifying screen at -60°C (the exposure time varied according to the radioactive intensity of the dried gel).

2.13 Preparation of Gapped Heteroduplex Plasmids

2.13.1 Preparation and Isolation of Small *Bam*HI or *Eco*RI Fragments

Enzyme digest: 192 μl (500 μg) pHR307a
                              30 μl 10x buffer 3 (BRL)
                              30 μl *Bam*HI or EcoRI (50 u/μl)
                              48 μl sterile double-distilled H_2O

TE buffer: see section 2.7.1

29:1 (w/w) acrylamide/bisacrylamide:
                              29 g acrylamide
                              ·1 g bisacrylamide
                              Ajusted with double-distilled H_2O to a final volume of 100 ml.

10x TEB buffer: see section 2.10

5% acrylamide gel: 2.5 ml 10x TEB buffer
                              4.17 ml 29:1 acrylamide/bisacrylamide
                              18.33 ml double-distilled H_2O
                              25 μl TEMED
                              50 μl 25% ammonium persulfate
                              The gel was allowed to polymerize at room temperature for at least 30 min before use.

Plasmid pHR307a was digested with *Bam*HI or *Eco*RI at 37°C for 3 h to release a 24 bp *Bam*HI fragment or a 42 bp *Eco*RI fragment, respectively. The resulting fragments were separated on a 5% acrylamide gel. Electrophoresis was for 0.5 h at 5 volt/cm and then the gel, supported on one plate, was stained for 10 to 30 min in 0.5 μg/ml ethidium bromide, followed by destaining in water for 10 to 30 min. The band for the 24 bp *Bam*HI or 42 bp *Eco*RI fragment was visualized on a UV transilluminator, excised from the gel and the gel slice was transferred into a 1.5-ml microfuge tube. Then, TE buffer was added to the tube and 10% SDS was added to a final concentration of 0.1% (V/V). The tube was kept at room temperature overnight, centrifuged for (Brinkman 5415C, 1.5 h, 4°C), and the supernatant was transferred to a fresh tube. Then 1 M MgCl, 10 mM ATP and 3 M Na acetate were added to final concentrations of 1%, 10% and 10% (v/v), respectively. Ethanol (2.5 volumes, 95%) was added

next and the tube was held at -20°C overnight. The DNA fragments were then

pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), dried, dissolved

in 30 μl sterile double-distilled H_2O and stored at -20°C.

2.13.2 Construction of Plasmids Containing a Small *Bam*HI or *Eco*RI Fragment

Enzyme digest:	10 μl (10 μg) YCpLK1E(32T) or YCpLK3EB 2 μl 10x buffer 3 (BRL) 1 μl *Bam*HI or *Eco*RI (50 u/μl) 7 μl sterile double-distilled H_2O Incubated at 37° for 2 h.
AP:	Phosphatase, alkaline, from calf intestine (1 u/μl) (Boehringer Mannheim)
Ligation reaction:	1 μl (0.1 μg) YCpLK1E(32T) or YCpLK3EB (linearized by *Bam*HI or *Eco*RI, then treated with AP) 5 μl *Bam*HI or *Eco*RI fragment 4 μl 5x T4 DNA Ligation Buffer (BRL) 1 μl (1 u/μl) T4 DNA ligase (BRL) 9 μl sterile double-distilled H_2O Incubated at room temperature for 3 h.

The plasmids YCpLK1E(32T) and YCpLK3EB, were linearized with *Bam*HI or

*Eco*RI, respectively. Then, 1 μl of AP was added to each digestion reaction

and incubation was continued at 37°C for 1 h. Following extractions with

TE-saturated phenol and TE-saturated chloroform, the fragments were

precipitated with 95% ethanol, washed with 70% ethanol, dried and dissolved

in 10 μl sterile double-distilled H_2O. The concentration of the DNA was

determined by A_{260} and the DNA was stored at -20°C.

*Bam*HI or *Eco*RI-linearized and AP-treated YCpLK1E(32T) or YCpLK3EB was

ligated to the 24 bp *Bam*HI or 42 bp *Eco*RI fragment, respectively. The ligation

mixes were transformed into *E. coli* JF1754 (see section 2.9.1) and

transformants were selected on YT + amp plates. Plasmids were then isolated

from the transformants by a rapid alkaline procedure (see section 2.7.3)

and the presence of the *Bam*HI or *Eco*RI fragments was confirmed by sizing

on agarose gels after linearizing the plasmids with *Pst*I. YCpLK1E(32T) or

YCpLK3EB containing the *Bam*HI or *Eco*RI fragments were named YCpLK1E(32T)-B, YCpLK1E(32T)-E, YCpLK3EB-B or YCpLK3EB-E, respectively.

2.13.3 Preparation of Gapped Heteroduplex Plasmids

Heteroduplexes with a transition mismatch (C/A) located at site 32 within the *SUP4*-o gene, and with a small gap positioned 112 bp 5′ to site 32 in *SUP4*-o or 119 bp 3′ to site 32, were prepared essentially as described in section 2.8. Single-stranded DNA was obtained from YCpLK1E(32T)-B and YCpLK1E(32T)-E or YCpLK3EB-B and YCpLK3EB-E, which carry a 24 bp insert at the *Bam*HI site, or a 42 bp insert at the *Eco*RI site, respectively. The double-stranded YCpLK1E or YCpLK3EB(32A) DNA used to construct the heteroduplexes was linearized by digestion with *Bam*HI or *Eco*RI. By using the appropriate plasmid and restriction enzyme combinations, the gap could be placed 5′ or 3′ to *SUP4*-o, on the transcribed or non transcribed strand (Figures 5 and 6).

2.14 Statistical Analysis

Chi-square contingency tests employing Yates' correction for continuity (Sokal and Rohlf 1969) were used to evaluate differences in a variety of parameters. The Monte Carlo estimate of the P value of the hypergeometric test (Adams and Skopek 1987) was calculated to assess the significance of differences in the distributions of base-pair substitutions in *SUP4*-o (1,500 simulations were run). The original VAX/VMS based program was incorporated into a PC based program by Cariello (1994). The simulations were run on an Intel 486 DX2-66 based personal computer. For both types of test, values of $P < 0.05$ were considered significant.

Figure 5. Construction of gapped heteroduplex plasmids having a C/A mismatch at site 32 in *SUP4*-o and the gap on the transcribed strand. Grey boxes represent functional *SUP4*-o alleles, while black boxes represent mutant alleles. White boxes represent a 24 bp *Bam*HI insert and hatched boxes represent a 42 bp *Eco*RI insert. The arrows adjacent to *SUP4*-o indicate the direction of transcription and are positioned beside the template strand for transcription. ss: single-stranded, ds: double stranded.

GAPPED HETERODUPLEX CONSTRUCTION
(gap on the transcribed strand)

Heteroduplex plasmids with a small gap on the transcribed strand
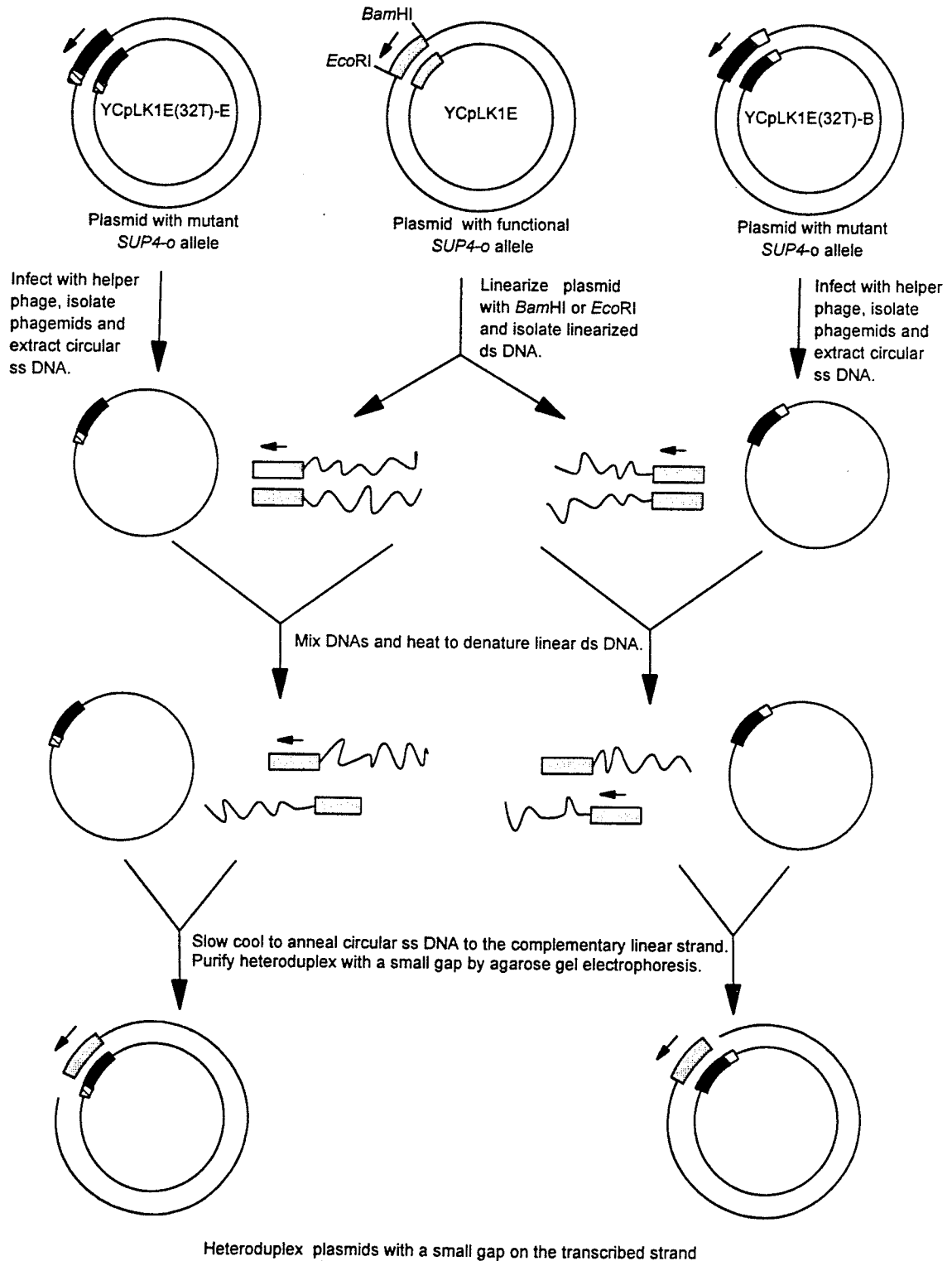
Figure 6. Construction of gapped heteroduplex plasmids having a C/A mismatch at site 32 in *SUP4*-o and the gap on the nontranscribed strand. Grey boxes represent functional *SUP4*-o alleles, while black boxes represent mutant alleles. White boxes represent a 24 bp *Bam*HI insert and hatched boxes represent a 42 bp *Eco*RI insert. The arrows adjacent to *SUP4*-o indicate the direction of transcription and are positioned beside the template strand for transcription. ss: single-stranded, ds: double stranded.

GAPPED HETERODUPLEX CONSTRUCTION
(gap on the nontranscribed strand)

EcoRI

BamHI

YCpLK3EB-B

YCpLK3EB(32A)

YCpLK3EB-E

Plasmid with functional
SUP4-o allele

Plasmid with mutant
SUP4-o allele

Plasmid with functional
SUP4-o allele

Infect with helper
phage, isolate
phagemids and
extract circular
ss DNA.

Linearize plasmid
with *Bam*HI or *Eco*RI
and isolate linearized
ds DNA.

Infect with helper
phage, isolate
phagemids and
extract circular
ss DNA.

Mix DNAs and heat to denature linear ds DNA.

Slow cool to anneal circular ss DNA to the complementary linear strand.
Purify heteroduplex with a small gap by agarose gel electrophoresis.

Heteroduplex plasmids with a small gap on the nontranscribed strand

3 RESULTS

3.1 Characterization of the *pms1Δ* and *msh2Δ* Mutators

To investigate the specificity of the mutator effect conferred by mismatch correction deficiency in yeast, strains isogenic to MKP-o but having mismatch correction genes mutated were required. The yeast genes *PMS1* and *MSH2* function in mismatch repair (see sections 1.2.1 and 1.2.2 of the Introduction). Thus, the first stage of the project involved the construction of MKP-o derivatives in which *PMS1* or *MSH2* were deleted (*pms1Δ* or *msh2Δ*, respectively).

3.1.1 Construction of a *pms1Δ* Strain

YY1 (*pms1Δ*), an isogenic derivative of MKP-o having the entire ORF of the *PMS1* gene deleted, was constructed as diagrammed in Figure 7. First, MKP-o was transformed with the *Bst*XI-linearized plasmid pWBK4 (Kramer *et al.* 1989b). pWBK4 is a yeast integrating plasmid which contains *URA3* and carries a 5.2 kb *Kpn*I yeast chromosomal DNA fragment encompassing the *PMS1* locus and flanking DNA (Figure 7A). However, the 2.7 kb *Mlu*I-*Sst*I fragment carrying the entire ORF of the *PMS1* gene has been deleted from the chromosomal DNA fragment (Figure 7A). Digestion of pWBK4 with *Bst*XI targets plasmid integration to the *Bst*XI site 3′ to the *PMS1* gene (Figure 7B) via homologous recombination (Rothstein 1991). Since MKP-o requires uracil due to a defective *URA3* allele (*ura3-52*), the *URA3* gene in the plasmid allows integration to be detected by selection for growth on medium lacking uracil. Integration of pWBK4 in Ura⁺ transformants was confirmed as follows. Genomic DNA from the Ura⁺ transformants YYU1, YYU2, YYU3, YYU4 as well as MKP-o, was digested with *Sal*I and electrophoresed on an agarose gel. The DNA in the gel was denatured, and the gel was dried

Figure 7. Construction of YY1 (*pms1*∆). Step one: *Bst*XI-linearized pWBK4 (A) was integrated at the *Bst*XI site adjacent to the chromosomal *PMS1* gene (B) as shown in (C). Step two: *URA3* and *PMS1* were excised by reciprocal intrachromosomal recombination between the repeated 1.3 kb *KpnI-MluI* fragments resulting in the structure shown in D. The dashed line indicates bacterial plasmid DNA. The solid and dotted lines represent yeast chromosomal DNA with the dotted segments indicating a region not drawn to scale. The boxes indicate the yeast genes.

A

URA3

Sal I
Kpn I —
5.1 kb
pWBK4
5.2 kb
Mlu I/Sst I
Sal I
— Kpn I
Bxt XI

B

Bam HI   Kpn I   Mlu I   PMS1   Sst I   Sal I   Bst XI   Kpn I   Bam HI

1.3 kb   2.7 kb   0.4 kb   2.2 kb   1.3 kb

19.2 kb

C

Kpn I   Mlu I   PMS1   Sst I   Sal I   Bst XI   Kpn I   URA3   Sal I   Kpn I   Mlu I/Sst I   Sal I   Bst XI   Kpn I   Sal I

8.6 kb

12.9 kb

D

Bam HI   Kpn I   Mlu I/Sst I   Sal I   Bst XI   Kpn I   Bam HI

16.5 kb
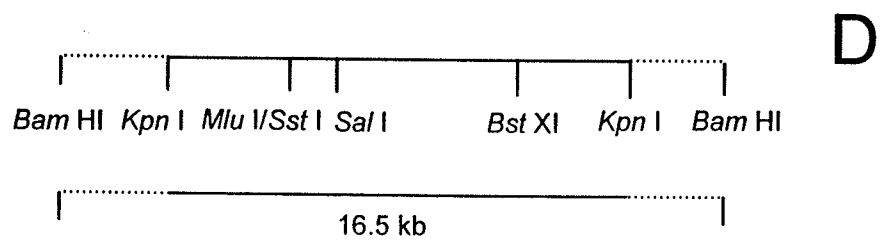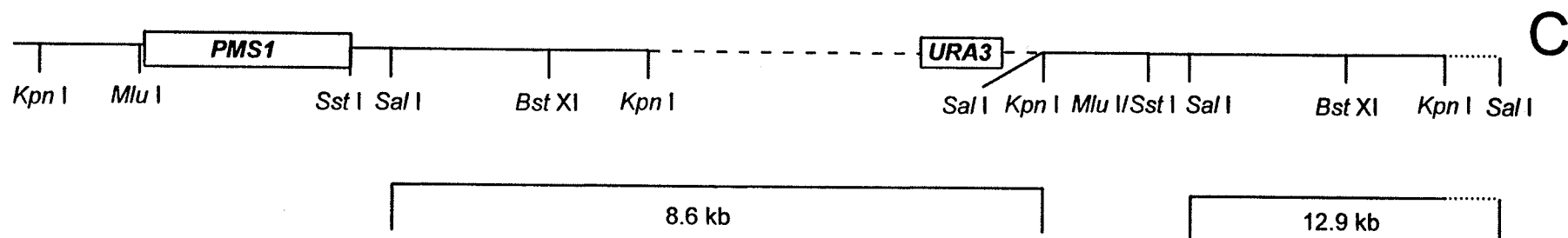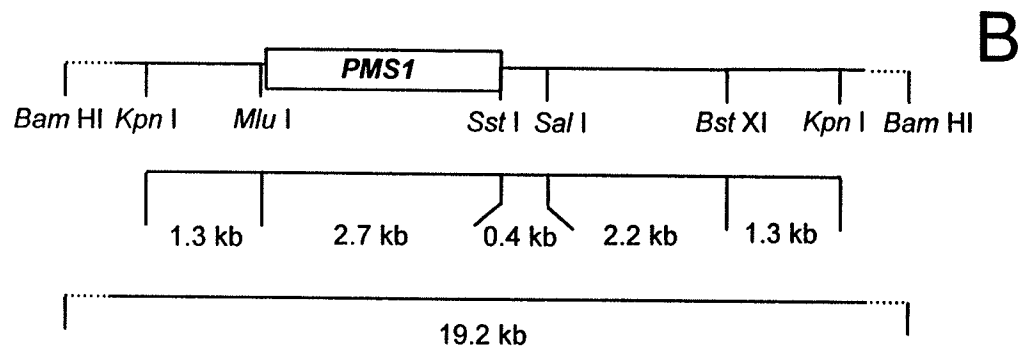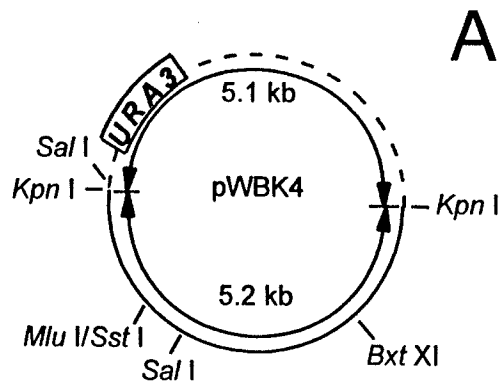
and probed with the pWBK4 1.3 kb *Bst*XI-*Kpn*I yeast fragment (Figure 7A). The integration of pWBK4 at the *Bst*XI site 3' to *PMS1* should result in the appearance of a novel 8.6 kb band in addition to the 12.9 kb band expected for genomic DNA from MKP-o (Figure 7C). The 8.6 kb band was detected for all four transformants (Figure 8A).

Intrachromosomal recombination between the repeated 1.3 kb *Kpn*I-*Mlu*I fragments in the construct (Figure 7C) deletes *PMS1* and *URA3*. Thus, loss of *PMS1* can be monitored by scoring for elimination of *URA3*. Since the *Bam*HI chromosomal DNA fragment encompassing *PMS1* is 19.2 kb, and *PMS1* is approximately 2.7 kb (Figure 7B), the recombination event that deletes most of the insert including *PMS1* reduces the 19.2 kb *Bam*HI fragment to 16.5 kb (Figure 7D). YYU1 was grown to stationary phase in supplemented minimal medium and then plated on 5-fluoro-orotic acid (FOA)-containing medium to select isolates which had lost *URA3* (Boeke *at al*. 1984). FOA has a chemical structure similar to orotidylic acid which is a precursor involved in UMP and dTMP synthesis. In a Ura$^+$ cell, FOA is converted into 5-fluoro-orotidine-5'-monophosphate by the *URA5* gene product, orotate phosphoribosyltranferase. The latter compound is decarboxylated to 5-fluoro-uridine-5'-monophosphate (FUMP) by the *URA3* gene product, orotidine-5'-phosphate decarboxylase. The FUMP is then converted in a series of steps to 5-fluoro-2'-deoxy-5'-monophosphate (FdUMP), a potent inhibitor of dTMP synthetase. Thus, FOA inhibits DNA synthesis by preventing dTMP formation. Given the role of *URA3* in the formation of FdUMP from FOA, it is evident that inactivation of *URA3* will render yeast cells immune to the toxic effects of FOA. Genomic DNA from several Ura$^-$ isolates (YY1-4), as well as MKP-o, was digested with *Bam*HI, and electrophoresed on an agarose gel. The DNA in the gel was denatured and

Figure 8. Hybridization analysis of Ura$^+$ transformants and Ura$^-$ isolates.
A. Step one: yeast genomic DNA from Ura$^+$ transformants was digested with
SalI and electrophoresed on a 0.7% agarose gel. The gel was denatured,
dried and probed with the ^{32}P-labelled 1.3 kb BstXI-KpnI fragment from plasmid
pWBK4 (Figure 7A). Lane 1: MKP-o; lane 2: YYU1; lane 3: YYU2; lane 4: YYU3;
lane 5: YYU4. B. Step two: yeast genomic DNA from Ura$^-$ isolates was digested
with BamHI and electrophoresed and probed as described above. Lane 1: MKP-o;
lane 2: YY1; lane 3: YY2; lane 4: YY3; lane 5: YY4. The DNA size markers
are in kb.
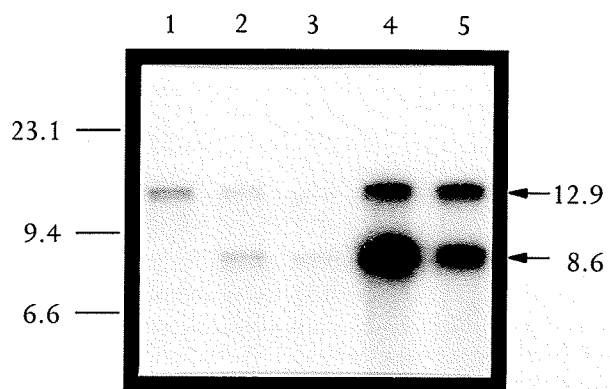
A
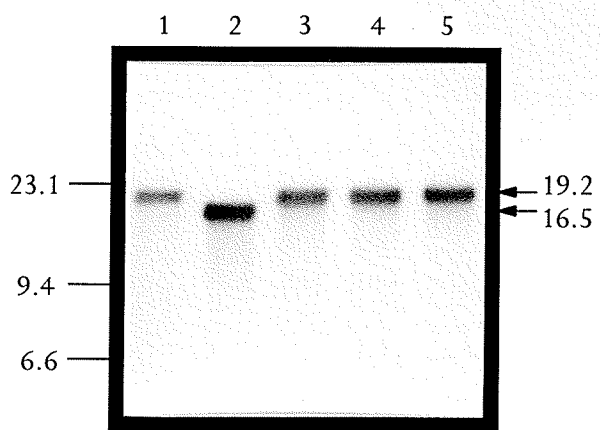
B

the gel dried and probed with the pWBK4 1.3 kb BstXI-KpnI fragment. Of the Ura⁻ isolates tested, only YY1 exhibited the change in fragment size from 19.2 kb to 16.5 kb (Figure 8B). The loss of PMS1 in YY1 was confirmed phenotypically by assaying for mismatch correction deficiency (see section 3.1.4). YY1 was then transformed with YCpMP2 to make YY1-p.

3.1.2 Construction of a msh2Δ Strain

YY2 (msh2Δ), having almost the entire ORF of the MSH2 gene deleted, was constructed as follows. First, MKP-o was transformed with a 5.4 kb AatII-PvuII DNA fragment (Figure 9A) which was isolated from plasmid pEN63. This fragment differs from the 3.6 kb AatII-PvuII yeast DNA fragment encompassing the MSH2 locus (Figure 9B) in that the EcoNI-HpaI fragment encoding 898 of the 965 amino acids in the Msh2 protein has been replaced by a 3.8 kb hisG-URA3-hisG cassette (Alani et al. 1987). As the cassette is embedded in sequences that flank MSH2 (Figure 9A and 9B), the 5.4 kb AatII-PvuII DNA fragment should integrate at the chromosomal MSH2 locus via recombination between the homologous fragment and chromosomal DNA sequences (Rothstein 1991). Ura⁺ transformants were selected and replacement of the MSH2 gene with the 5.4 kb AatII-PvuII pEN63 DNA fragment was confirmed by DNA hybridization analysis. Total chromosomal DNA was isolated from MKP-o and a number of Ura⁺ isolates, digested with EcoRI, and the resulting fragments were separated by agarose gel electrophoresis. Following denaturation of the DNA in the gel, the gel was dried and probed with a mixture of 1.7 kb and 1.8 kb EcoRI fragments, which overlap the MSH2 gene (Figure 9B), and were isolated from plasmid pII-2 (Reenan and Kolodner 1992a). From Figure 9B, it can be seen that the probes should detect 1.7 kb and 1.8 kb EcoRI fragments when hybridized to

Figure 9. Construction of YY2 *(msh2Δ)*. Step one: the 5.4 kb *Aat*II-*Pvu*II pEN63 fragment containing the *hisG-URA3-hisG* cassette surrounded by sequences that flank *MSH2* (A) was integrated at the *MSH2* locus (B). Integration occurs via homologous recombination and deletes the chromosomal *MSH2* gene as shown in C. Step two, the *URA3* gene was excised via reciprocal intrachromosomal recombination between the *hisG* repeats to leave a single copy of *hisG* as shown in (D). The lines represent yeast chromosomal DNA. The boxes indicate the genes involved.
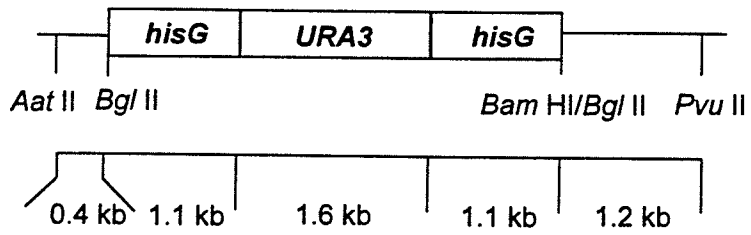
A
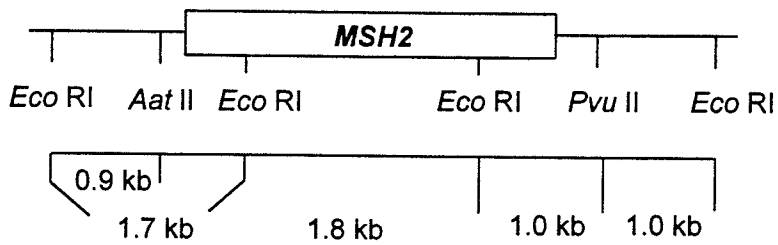
B
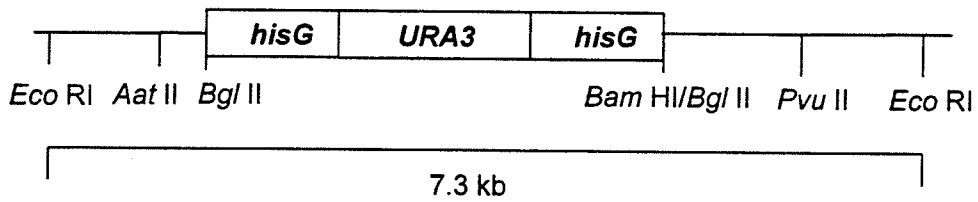
C
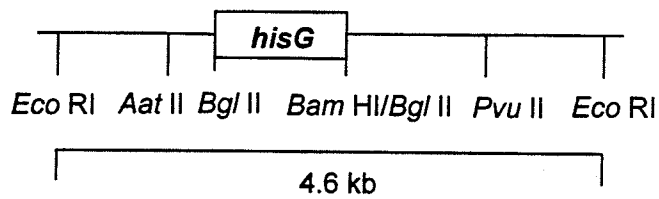
D

MKP-o DNA. Figure 10 lane 1 shows that MKP-o has one band at 1.7 kb and another band at 1.8 kb as expected. Integration of the 5.4 kb *Aat*II-*Pvu*II pEN63 fragment at the chromosomal *MSH2* locus changes this pattern because the two *Eco*RI sites within *MSH2* are eliminated (Figure 9C). Hence, the 1.7 kb and 1.8 kb *Eco*RI bands are replaced by a 7.3 kb *Eco*RI band (Figure 9C). This replacement was observed in one of the Ura⁺ transformants, designated YYUC2 (Figure 10, lane 2).

In order to allow selection for YCpMP2, it was necessary to delete *URA3* from YYUC2. *URA3* can be excised from the construct via recombination between the 1.1 kb *hisG* repeats surrounding *URA3* (Figure 9C; Alani *et al.* 1987). Thus, YYUC2 was grown to stationary phase in supplemented minimal medium and then plated on FOA-containing medium to select Ura⁻ isolates. Genomic DNA from several Ura⁻ isolates was digested with *Eco*RI and the fragments were separated by gel electrophoresis and hybridized to the same probes used in the initial stage of the construction. Loss of *URA3* and one *hisG* repeat reduces the 7.3 kb *Eco*RI band to a 4.6 kb *Eco*RI band (Figure 9D). All of the Ura⁻ transformants tested showed the expected 4.6 kb band (Figure 10, lanes 3-9). Inactivation of *MSH2* was confirmed phenotypically in an appropriate isolate, designated YY2, by assaying for mismatch correction deficiency (see section 3.1.4). YY2 was then transformed with YCpMP2 to make YY2-p.

3.1.3 Specificities of the *pms1*Δ and *msh2*Δ Mutators

The specificities of the *pms1*Δ and *msh2*Δ mutators were determined by DNA sequence characterization of spontaneous mutations arising in the *SUP4*-o gene in the *pms1*Δ or *msh2*Δ backgrounds.

Figure 10. Hybridization analysis of Ura$^+$ transformants and Ura$^-$ isolates. Yeast genomic DNA samples from a single Ura$^+$ transformant or several Ura$^-$ isolates were digested with *EcoRI* and electrophoresed on a 0.7% agarose gel. The gel was denatured, dried and probed with the ^{32}P-labelled 1.7 kb and 1.8 kb *EcoRI* fragments shown in Figure 9B. Lane 1: MKP-o; lane 2: Ura$^+$ transformant (YYUC2); lanes 3-9: Ura$^-$ isolates of YYUC2. The DNA size markers are in kb.

1 2 3 4 5 6 7 8 9

9.4

6.6 — ← 7.3

4.4 — ← 4.6

2.3
2.0

← 1.8
← 1.7

0.6
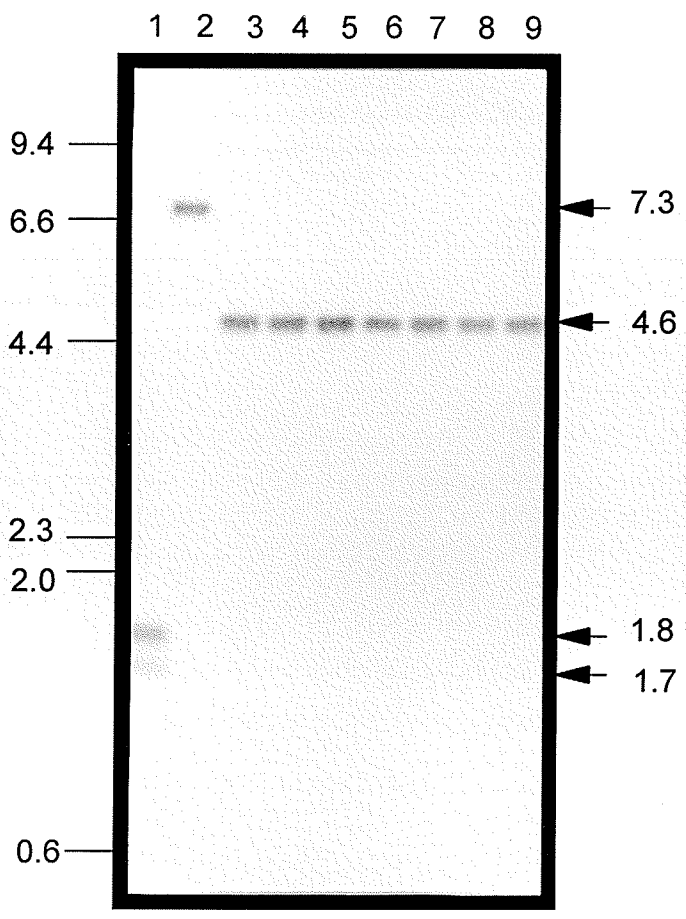
3.1.3.1 Plasmid Retention, *SUP4*-o Mutation Rate and *lys2-1* → Lys$^+$ Reversion/Suppression

The yeast strains (MKP-op: *PMS1 MSH2*; YY1-p: *pms1Δ*; YY2-p: *msh2Δ*) used in this study are isogenic except for the *PMS1* or *MSH2* loci. Hence, any differences detected among them should reflect the properties of the individual *PMS1* or *MSH2* mutators, rather than variation in genetic background. Since *SUP4*-o is carried on the yeast centromere plasmid YCpMP2, cultures of the three strains were grown from low titre inocula to stationary phase in medium selective for YCpMP2. They were then plated to measure plasmid retention and isolate *SUP4*-o mutations. Plasmid retention was determined by comparing the number of colonies that emerged on medium selective for the plasmid with the corresponding number for nonselective medium. Of the *pms1Δ* and *msh2Δ* cells, 91% and 82%, respectively, carried YCpMP2 (Table 2). These values were similar to 88% for the wildtype, indicating that the defects in the *PMS1* and *MSH2* genes did not alter plasmid retention. Relative to the wildtype parent, the rates of spontaneous plasmid *SUP4*-o mutation were increased by 7- and 6-fold for the *pms1Δ* and *msh2Δ* strains, respectively (Table 3). Additionally, the rates of spontaneous *lys2-1* → Lys$^+$ reversion were 19- and 23-fold greater, while those of the *lys2-1* → Lys$^+$ suppression were increased by 3- and 4-fold for the *pms1Δ* and *msh2Δ* strains, respectively, compared to the wildtype parent (Table 3). These increases reflect the *pms1Δ* and *msh2Δ* mutator effects.

3.1.3.2 Mutational Classes Detected

Two collections of 210 (*pms1Δ*) and 213 (*msh2Δ*) spontaneous *SUP4*-o mutations, each isolated from an independent culture of the *pms1Δ* or *msh2Δ*

TABLE 2. Plasmid retention

Strain	Colonies on selective medium	Colonies on nonselective medium	Percent plasmid retention
PMS1 MSH2	37,299	42,231	88.3
pms1Δ	19,976	21,957	91.0
msh2Δ	22,329	27,142	82.3

TABLE 3. Spontaneous mutation in isogenic wildtype and mismatch correction deficient strains

Location and type of mutation scored	Strain	Mutation frequency[a]	Mutation rate
Plasmid: *SUP4*-o → *sup4*⁻	*PMS1 MSH2*	9.2×10^{-7}	2.8×10^{-7}
	pms1Δ	9.9×10^{-6}	1.9×10^{-6}
	msh2Δ	8.7×10^{-6}	1.7×10^{-6}
Chromosome: *lys2-1* → Lys⁺ (reversion)	*PMS1 MSH2*	4.7×10^{-9}	1.5×10^{-9}
	pms1Δ	7.3×10^{-8}	2.8×10^{-8}
	msh2Δ	9.3×10^{-8}	3.5×10^{-8}
Chromosome: *lys2-1* → Lys⁺ (suppression)	*PMS1 MSH2*	1.5×10^{-7}	2.5×10^{-8}
	pms1Δ	3.3×10^{-7}	8.7×10^{-8}
	msh2Δ	4.3×10^{-7}	1.1×10^{-7}

[a] Frequencies are the medians for 20 (*PMS1 MSH2*) or 30 (*pms1Δ* and *msh2Δ*) independent cultures. Frequencies and rates for *SUP4*-o mutation and *lys2-1* reversion or suppression in *PMS1 MSH2* are taken from Kang *et al.* (1992) and Roche *et al.* (1995), respectively.

strains, were characterized and the resulting mutational spectra werecompared to that for 354 mutants selected in the wildtype parent. The data for the wildtype strain have been published (Kang et al. 1992) but are presented here to facilitate critical comparisons.

Only 5 and 4 of the 8 classes of mutation detected in the wildtype strain also were recovered in the pms1Δ and msh2Δ mutators, respectively (Table 4). The relative fraction of single base-pair substitutions was reduced from the 81.9% in the PMS1 MSH2 background to 60.0% or 63.8% in the pms1Δ or msh2Δ, respectively (P < 0.001 in both cases). However, more single base-pair deletions were recovered in the pms1Δ (34.7%) and msh2Δ (33.4%) strains than in the parental wildtype (6.8%) (P < 0.001 in both cases). The relative fraction of single base-pair insertions was also increased from 0.3% for PMS1 MSH2 to 4.3% and 2.3% for pms1Δ and msh2Δ, respectively (P < at least 0.05 in both cases) (the difference between the two mutators was not significant, P > 0.10). The rates of single base-pair substitution were increased only by 5-fold in both mutators. The rates of single base-pair deletions were 35- and 30-fold greater, and those of single base-pair insertions were 82- and 41-fold greater in the pms1Δ and msh2Δ mutators, respectively (Table 4).

Non-tandem substitutions and duplications were not detected in either mutant strain (Table 4). A multiple base-pair deletion, and a complex change, occurred in the pms1Δ mutator, and a single Ty insertion was detected in the msh2Δ mutator (Tables 4 and 5). Perhaps the preferential increases in the rates of single base-pair substitution, deletion and insertion reduced the probability of detecting the rarer mutational classes in both mutator strains. Consistent with this possibility, the relative fractions of multiple base-pair deletion and complex change were

TABLE 4. Sequence alterations in *SUP4-o* mutants

Alteration	PMS1 MSH2			pms1Δ			msh2Δ		
	Number detected		Rate (x 10^{-8})	Number detected		Rate (x 10^{-8})	Number detected		Rate (x 10^{-8})
Substitution									
Single bp	290	(81.9)[a]	22.8	126	(60.0)	115.0	136	(63.8)	110.0
Non-tandem bp	2	(0.6)	0.2	-			-		
Deletion									
1 bp	24	(6.8)	1.9	73	(34.7)	66.5	71	(33.4)	57.7
>1 bp	7	(2.0)	0.6	1	(0.5)	0.9	-		
Insertion									
1 bp	1	(0.3)	0.1	9	(4.3)	8.2	5	(2.3)	4.1
Ty element	26	(7.3)	2.0	-			1	(0.5)	0.8
Duplication	1	(0.3)	0.1	-			-		
Complex change	3	(0.8)	0.2	1	(0.5)	0.9	-		
Total	354		27.9	210		191.5	213		172.6

[a]Per cent of total events.

TABLE 5. Multiple mutations, deletions and insertions

Sites[a]	Change[b]	PMS1 MSH2	pms1Δ	msh2Δ
		Number detected		
-2 → -249	-248	1	-	-
8, 10	A → G, C → T	1	-	-
12, 14	T → C, G → A	1	-	-
13 → -122	-135	1	-	-
15	-1	1	-	-
17 ↔ 18	Ty	2	-	-
18 → 19	-1	1	-	-
37 ↔ 38	Ty	23	-	1
43 ↔ 44	Ty	1	-	-
58 → 64	-7, +GGGCC	2	-	-
61 → 64	-4	-	1	-
62 → 89	-27	1	-	-
70 → -555	-625	1	-	-
72	-1	1	-	-
72 → 78	Direct duplication	1	-	-
78	-1	-	-	1
79 → 83	-1	19	72	66
79 → 83	+1	1	9	5
79 → 83, 88 → 119	-1, -32	1	-	-
81 → -726	-807	1	-	-
84 → 86	-1	2	1	4
88, 89	-1, T→A or C→A, -1	-	1	-
89 → 96	-8	1	-	-
89 → 97	-9	1	-	-

[a]Sites for deletions of 9, 27, 248, or 807 bp in PMS1 MSH2 and 4 bp in pms1Δ are estimates because sequence repeats are at the deletion termini. The symbol ↔ indicates that Ty is presumed to have inserted between the two sites. For deletions or insertions in runs of base pairs, the position of the run is given since the precise base pair eliminated or added cannot be determined.

[b]Changes are given for the transcribed strand (see Figures 11 and 12).

lower for *pms1Δ* than *PMS1 MSH2*, as was the case for the relative fractionof Ty insertion in *msh2Δ* (Table 4). Furthermore, the one Ty insertion recovered in the *msh2Δ* strain was at position 37, which is a hotpot for Ty transposition in the wildtype parent (Tables 4 and 5).

3.1.3.3 Single Base-Pair Deletion/Insertion

In the wildtype strain, 19/24 single base-pair deletions occurred in the run of five G•C pairs at 79 → 83 (Table 5). Three were in runs of three (84 → 86: 2) or two (18 → 19: 1) base-pairs and the other two were at sites 15 and 72. The only single base-pair insertion recovered in the wildtype strain also was found at 79 → 83 (Table 5). Among the 73 single base-pair deletions recovered in the *pms1Δ* mutator, 72 were in the run of five G•C pairs at 79 → 83, and the remaining deletion was in the triple G•C run at 84 → 86. All nine single base-pair insertions detected in the *pms1Δ* mutator also were in the run at 79 → 83 (Table 5). Similar results were observed for the *msh2Δ* strain. Most (66) of the 71 single base-pair deletions were in the run at 79 → 83, four were in the run at 84 → 86, and one was at site 78, with all five single base-pair insertions in the five bp run at 79 → 83 (Table 5). Thus, more of the single base-pair deletions occurred within the run at 79 → 83 in the mutator strains (*pms1Δ*: 99%; *msh2Δ*: 93%) than the wildtype parent (79%) ($P < 0.05$ in both cases).

3.1.3.4 Single Base-Pair Substitution

All six possible types of single base-pair change were recovered in each of the three strains (Table 6). Yet, the fractions of total transitions decreased somewhat whereas the fractions of total transversions increased in the mutator backgrounds. These small differences resulted in slight reductions in the ratios of transitions to transversions in the *pms1Δ*

TABLE 6. Single base-pair substitutions

Substitution	PMS1 MSH2		pms1Δ		msh2Δ	
	Number detected	Rate (x 10^{-8})	Number detected	Rate (x 10^{-8})	Number detected	Rate (x 10^{-8})
Transitions						
G·C → A·T	74 (25.5)[a]	5.8	31 (24.6)	28.2	20 (14.7)	16.2
A·T → G·C	42 (14.5)	3.3	10 (7.9)	9.1	18 (13.2)	14.6
Total	116 (40.0)	9.1	41 (32.5)	37.3	38 (27.9)	30.8
Transversions						
G·C → T·A	99 (34.1)	7.8	58 (46.0)	52.8	80 (58.8)	65.0
G·C → C·G	59 (20.4)	4.6	19 (15.1)	17.3	9 (6.7)	7.3
A·T → C·G	5 (1.7)	0.4	6 (4.8)	5.5	5 (3.7)	4.1
A·T → T·A	11 (3.8)	0.9	2 (1.6)	1.8	4 (2.9)	3.2
Total	174 (60.0)	13.7	85 (67.5)	77.4	98 (72.1)	79.6

[a]Per cent of total substitutions.

(27%) and *msh2Δ* (41%) mutators relative to the parental wildtype (Table 6), but only the latter decrease was significant ($P < 0.01$). The ratio change between the two mutators was not significant ($P > 0.05$). Consistent with the changes in the transition:transversion ratios, the transition rate increased 4-fold in *pms1Δ* and 3-fold in *msh2Δ*, but the transversion rate increased by about 6-fold for both the *pms1Δ* and *msh2Δ* mutators, relative to the wildtype parent (Table 6).

Among the transitions, fewer G·C → A·T events were detected in the *msh2Δ* (14.7%) mutator than in the wildtype (25.5%) or *pms1Δ* (24.6%) strains ($P < 0.01$) (Table 6). Although there appeared also to be fewer A·T → G·C transitions in the *pms1Δ* strain relative to the other two, the difference was not significant ($P > 0.05$ in both cases). The rates of the transitions increased by about 3- to 5-fold in both the *pms1Δ* and *msh2Δ* mutators.

The relative fractions of G·C → T·A transversions increased progressively from 34% in the wildtype parent to 46% and 59% in the *pms1Δ* and *msh2Δ* strains, respectively ($P <$ at least 0.05 in both cases), but the difference between the two mutators was not significant ($P > 0.05$). In contrast, the relative fractions of the G·C → C·G transversions appeared to decrease from 20% for the wildtype parent to 15% and 7% for the *pms1Δ* and *msh2Δ* mutators, respectively. However, only the difference between the *msh2Δ* mutator and the wildtype parent was significant ($P < 0.001$). Relative to the wildtype parent, the rates of G·C → T·A transversions were 7- to 8-fold greater in the *pms1Δ* and *msh2Δ* strains, whereas the rates of G·C → C·G transversions were increased by only 2- to 4-fold (Table 6).

Although there were small fluctuations in the fractions of transversions recovered at A·T pairs in the three strains, the significance of these differences is questionable because relatively few events were detected.

Even with this caveat in mind, it is interesting to note that the rate of A·T → C·G transversion was 10- to 14-fold greater in the *pms1Δ* and *msh2Δ* strains, and this is the largest increase observed in the two mutator strains. By comparison, the rate of A·T → T·A transversion was only 2- to 4-fold greater in the *pms1Δ* and *msh2Δ* backgrounds (Table 6).

3.1.3.5 Distributions of Single Base-Pair Substitutions

The distributions of the single base-pair changes arising in the wildtype strain and the two mutators are presented in Figures 11 and 12. Substitutions were distributed throughout *SUP4*-o with the exception of the intron where changes occurred at only two positions (sites 43 and 51). No single base-pair changes were detected in the regions immediately flanking *SUP4*-o.

In total, 66 different sites were mutated with 41 or 47 common to both the wildtype and *pms1Δ* or *msh2Δ* strains, respectively (Figures 11 and 12). For the wildtype vs. *pms1Δ*, 23 of the remaining 25 sites were mutated only in the parent and 2 only in the mutator strain. Similarly, 17 of the 19 sites not common to both the wildtype and *msh2Δ* were mutated only in the former, and the remaining 2 only in the latter background. Neither *pms1Δ* nor *msh2* uniformly increased mutation rates at the sites common to the wildtype and mutator strains. The degree of enhancement ranged from 2- to 58-fold, with a mean increase of 6.7-fold, for *pms1Δ* vs. wildtype, and from 2- to 82-fold with a mean increase of 5.6-fold, for *msh2Δ* vs. wildtype. For each mutator, however, there was one position where the substitution rate actually was lower than in the wildtype strain (47% at site 18 for *pms1Δ*; 36% at site 51 for *msh2Δ*). Nevertheless, it is clear that the mismatch correction deficiencies had much larger effects at

Figure 11. Distribution of spontaneous base substitutions in the *SUP4*-o gene in the *pms1∆* strain. For simplicity, only the region of the transcribed strand encoding the tRNA is shown. The anticodon is at 36 to 38 and the 14 bp intron extends from 40 through 53 (Knapp *et al*. 1978). Mutations isolated in MKP-o (*PMS1 MSH2*) and YY1 (*pms1∆*) are presented above and below the transcribed strand, respectively.

PMS1 MSH2

```
            T                AAAGTT                                          AAAAG                              TTTTT TTTT
            T                AAAAAA                                          A                                  TT    T
            G                     A                                          A                                  TT    T
    T       G                     A                                          A        T                         TT    T
    T       A                     A                            C             A        T                         CG    T
    T       A                     A                T    C      A             A        T              C          CG  T TC
    C       TA                    A                T    C      T    A        A   C A  T       T       G    C     CG  T TC
    A       CAT          C        A                TT AC       T    A        A   C A  T   C   T       G    C     CA  G TC
    A       AAA      C   C        AT               CAGTAC      T  A GC       A   C A  A   C CGTGTT    G    A T   CA  G TA
   AGT      AAA      C   AA       AA               CAGCAC      G AG GC       A   C A  A   C CGGACG    G    A C   AAGG TA
   AGA  AAAGCT       A   AA       AA               CCAGAACG    AACAGTGCA     A   CTAGCAG  TC CAAACA   AC   A CT  AAGG AA
                                                                                                               TTAAGAAAA

    1          10            20            30            40            50            60            70            80            89
3' GAGAGCCATC   GGTTCAACCA   AATTCCGCGT   TCTGAAATTA   AATAGTGATG   CTTTAGAACT   CTAGCCCGCA   AGCTGAGCGG   GGGCCCTCT  5'

    T AAAG A    AC CA   TA        A A AA     GA A   G G        A        G        C AATATAG    AA      GCT    TCA AGAA
    T AA G A    A T     A         C A AA     GA C              A        G        CA ATG       AA      C      CA  GA
      CT   A    T       A         C A AA     T               A                  T AT         AG             CA   A
         T T                      G A AA                     G                  T            T              CA   A
         T                        A A T                                                      T              CG   A
         T                        A A                                                                       CT   T
         T                        A                                                                         TT
                                  A                                                                         TT
                                                                                                            T
                                                                                                            T
```

pms1Δ

Figure 12. Distribution of spontaneous base substitutions in the *SUP4*-o gene in the *msh2*Δ strain. Mutations isolated in MKP-o (*PMS1 MSH2*) and YY2 (*msh2*Δ) are presented above and below the transcribed strand, respectively. Legend as for Figure 11.

PMS1 MSH2

```
         T                    AAAGTT                                                                  AAAAG                                                                        TTTTT   TTTT
         T                    AAAAAA                                                        C          A                                                                          TT     T
         G                    A                                                             A          A         T                                                               TT     T
    T    G                    A                                                             A          A         T                                                               TT     T
    T    A                    A                                                             C          A         T                                                               CG     T
    T    A                    A                                                             A          A         T                                                     C         CG   T  TC
    T    A          C         A                              T   C                  T   A   A     C A  T         T                              G     C                CG   T  TC
    C    TA         C         A                              T   C                  T   A   A     C A  T         T   T                          G     C                CA   G  TC
    A    CAT        C         AT                             TT AC                  T A GC  A     C A  A    C T   G T T                         G     A  T             CA   G  TA
    A    AAA     C  C   AA    AA                             CAGTAC                 G AG GC A     C A  A    C CGTGTT                            G     A  C             AAGG TA
    AGT  AAA     C  C   AA    AA                             CAGCAC                 GGCAG GCC A   C A  A    C CGGACG                            AC    A  CT            AAGG AA
    AGA  AAAGCT  A  AA  AA    AA                             CCAGAACG               AACAGTGCA  A  CTAGCAG   TC CAAACA                           AAC CAGCC              TTAAGAAAA

    1          10            20              30             40          50             60           70            80            89
3'  GAGAGCCATC    GGTTCAACCA   AATTCCGCGT   TCTGAAATTA   AATAGTGATG   CTTTAGAACT   CTAGCCCGCA   AGCTGAGCGG   GGGCCCTCT  5'

    ACT    AAG A   A  AA   AA    C AAAAC      GAGCGCGC      A           G  C ATG     C A T T G    GC   C  T    TTCAGA AC
    TTT    AA      C  AA   AA    C A AA       GA C                         T G          T   T          T  T   TCA A  AC
     T     AA      C  A    A       A TA       A                            G                                  TTA T  A
           A            A  A       A   A A                                 G                                  TTA T  A
           A            A          A     A                                                                    TTA T  A
           T            A                                                                                     TTA    T
                                                                                                             T A    T
                                                                                                             T  A
                                                                                                                A
                                                                                                                A
                                                                                                             AAAAAA
                                                                                                             AATTTTTT
```

msh2Δ

specific sites than might have been expected on the basis of the magnitudeof the increase in the total mutation rate.

A statistical comparison (Adams and Skopek 1987) of the distributions for the *pms1Δ* or *msh2Δ* mutators vs. the wildtype strain indicated that the chance of random sampling error accounting for differences was 1 in 500 (with 1,500 simulated comparisons, the upper limit on the 90% confidence interval for the estimate of *P* was 0.002). However, application of the same analysis to the distributions for the *pms1Δ* and *msh2Δ* mutators revealed that for this comparison, differences were probably attributable to sampling error (with 1,500 simulated comparisons, the upper limit on the 90% confidence interval for the estimate of *P* was 0.0653). Thus, it seems reasonable to think that the differences in the relative fractions of certain of the base-pair substitutions in the *pms1Δ* vs. *msh2Δ* backgrounds (Table 6) also were artifactual.

3.1.4 Heteroduplex Repair in the *pms1Δ* and *msh2Δ* Mutators

Both the *pms1Δ* and *msh2Δ* mutators increased the rates of single base-pair transitions, transversions, deletions, and insertions. These increases suggested that a general reduction in the efficiency of mismatch correction was responsible for the mutator effects. To confirm this possibility, the abilities of the two mutator strains and their wildtype parent to repair defined mismatches in *SUP4*-o were compared.

The base mismatches selected for analysis were G/T and A/C transition mismatches at site 27, G/G transversion mispairs at positions 18 and 51, and A/A and T/T transversion mismatches at site 55. The rates of the substitutions potentially due to failure to repair the mispairs at sites 27 and 51 were 10- to 47-fold greater in the *pms1Δ* and *msh2Δ* strains

relative to the wildtype parent (Table 6, Figures 11 and 12). The transversion mismatches at sites 18 and 55 were used as internal controls because the substitutions expected to arise from these mispairs were not detected at positions 18 and 55 in the *pms1Δ* and *msh2Δ* strains (Figures 11 and 12). Two single nucleotide loop mismatches also were chosen. One (-/C) has a loop that results from deletion of a single G within the run at 79 → 89 on the transcribed strand of *SUP4*-o, whereas the other loop mismatch (+/C) is due to insertion of a single G within the same run on the same strand. The rates of single base-pair deletion or insertion potentially attributable to failure to repair the -/C or +/C mismatches were 36- to 44-fold or 41- to 82-fold greater, respectively, in the *pms1Δ* and *msh2Δ* strains (Tables 4 and 5).

A series of heteroduplex plasmids each carrying one of the selected mismatches was constructed. Circular, single-stranded DNA was prepared from YCpMP2 or derivatives carrying mutant *SUP4*-o alleles with defined base-pair changes and annealed to linearized molecules of these derivatives or YCpMP2, respectively. The resulting heteroduplexes carried a functional *SUP4*-o allele on one strand and a defective *SUP4*-o allele on the other. These heteroduplex plasmids were then transformed into the wildtype, *pms1Δ* and *msh2Δ* strains. Prior to the first round of DNA replication in the transformed cells, the mismatch can be corrected to the normal base-pair to give a functional *SUP4*-o allele. Alternatively, it might not be repaired. The *ade2-1* allele present in the yeast strains causes red colouring in the absence of a functional *SUP4*-o gene. Thus, under conditions selective for the plasmid, three types of transformant colony can emerge: (1). white (indicating correction to the normal base-pair); (2) red (indicating restoration to the incorrect base-pair); (3)

sectored red/white (indicating failure to repair the mismatch). The sectored colony appears in the absence of mismatch correction because one of the initial two daughter cells produced following the first round of DNA replication bears a plasmid with a functional *SUP4*-o allele while the other carries a plasmid with a defective copy. A sectored colony also might result from cotransformation of a cell with two heteroduplex plasmids because multiple copies of yeast centromere plasmids are mutually unstable in haploid yeast cells (Newlon 1988). By transforming strains with mixtures of YCpMP2 and mutant derivatives, it was demonstrated that less than 1% of the sectored transformants are due to cotransformation (Kunz *et al*. 1991). Furthermore, it was verified that colony colouring is associated with mismatch resolution by sequencing *SUP4*-o genes on plasmids derived from cells in red or white colonies or sectors (Kunz *et al*. 1991).

In this system, the fraction of sectored colonies gives the relative efficiency of mismatch repair whereas the ratio of red to white colonies is a measure of the direction of mismatch correction. Table 7 shows that compared to the wildtype parent, there were substantial increases in the fractions of sectored colonies in both mutators (P < at least 0.001 in all cases) for all mismatches tested. The sole exception was the T/T mismatch which was repaired poorly in the wildtype parent. These results indicate that defects in the *PMS1* or *MSH2* genes severely impair correction of mismatches in a plasmid-borne copy of *SUP4*-o. In addition, it is clear that the G/T transition mispair and the single nucleotide loop mismatches are the most efficiently corrected substrates assessed in the wildtype strain (Table 7). Furthermore, the loop due to a G insertion (+/C) is corrected twice as efficiently as the loop which results from a G deletion (-/C) (P < 0.001). Although changes in certain of the red:white ratios

TABLE 7. Analysis of mismatch correction

Mismatch[a]	PMS1 MSH2			pms1Δ			msh2Δ		
	No. of colonies[b]	R:W[c]	Sectored colonies (%)	No. of colonies	R:W	Sectored colonies (%)	No. of colonies	R:W	Sectored colonies (%)
T/T 55	1608	1.54	61	1302	1.13	72	838	1.32	72
A/A 55	3142	0.73	26	1906	1.39	69	1362	0.87	72
G/G 18	2301	1.89	29	1271	1.63	65	1014	1.22	68
G/G 51	1708	1.10	23	1693	1.43	69	933	0.99	72
A/C 27	1729	0.95	25	1411	1.20	72	1158	1.04	71
G/T 27	1545	0.46	12	1423	0.66	64	568	0.63	74
-/C 79-83	2726	0.73	12	2280	1.39	77	1373	0.76	73
+/C 79-83	3061	0.76	6	2863	1.24	67	2083	1.24	66

[a] The first base listed is on the nicked strand; the second is on the continuous strand. - indicates a single base deletion, + indicates a single base insertion. The number(s) indicates the position of the mismatch or the base-pair run in SUP4-o. The functional SUP4-o allele is on the nicked strand for A/A 55 and G/T 27. For the other mismatches the functional allele is on the continuous strand.

[b] The values are the totals for 2 or more independent transformations.

[c] Nonsectored red (R) colonies:nonsectored white (W) colonies.

were noted, no consistent pattern was discernable (Table 7).

3.2 Characterization of the *rad3-1* Mutator

3.2.1 Rationale for Selection of *rad3* Strains

Most *rad3* mutant alleles tested increase the rate of spontaneous reversion and forward mutation (Song *et al*. 1990). On the basis of this and other phenotypic effects, the *RAD3* gene product was suggested to play a role in the fidelity of DNA replication or in postreplicative mismatch correction in yeast (Montelone *et al*. 1988; Song *et al*. 1990). The characterization of spontaneous *SUP4*-o mutations arising in a *rad3-102* mutator strain was viewed as favouring the latter possibility (Montelone *et al*. 1992). If *RAD3* functions in mismatch correction, one would expect the mutational specificity of the *rad3* mutator to reflect changes in the direction or efficiency of mismatch correction in yeast. Moreover, the mutator phenotype should be associated with a defect in heteroduplex repair.

For these reasons two approaches were taken to determine whether the *RAD3* gene product functions in mismatch correction. First, the influence of the *rad3-1* mutator allele on the specificity of spontaneous mutagenesis at *SUP4*-o was examined and the resulting mutational spectrum was compared to that for the parental wildtype (*RAD3*) strain, Second, the mutational specificity data for *rad3-1* were employed to design heteroduplex plasmids carrying defined mismatches whose repair was monitored in the *rad3-1* and *RAD3* strains. Certain of these parameters also were assayed in a *rad3-25* background.

The two *rad3* alleles were chosen because they were found to have different phenotypic consequences (Song *et al*. 1990). In particular,

rad3-1 conferred high UV sensitivity, and increased the rate of spontaneous *lys2-1* reversion and forward mutation at *CAN1*. On the other hand, *rad3-25* caused only moderate UV sensitivity and had essentially no effect on spontaneous mutagenesis. Thus, the role of *RAD3* in the avoidance of spontaneous mutation is reduced by *rad3-1* but not by *rad3-25*, suggesting that if the Rad3 protein functions in mismatch correction, then this process is not affected by the *rad3-25* mutation, which can serve as a negative control. Presumably, the different phenotypic features associated with the two *rad3* alleles are due to their individual alterations (Song *et al.* 1990). Both *rad3-1* and *rad3-25* have missense mutations in the *RAD3* ORF leading to Glu → Lys substitutions. However, the affected codon (236) in *rad3-1* lies within a consensus motif for DNA helicases designated domain II in the Rad3 protein whereas the altered codon (548) in *rad3-25* is positioned downstream of the domain V helicase consensus motif. Whether either change modifies the Rad3 helicase activity remains to be determined.

3.2.2 Specificity of the *rad3-1* Mutator

The specificity of the *rad3-1* mutator was investigated by characterizing spontaneous mutations arising in the *SUP4*-o gene in the *rad3-1* background.

3.2.2.1 Plasmid Retention and *SUP4*-o Mutation Rate

To determine spontaneous mutation rates, cultures of three yeast strains (MKP-op: *RAD3*; WS3-1p: *rad3-1*; WS3-25p: *rad3-25*) carrying YCpMP2 were grown from low titre inocula to stationary phase in medium selective for the plasmid. They were then plated to measure plasmid retention (*rad3-1* and *rad3-25* only) and isolate *SUP4*-o mutations (all three strains). For each strain, ca. 90% of the cells retained YCpMP2 indicating that the

effects of the two *rad3* alleles do not include a reduction of plasmid stability (Table 8).

Relative to the wildtype parent, the *rad3-1* mutator increased the rate of spontaneous *SUP4*-o mutation 3-fold (Table 8). The degree of the increase was near to that (4.8-fold) reported for spontaneous mutation at the *CAN1* locus in an unrelated *rad3-1* strain (Song *et al*. 1990), but less by about half than expected for a defect in mismatch repair (see section 3.1.3). In agreement with previous results (Song *et al*. 1990), the *SUP4*-o mutation rate in the *rad3-25* strain was estimated to be the same as that for the *RAD3* parent (Table 8).

3.2.2.2 Mutational Classes Detected

A total of 225 *SUP4*-o mutants, each isolated from an independent culture of *rad3-1*, was characterized and the mutational spectrum was compared to that for 354 mutants selected in the *RAD3* background. The *RAD3* spectral data (excluding the rates, which were determined again) are those shown in the study of the *pms1Δ* and *msh2Δ* mutators (see section 3.1), but are presented again here for ease of comparison. Increases in the rates of single base-pair substitution (3-fold), deletion (4.6-fold), and insertion (19.3-fold) accounted for 99% of the total mutation rate enhancement attributable to the *rad3-1* mutator (Table 9). However, the magnitude of the increase was larger in the *pms1Δ* and *msh2Δ* strains by almost 2-fold for the single base-pair substitutions, and by about 6- to 8-fold and 2- to 4-fold for the single base-pair deletions and insertions, respectively (see section 3.1.3.3).

Of the 22 single base-pairs deletion recovered in *rad3-1* strain, 16 were in runs of two or more base pairs (25 → 26: 1; 65 → 67: 5; 79 → 83: 10) as

TABLE 8. Plasmid retention and *SUP4*-o mutation frequencies and rates

Strain	Colonies on selective medium	Colonies on nonselective medium	Percent plasmid retention[a]	Mutation frequency[b] (x 10^{-6})	Mutation rate (x 10^{-7})
RAD3	37,299	42,231	88.3	1.96	4.8
rad3-1	9,006	9,616	93.6	10.34	15.5
rad3-25	11,371	12,488	91.1	1.97	4.8

[a] Plasmid retention was assessed by comparing the number of colonies that formed on medium selective for the plasmid with the corresponding number for nonselective medium. Plasmid retention data for the *RAD3* strain are taken from Table 2.

[b] Frequencies are the medians for at least 30 independent cultures.

TABLE 9. Sequence alterations in *SUP4*-o mutants

DNA sequence alteration	RAD3		rad3-1	
	No. detected (% of total)	Rate (x 10^{-8})	No. detected (% of total)	Rate (x 10^{-8})
Substitution				
Single	290 (81.9)	39.3	190 (84.4)	131.0
Tandem bp	-		2 (0.9)	1.4
Non-tandem bp	2 (0.6)	0.3	1 (0.4)	0.7
Deletion				
1 bp	24 (6.8)	3.3	22 (9.9)	15.2
>1 bp	7 (2.0)	1.0	2 (0.9)	1.4
Insertion				
1 bp	1 (0.3)	0.14	4 (1.8)	2.7
Ty element	26 (7.3)	3.5	3 (1.3)	2.1
Duplication	1 (0.3)	0.14	-	
Complex change	3 (0.8)	0.4	1 (0.4)	0.7
Total	354	48.08	225	155.2

were all 4 single-base pair insertion (35 → 37: 1; 79 → 83: 3) (Table 10). However, the proportion of deletions in the run of 5 G•C pairs at 79 → 83 was significantly less (45%) for the rad3-1 strain than for the pms1Δ (99%) and msh2Δ (93%) (P < 0.001 in both cases). The remaining 6 deletions in the rad3-1 strain were within the first 20 base pairs on either end of SUP4-o (sites 5, 8, 9, 10, 69, 87) (Table 10 and Figure 13).

Two tandem double substitutions were found only in the rad3-1 strain, and a direct duplication was detected only in the RAD3 background (Tables 9 and 10). Several other mutational classes, including non-tandem double base pair substitution, multiple base pair deletion, insertion of the yeast retrotransposon Ty, and more complex changes, occurred in both strains at similar rates (Tables 9 and 10). Thus, these latter events probably arose via spontaneous processes independent of RAD3.

3.2.2.3 Single Base-Pair Substitution

In contrast to the pms1Δ and msh2Δ mutators, the relative fractions of the substitutions recovered in the rad3-1 mutator were similar to those for the wildtype strain (Tables 6 and 11). Furthermore, the G•C → T•A : G•C → C•G transversion ratio was reduced by 50% in the rad3-1 background relative to the wildtype parent (P < 0.01). However, this ratio was 3.6- and 10.6-fold greater in the isogenic pms1Δ and msh2Δ strains, respectively, than in the rad3-1 background (P < 0.001 in both cases). The rates of all 6 types of single base-pair substitution were increased by 2.5- to 8-fold in the rad3-1 strain over the corresponding RAD3 values. This was similar to the situation in pms1Δ or msh2Δ backgrounds, except that the rate increases for the G•C → T•A and A•T → C•G transversions were 2- to 3-fold greater in the latter two strains (Tables 6 and 11).

TABLE 10. Deletions, insertions and multiple concurrent mutations

		No. detected	
Sites[a]	Changes[b]	RAD3	rad3-1
-2 → -249	-248	1	-
5	-1	-	1
8	-1	-	1
9	-1	-	1
8, 10	A → G, C → T	1	-
10	-1	-	1
12, 14	T → C, G → A	1	-
13 → -122	-135	1	-
15	-1	1	1
15, 16	C → A, A → G	-	1
17 ↔ 18	Ty	2	1
18 → 19	-1	1	-
18, 21	C → T, A → C	-	1
23 → -145	-168	-	1
25 → 26	-1	-	1
35 → 37	+1	-	1
37 ↔ 38	Ty	23	2
43 ↔ 44	Ty	1	-
58 → 64	-7, +GGGCC	2	1
62 → 89	-27	1	-
65 → 67	-1	-	5
69	-1	-	1
70 → -555	-625	1	-

TABLE 10. (Continued)

Sites[a]	Changes[b]	No. detected	
		RAD3	rad3-1
71, 72	A → C, G → A	-	1
72	-1	1	-
72 → 78	Direct duplication	1	-
79 → 83	-1	19	10
79 → 83	+1	1	3
79 → 83, 84	+1, C → A	-	1
79 → 83, 88 → 119	-1, -32	1	-
81 → -726	-807	1	-
84 → 86	-1	1	-
87	-1	-	1
87, 88	T → C, C → T	-	1
89 → 96	-8	1	-
89 → 97	-9	1	1

[a] Sites for deletions of 9, 27, 248, or 807 bp in MKP-op (RAD3) and 9 or 168 bp in WS3-1p (rad3-1) are estimates because sequence repeats are at the deletion termini. The symbol → indicates that Ty is presumed to have inserted between the two sites. For deletions or insertions in runs of base pairs, the position of the run is given since the precise base pair eliminated or added cannot be determined.

[b] Changes are given for the transcribed strand (see Figure 13).

Figure 13. Distribution of spontaneous base substitutions in the *SUP4*-o gene in the *rad3-1* strain. Mutations isolated in MKP-o (*RAD3*) and WS3-1 (*rad3-1*) are presented above and below the transcribed strand, respectively. Legend as for Figure 11.

RAD3

```
            T                      AAAGTT                                                        AAAAG                                                  TTTTT  TTTT
            T                      AAAAAA                                                        A                                                      TT     T
            G                      A                                                             A                                                      TT     T
   T        G       A              A                                                             A      T                                               TT     T
   T        A       A              A                                                             A      T                                               CG     T
   T        A       A              A                        C                                    A      T                                     C        CG   T TC
   C        TA      C              A            T   C       A                                    A   C A T           T                        C        CG   T TC
   A        CAT     C     AT       T   C        T   A       A                                    A   C A  T       C  T                      G C         CA   G TC
   A        AAA   C C     AA       TT AC        T A GC      A                                    A   C A   A      C G T T                    G A T       CA   G TA
   AGT      AAA   C AA    AA       CAGTAC       G AG GC     A                                    A   C A   A      C CGTGTT                   G A C       AAGG TA
   AGA    AAAGCT  A AA    AA       CAGCAC       GGCAG GCC   A                                    A   C A   A      C CGGACG                   AC A CT      AAGG AA
                                   CCAGAACG     AACAGTGCA   A                                    A   CTAGCAG     TC CAAACA                   AAC CAGCC    TTAAGAAAA

   1        10               20              30          40          50               60              70              80              89
3' GAGAGCCATC      GGTTCAACCA      AATTCCGCGT      TCTGAAATTA      AATAGTGATG      CTTTAGAACT      CTAGCCCGCA      AGCTGAGCGG      GGGCCCTCT 5'

   AG     AGAG A   AT  A  AG       CAAGAACG      GGA C C                  A      CAGCT       A  AGGATGG      CAAC   A CC          CG A TA
   AT     AGTG A                   CCTGAAC       G A   G                  A      TAGT        A  AGGAT        CGG    C CT          CG A TC
          AT G A                   C TTA C       T C                      A      TTGT        C  AGTG         TG     T             CG G TC
          AT                         T C C       T T                      A      TT             C           TG                   C  T TC
          C                          T T                                  A                     C           G                    C    TC
          C                                                               A                     C           G                    T    TC
          C                                                               A                     C           G                    T    TC
          T                                                               G                     T           G                         T
          T                                                               G                     T           G                         T
          T                                                               G
          T                                                               G
          T                                                               TT
                  GTT
```

rad3-1

TABLE 11. Single base-pair substitutions

Substitution	RAD3		rad3-1	
	No. Detected (% of total)	Rate (x 10^{-8})	No. Detected (% of total)	Rate (x 10^{-8})
Transitions				
G·C → A·T	74 (25.5)	10.0	44 (23.2)	30.3
A·T → G·C	42 (14.5)	5.7	21 (11.0)	14.5
Total	116 (40.0)	15.7	65 (34.2)	44.8
Transversions				
G·C → T·A	99 (34.1)	13.4	49 (25.8)	33.8
G·C → C·G	59 (20.4)	8.0	58 (30.5)	40.0
A·T → C·G	5 (1.7)	0.7	8 (4.2)	5.5
A·T → T·A	11 (3.8)	1.5	10 (5.3)	6.9
Total	174 (60.0)	23.6	125 (65.8)	86.2

3.2.3 Heteroduplex Repair in *rad3* Strains

Alone, analysis of the specificity of spontaneous mutagenesis in the *rad3-1* strain did not provide convincing evidence for or against involvement of the Rad3 protein in mismatch correction. To explore this possibility further, we measured the efficiency of mismatch correction directly in the *RAD3*, *rad3-1* and *rad3-25* strains. This was accomplished by first constructing heteroduplex plasmids carrying specific mispairs at defined sites in *SUP4*-o. In order to choose appropriate combinations of sites and mismatches, it was necessary to know the rates of the base-pair changes that could arise from particular mismatches at different positions within the gene. Large substitution rates at individual *SUP4*-o sites in the *rad3-1* background might possibly signal reduced mismatch repair at those locations.

Site-specific substitution rates were determined by comparing the distributions of the single base-pair changes at *SUP4*-o in the *rad3-1* and *RAD3* strains (Figure 13). In total, 66 different sites were mutated with 49 common to both strains. Of the remaining 17 sites, 14 were mutated only in the wildtype and 3 only in the *rad3-1* mutator. Mutations were found at just one position (51) with the 14 bp intron. In the *rad3-1* strain, the mutation rate was at least 2-fold higher at 44 of the common sites, with a mean increase of 5.8-fold, although at certain positions the rates of specific substitutions were as much as 20-fold higher. A statistical comparison (Adams and Skopek 1987) of the distributions indicated that the chance of random sampling error accounting for differences was 1 in 100 (P = 0.01). On the basis of the site-specificity data, G/T and A/C mismatches at site 27, G/G mispairs at positions 18 and 51, and A/A and T/T mismatches at site 55 were selected. The rates of the substitutions

potentially attributable to failure to repair these mismatches were 16- to 20-fold higher in *rad3-1* than *RAD3*. For internal controls, G/T and A/C mismatches at sites 32 and 35 were used because the transition rates at these sites either were just slightly higher (32: 3-fold) or did not appear to increase (35: < 2-fold) in the *rad3-1* strain.

Heteroduplex plasmids each carrying one of the selected transition or transversion mismatches were constructed and transformed into the *RAD3* and *rad3* strains. Conceivably, the *rad3-1* mutator phenotype might be due to restoration of base mismatches to the incorrect base-pairs or to a decreased efficiency of mismatch correction. If so, an appropriate shift in the red:white colony ratio or an increase in the proportion of sectored colonies, would be expected. When heteroduplex repair was compared in the *RAD3* and *rad3* strains, some changes in the red:white ratios were noted but not for all the mismatches and, where detected, the alterations were insufficient to account for the magnitude of the site-specific mutator effect (Table 12). Furthermore, there was no increase in the fractions of sectored colonies in the *rad3* background. Thus, neither *rad3-1* nor *rad3-25* modified the directionality, or reduced the efficiency of mismatch correction. Surprisingly, comparison of the relative fractions of sectored colonies revealed that mismatch repair was 2.5- to 5-fold more efficient in *rad3-1* than the wildtype parent for all mismatches tested except the T/T mispair (1.6-fold) ($P < 0.001$ in all cases) (Table 12). Although the difference was much smaller and of questionable significance, it is still interesting to note that mismatch repair also was consistently more efficient in the *rad3-25* strain.

TABLE 12. Analysis of mismatch correction in wildtype and *rad3* strains

Mismatch[a]	Allele on nicked strand[b]	RAD3			rad3-1			rad3-25		
		No. of colonies[c]	Ratio, red:white[d]	Sectored colonies (%)	No. of colonies	Ratio, red:white	Sectored colonies (%)	No. of colonies	Ratio, red:white	Sectored colonies (%)
18 G/G	-	2301	1.89	29	1676	2.11	6	3293	1.98	23
27 G/T	+	1545	0.46	12	1827	0.47	3	2603	0.41	9
27 A/C	-	1729	0.95	25	3228	1.00	8	3227	1.09	21
32 C/A	+	3110	0.68	41	1192	0.72	16	1593	0.69	35
32 T/G	-	1485	0.56	15	2190	0.79	3	1349	0.69	10
35 A/C	+	2086	1.09	33	979	1.20	10	1775	1.21	21
35 G/T	-	3894	1.84	15	1173	2.13	5	1561	1.96	11
51 G/G	-	1708	1.10	23	2089	1.31	6	2394	0.90	21
55 A/A	+	3142	0.73	26	2961	0.73	9	3976	0.71	22
55 T/T	-	1608	1.54	61	2806	1.85	39	2826	1.88	57

[a] The number indicates the position of the mismatch in *SUP4*-o (see Fig. 13). The first base listed is on the nicked strand; the second is on the continuous strand.

[b] "+" represents a functional *SUP4*-o allele; "-" represents an inactive *sup4*-o allele.

[c] The values are the totals for 2 or more independent transformations.

[d] Nonsectored red colonies:nonsectored white colonies.

3.3 Influence of Strand Nicks on Mismatch Correction

In *E. coli* cells and *D. melanogaster* and human cell extracts, strand breaks or nicks determine the strand specificity of mismatch correction (Welsh *et al.* 1987; Hare and Taylor 1985, 1988; Lahue *et al.* 1989; Holmes *et al.* 1990; Thomas *et al.* 1991). However, none of the eukaryotic systems used allowed the direction of intracellular mismatch repair to be assessed in the cells where repair occurred. Instead, the heteroduplex substrates were isolated and the direction of repair established *in vitro* by biochemical analysis. Thus, it was not certain that mismatch correction actually is directed by strand nicks within intact eukaryotic cells.

In order to assess the role of nicks in directing mismatch correction in yeast cells, heteroduplexes with transition mismatches (C/A, T/G, A/C and G/T), located at site 32 or 35 within the *SUP4*-o gene were constructed. The substrates for construction were obtained using plasmids YCpLK1E, YCpLK3EB, and their derivatives having a G·C → A·T or A·T → G·C transition at *SUP4*-o position 32 or 35, respectively. These plasmids contain single *Bam*HI and *Eco*RI recognition sites at the 3′ or 5′ ends of the 249 bp yeast chromosomal DNA fragment carrying the *SUP4*-o gene (Figure 3). Recall that the heteroduplexes are assembled by annealing linearized double-stranded plasmid DNA to circular single-stranded plasmid DNA. Furthermore, it is the nontranscribed strand of *SUP4*-o that is amplified as part of the circular single-stranded YCpLK1E DNA, but the transcribed strand of *SUP4*-o that is amplified as part of the circular single-stranded YCpLK3EB DNA. Thus, by linearizing YCpLK1E, YCpLK3EB or a derivative carrying a mutated *SUP4*-o allele with *Bam*HI or *Eco*RI, and using the mutant derivatives, YCpLK1E or YCpLK3EB (as required) to produce the circular single-stranded molecules, strand nicks can be placed 5′ or 3′ to the mismatch on either

strand encompassing the *SUP4*-o gene. For simplicity, the two strands will be designated the transcribed or nontranscribed strands, and the polarity referred to will pertain to the individual strands. On this basis, Figure 14 shows that a nick can be selectively placed at position **a** or **b**, 112 bp 5' or 119 bp 3', respectively, to a mismatched base at site 32 on the nontranscribed strand of the *SUP4*-o gene. Alternatively, the nick can be positioned at **c** or **d**, 112 bp 3' or 119 bp 5', respectively, to the mismatched base on the transcribed strand of *SUP4*-o. Heteroduplex controls can be constructed by linearization with *Xho*I which incises approximately 4 kb away in either direction from *SUP4*-o, and does not influence the direction of mismatch correction (Kunz *et al*. 1991). This lack of effect is consistent with evidence suggesting that mismatch repair tracts in yeast are shorter than 1 kb (Bishop and Kolodner 1986, Borts and Haber 1987; Judd and Petes 1988).

As shown in Tables 13 and 14, the single strand breaks had no effect on the efficiency of mismatch correction at either site, as determined by the relative fractions of sectored colonies. However, small changes in the ratios of red:white colonies (except for the A/C mismatch at site 32 where the nick was 3' to the mismatch on the nontranscribed strand, Table 14) suggested that there was a slight strand preference consistent with the base on the nicked strand being removed. Furthermore, it appeared that a nick on the 5' side of the mismatch might have had more effect than a 3' nick as indicated by the slightly greater changes in the ratios of red:white colonies for the 5' nicks. However, this bias was much smaller than might be expected on the basis of results using eukaryotic cell extracts (Holmes *et al*. 1990; Thomas *et al*. 1991).

One potential explanation for the minor (at best) effect of the strand

Figure 14. Diagram of gap positions relative to the mismatched base at site 32 in *SUP4*-o. The solid line represents the transcribed strand (T) of *SUP4*-o, while the dotted line represents the nontranscribed strand (NT). **a**, **b**, **c** and **d** represent the gap positions relative to the mismatched base-pair (see text for additional details).
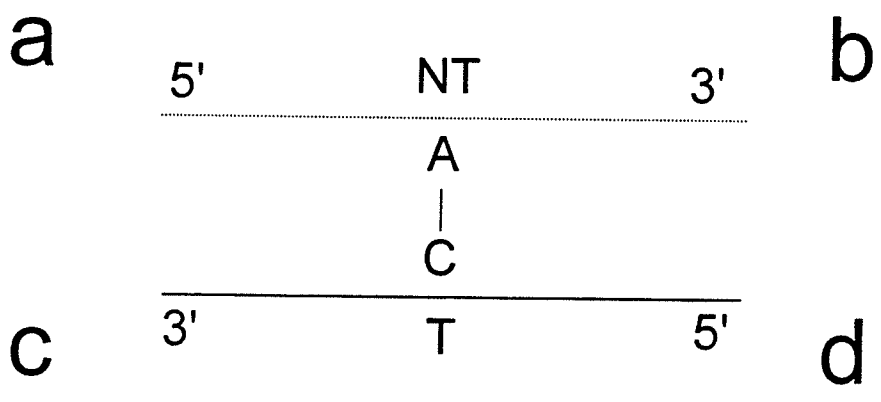
a

5'   NT   3'

b

A
|
C

c

3'   T   5'

d

TABLE 13. Influence of nicks on the transcribed strand on mismatch correction

Mismatch[a]	Site	Location of nick[b]	Allele on nicked strand[c]	No. of colonies[d]	Ratio, red/white[e]	Percent sectored colonies
C/A	32	X	+	2839	0.68	35
		5′	+	1067	1.27	28
		5′+ AP	+	1577	1.35	30
		3′	+	1560	0.88	29
		3′+ AP	+	992	0.95	31
T/G	32	X	-	1413	0.56	11
		5′	-	1748	0.40	10
		3′	-	1723	0.46	10
A/C	35	X	+	1909	1.09	25
		5′	+	2982	1.42	23
		3′	+	2165	1.19	17
G/T	35	X	-	3774	1.84	12
		5′	-	3890	1.35	11
		3′	-	2325	1.63	16

[a] The first base is on the nicked strand, the second on the continuous strand.

[b] X: nicked at the *XhoI* site approximately 4 kb in either direction from the site of the mismatch; 5′: nicked 119 bp (site 32) or 116 bp (site 35) 5′ to the mismatched base; 5′+AP: as for 5′ but treated with alkaline phosphatase prior to transformation into MKP-o; 3′: nicked 112 bp (site 32) or 115 bp (site 35) 3′ to the mismatched base; 3′+AP: as for 3′ but treated with alkaline phosphatase prior to transformation into MKP-o.

[c] "+" represents a functional *SUP4*-o allele; "-" represents an inactive *sup4*-o allele.

[d] The values are the totals for at least two independent transformations.

[e] Nonsectored red colonies/nonsectored white colonies.

TABLE 14. Influence of nicks on the nontranscribed strand on mismatch correction

Mismatch[a]	Site	Location of nick[b]	Allele on nicked strand[c]	No. of colonies[d]	Ratio, red/white[e]	Percent sectored colonies
A/C	32	X	-	3233	0.62	25
		5′	-	3199	0.57	23
		5′+ AP	-	2539	0.55	24
		3′	-	3417	0.76	25
		3′+ AP	-	2276	0.80	23
G/T	32	X	+	2901	0.17	5
		5′	+	1090	0.37	4
		3′	+	1519	0.32	5
C/A	35	X	-	2802	1.45	21
		5′	-	1657	1.10	22
		3′	-	2243	1.39	23
T/G	35	X	+	3461	1.50	9
		5′	+	3248	1.76	9
		3′	+	1352	1.66	9

[a] The first base is on the nicked strand, the second on the continuous strand.

[b] X: nicked at the XhoI site approximately 4 kb in either direction from the site of the mismatch; 5′: nicked 112 bp (site 32) or 115 bp (site 35) 5′ to the mismatched base; 5′+ AP: as for 5′ but treated with alkaline phosphatase prior to transformation into MKP-o; 3′: nicked 119 bp (site 32) or 116 bp (site 35) 3′ to the mismatched base; 3′+ AP: as for 3′ but treated with alkaline phosphatase prior to transformation into MKP-o.

[c] "+" represents a functional SUP4-o allele; "-" represents an inactive sup4-o allele.

[d] The values are the totals for 3 or 4 independent transformations.

[e] Nonsectored red colonies/nonsectored white colonies.

nicks is that when the heteroduplex enters the cell, the nick is very quickly ligated. To test this possibility, nicked heteroduplexes were treated with alkaline phosphatase prior to transformation into MKP-o in an effort to inhibit the ligation reaction. The results with the alkaline phosphatase-treated nicked heteroduplexes were not consistently different from those observed for the nicked heteroduplexes that were not treated with alkaline phosphatase (Tables 13 and 14). This was true even if the linear double-stranded plasmid DNA was treated with alkaline phosphatase prior to heteroduplex construction in order to make sure that the DNA strand ends were accessible to alkaline phosphatase (Table 15). These results suggested that: (1) alkaline phosphatase treatment does not prevent ligation from quickly restoring strand integrity; (2) the nicks on the naked heteroduplex molecules that enter the cells might not be recognized as well by the mismatch correction enzymes as nicks occurring in chromatin; or (3) strand breaks do not have a role in mismatch correction in yeast.

In an attempt to avoid rapid processing of the nick when the heteroduplex enters the cell, four heteroduplex plasmids having a C/A mismatch at site 32 of the SUP4-o gene and strand gaps instead of nicks were constructed (Figure 5, 6). The strategy used was to insert a 24 bp BamHI fragment or a 42 bp EcoRI fragment, each isolated from pHR307a, into the BamHI or EcoRI sites, respectively, of plasmids YCpLK1E, YCpLK3EB or their derivatives having a G·C → A·T transition at position 32 in SUP4-o. However, due to technical difficulties, two BamHI fragments or two EcoRI fragments were positioned 112 bp 5′ or 119 bp 3′, respectively, to the mismatched base on the nontranscribed strand of SUP4-o on YCpLK1EB. This results in heteroduplex plasmids having either a 48 or 84 bp gap on the

TABLE 15. Influence of nicks on the transcribed strand on mismatch correction

Mismatch[a]	Site	Location of nick[b]	Allele on nicked strand[c]	No. of colonies[d]	Ratio, red/white[e]	Percent sectored colonies
T/G	32	X	-	1413	0.56	11
		X + AP	-	1593	0.65	14
		5′	-	1748	0.38	10
		5′+ AP	-	1742	0.39	11
		3′	-	1723	0.45	10
		3′+ AP	-	2790	0.56	11

[a] The first base is on the nicked strand, the second on the continuous strand.

[b] X: nicked at the XhoI site approximately 4 kb in either direction from the site of the mismatch; X + AP: as for X but linearized double-stranded DNA was treated with alkaline phosphatase prior to being annealed to circular single-stranded DNA in order to construct heteroduplex DNA; 5′: nicked 119 bp 5′ to the mismatched base; 5′+ AP: as for 5′ but treated with alkaline phosphatase; 3′: nicked 112 bp 3′ to the mismatched base; 3′+ AP: as for 3′ but treated with alkaline phosphatase.

[c] "-" represents an inactive *sup4-o* allele.

[d] The values are the totals for 2 or 3 independent transformations.

[e] Nonsectored red colonies/nonsectored white colonies.

nontranscribed strand at position **a** or **b** in Figure 14. For ease of discussion, the positions of these gaps are henceforth designated 5'NT or 3'NT, respectively. A single *Bam*HI or *Eco*RI fragment was inserted 112 bp 3' or 119 bp 5', respectively, to the mismatched base on the transcribed strand of *SUP4*-o in YCpLK3EB. This allowed construction of heteroduplexes having a 24 bp or 42 bp gap on the transcribed strand at position **c** or **d** in Figure 14. In this case, the positions of the gaps are designated 3'T or 5'T, respectively. The control heteroduplex plasmids had a nick on the transcribed or nontranscribed strand at the *Xho*I site approximately 4 kb in either direction from the site of the mismatch. The locations of these nicks are designated XT or XNT, respectively.

Transformation of the heteroduplex plasmids into MKP-o revealed that the strand containing the gap was preferentially repaired (Table 16). This was indicated by the 11- and 4-fold increases in the ratios of red:white colonies resulting from transformation with the 5'T and 3'T gapped heteroduplexes, respectively ($P < 0.001$ in both cases). Increases are expected if the gaps direct mismatch repair to the gap-containing strand because the gaps in these plasmids are on the strand which carries a functional *SUP4*-o allele. Thus, the incorrect base-pair should be selectively restored. Conversely, a decrease in the red:white ratio is expected if the plasmids have gaps on the strand which carries a mutated *sup4*-o allele. In this case, the wildtype base-pair should be reinstated most often. Indeed, the ratios of red:white colonies decreased by 48% and 61%, respectively, for transformation with the 5'NT and 3'NT gapped heteroduplexes ($P < 0.001$ in both cases) (Table 16).

Interestingly, the presence of a gap also appeared to increase the efficiency of mismatch correction. For all four gapped heteroduplex

TABLE 16. Influence of gaps on correction of a C/A mismatch at site 32 in *SUP4*-o

Location of gap or nick[a]	Allele on gapped strain[b]	No. of colonies[c]	Sectored colonies (%)	Ratio, red:white[d]
X T	+	2216	41	0.69
5'T	+	2743	11	7.75
3'T	+	2925	26	2.90
X NT	-	3805	36	0.62
5'NT	-	1516	31	0.32
3'NT	-	1283	28	0.24

[a] X T: nicked at the XhoI site approximately 4 kb in either direction from the site of the mismatch; 5'T or 3'T: a gap 5' or 3' to the mismatched base on the transcribed strand of *SUP4-o*. 5'NT or 3'NT: a gap 5' or 3' to mismatched base on the nontranscribed strand of *SUP4-o*.

[b] "+" represents a functional *SUP4*-o allele; "-" represents an inactive *sup4*-o allele.

[c] Totals for 2 to 4 independent transformations.

[d] Nonsectored red colonies:nonsectored white colonies.

plasmids (5'T, 3'T, 5'NT, 3'NT), the relative fractions of sectored colonies were decreased by 14% to 73% relative to the corresponding control plasmids ($P < 0.001$ in all cases) (Table 16).

Finally, a gap on the transcribed strand seemed to have more of an effect on mismatch correction than a gap on the nontranscribed strand. The magnitudes of the increases in the ratios of red:white colonies or the efficiency of mismatch correction were greater (5.9- and 1.6-fold or 3.2- and 1.2-fold, respectively) for the 5'T and 3'T heteroduplexes than the increases in the ratios of white:red colonies and the efficiency of mismatch correction for the 5'NT and 3'NT heteroduplexes (Table 16). Furthermore, the presence of the transcribed strand gap 5' to the mismatch appeared to direct mismatch correction better than a gap 3' to the mismatch. The ratio of red:white colonies was almost three-fold greater ($P < 0.001$) and the fraction of sectored colonies more than 50% lower ($P < 0.001$) for the 5' gapped heteroduplex (Table 16). Essentially the opposite results were observed when the gaps were on the nontranscribed strand. Thus, it is important to bear in mind that the sites of the 5'T and 3'NT gaps are opposite each other on the double-stranded plasmid molecules.

4 DISCUSSION

The potential roles of the yeast *PMS1*, *MSH2* and *RAD3* genes in the correction of mismatches formed during intracellular DNA replication were examined. Two approaches were taken. First, the mutational specificities of the *pms1Δ*, *msh2Δ* and *rad3-1* mutators were characterized by sequencing mutations arising spontaneously in a plasmid-borne copy of the yeast *SUP4*-o gene. Second, repair of heteroduplex plasmids containing defined mismatches was assessed in the *pms1Δ*, *msh2Δ* and *rad3-1* backgrounds. In addition to these studies, the influence of strand breaks on mismatch correction also was investigated. Here, the possible links between these genes, as well as strand breaks, and the specificity, efficiency or direction of mismatch correction in yeast are considered.

4.1 Specificities of the *pms1Δ* and *msh2Δ* Mutators

Postreplicative mismatch repair mainly corrects DNA replication errors, which arise from nucleotide misinsertion errors and strand slippage on templates containing simple repeats (Leong *et al.* 1986; Strand *et al.* 1993). Thus, it is not surprising that disruption of the yeast mismatch repair genes *PMS1* and *MSH2* enhances spontaneous mutagenesis. My data suggest that a general reduction in the efficiency of mismatch correction is responsible for the mutator effects. DNA sequence analysis indicated that the rates of all six types of single base-pair substitution, plus single base-pair deletions and insertions, were increased in the *pms1Δ* and *msh2Δ* strains relative to the isogenic wildtype parent (Table 4). These are the classes of mutation expected to occur most often as the result of DNA replication errors (Morrison *et al.* 1993; Ramachandran *et al.* 1993).

A detailed analysis of mutational specificity revealed that the effects

of the mutators on the various mutational classes were not uniform. The rate of single base-pair deletion plus insertion was increased by 37- or 31-fold in the *pms1Δ* or *msh2Δ* strain, respectively (Table 4). However, the total rate of single base-pair substitution was increased only by 5-fold in each mutator background. These results suggest that when mismatches form naturally during DNA replication in yeast, the mutational intermediates that give rise to single base-pair deletions or insertions are produced more frequently or repaired much more efficiently than base mismatches (which lead to base-pair substitutions). This observation contrasts markedly with the situation for *E. coli*, in which it is the rate of single base-pair substitution that shows the greatest increase in mismatch correction deficient strains (Leong *et al.* 1986; Schaaper and Dunn 1987). Thus, my results indicate that the specificity of mismatch repair may differ considerably in eukaryotes compared to prokaryotes (see below for additional discussion of this point). On the other hand, since *SUP4*-o is carried on a plasmid, I caution that my findings may not be generally applicable to the effects of mismatch correction deficiency on spontaneous mutagenesis at chromosomal loci. Although plasmids such as YCpMP2 mimic chromosome behaviour and have chromatin structure, they usually are much smaller in size than bona fide yeast chromosomes, are circular rather than linear, and may be unable to achieve higher order chromatin structure. Such limitations may result in different responses for plasmid-borne genes and the same genes at their chromosomal loci if chromatin structure significantly influences the mutator effects due to a failure to repair mismatches.

Interestingly, in both mutators and the wildtype parent, most of the single base-pair deletions and insertions occurred in base-pair runs,

primarily the tract of five G·C pairs at 79 → 83 (Table 5). The locations of these events, plus the fact that in each strain the number of occurrences increased with the number of base-pairs in the run, argues that the formation of these mutations involved strand slippage and loop formation during DNA replication through the runs (Streisinger *et al.* 1966), as diagrammed in Figure 15. Additionally, the data implicate the length of the run as one potential factor affecting the frequency of loop formation and/or the efficiency of loop correction in eukaryotic cells.

Relative to the wildtype strain, the rate of single base-pair insertion increased to a greater extent (41- to 82-fold) in the *pms1Δ* and *msh2Δ* strains than the rate of single base-pair deletion (30- to 35-fold). The larger enhancement in the insertion rate suggests that loops which result in base-pair insertions either are produced more often or are corrected more efficiently than those which result in base-pair deletion in yeast. My investigation of heteroduplex repair supports the latter interpretation. A nucleotide loop involving a single base-pair insertion (+/C) within the run at 79 → 89 was corrected twice as efficiently as a loop due to a base-pair deletion (-/C) within the same run (Table 7).

The finding that the insertion loop is corrected more efficiently than the deletion loop may have important implications for expansion of simple nucleotide repeat sequences in eukaryote cells. Expansions of simple repetitive DNA have been associated with several human neurodegenerative diseases including the fragile X syndrome and Huntington's disease, as well as cancers including HNPCC (Loeb 1994). It has been inferred that expansion of repetitive stretches in both neurodegenerative diseases and cancers is the result of slippage in the repetitive sequences during DNA replication (Loeb 1994). *In vitro*, it has been clearly established that

Figure 15. Diagram of DNA polymerase slippage on the *SUP4*-o gene. For simplicity, only the region from site 76 to 86 of *SUP4*-o is shown. During DNA replication, one strand transiently dissociates from the other, and then reanneals in a misaligned configuration. In this way, strand slippage can form an intermediate containing an unpaired base stablized by flanking base-pairs. If the unpaired base is located in the newly synthesized strand, subsequent replication will result in a single base-pair insertion at the tract of five G·C pair at 79 → 83 position (A). If the unpaired base is located in the template strand, replication would lead to a single base-pair deletion at this site (B). This diagram is modified from Kunkel (1993) and Strand *et al*. (1993).

```
                   79      83
      3'   A G C G G G G G C C C   5'

                        ↓

           T C G C C C C C
      3'   A G C G G G G G C C C   5'

           ↓          Misalignment          ↓

              C
      T C G C C C C C                T C G C C C C
3'    A G C G G G G G C C C   5'     3'   A G C G G G G C C C   5'
                                              G

      Insertion ↓                    Deletion ↓

      T C G C C C C C C G G G        T C G C C C C G G G
3'    A G C G G G G G G C C C   5'   3'   A G C G G G G C C C   5'

              A                              B
```

slippage by DNA polymerases occurs during the copying of repetitive nucleotide sequences (Schlotterer and Tautz 1992). Thus, it is reasonable to think that the mismatch repair system plays a very important role in limiting DNA expansion in eukaryotes. Consistent with this idea, Strand *et al.* (1993) determined that inactivation of the yeast DNA mismatch repair genes *PMS1*, *MSH2* and *MLH1* leads to 100- to 700-fold increases in tract instability of simple repeats on plasmids, while mutations that eliminate the proofreading function of DNA polymerases have little effect. Hence, they suggested that DNA polymerases have a very high rate of slippage *in vivo* on templates containing single repeats, and that most of these errors are corrected by cellular mismatch repair systems. Taken collectively, my results further suggest that mismatch correction acts more efficiently on mutational intermediates that can result in repeat expansion than any other sort of mismatch. If so, this would provide an explanation for the specific association of mismatch correction defects in HNPCC cells with repeat expansion in the same cells (Parsons *et al.* 1993).

Generally speaking, transition mismatches are corrected more efficiently in *E. coli* than transversion mismatches (Radman and Wagner 1986; Lahue and Modrich 1988; Meselson 1988). Consequently, *E. coli* mutants defective in mismatch correction show much larger increases in the frequency of transition than transversion (Leong *et al.* 1986; Schaaper and Dunn 1987). Studies of heteroduplex plasmid repair in yeast suggested that among the base mispairs, at least the G/T, A/C, and G/G mismatches are efficiently corrected relative to other types (Bishop *et al.* 1989; Kramer *et al.* 1989a). Thus, one might expect the two base-pair transitions and the G·C → C·G transversion to exhibit the largest rate increases in response to a reduction in the overall efficiency of mismatch correction in this

organism. However, in contrast to the situation in *E. coli*, my sequence data indicated that naturally-occurring transition mismatches are not corrected more efficiently than transversion mismatches in yeast (Table 6). Furthermore, the mutation rate increases for the $G \cdot C \to T \cdot A$ and $A \cdot T \to C \cdot G$ transversions were much higher than those for the $G \cdot C \to C \cdot G$ and $A \cdot T \to T \cdot A$ transversions. These results provide additional evidence that the specificity of repairing naturally-occurring mismatches differs in eukaryotes and prokaryotes.

The analysis of the base-pair substitution rate changes in the *pms1Δ* and *msh2Δ* strains suggested that mismatches which give rise to $G \cdot C \to T \cdot A$ or $A \cdot T \to C \cdot G$ transversions (G/A and C/T or A/G and T/C, respectively, where the correct base in the mispair is given first) are repaired more efficiently in yeast cells than mismatches that produce $G \cdot C \to C \cdot G$ and $A \cdot T \to T \cdot A$ transversions (G/G and C/C or A/A and T/T, respectively). Thus, G/G, C/C, A/A, and T/T mismatches might be among the most poorly-repaired mismatches in yeast. The heteroduplex data (Table 7) showed that the T/T mismatch was repaired much less efficiently in the wildtype parent than all the other mispairs tested. However, the G/G and A/A mismatches were corrected twice as efficiently as the T/T mismatch. These results are consistent with previous reports by Bishop *et al.* (1989) and Kramer *et al* (1989a) that T/T and C/C mismatches are poorly repaired in yeast. Moreover, they suggest that T/T and C/C mismatches are infrequent errors during DNA replication in yeast, otherwise large increases in the rates of $G \cdot C \to C \cdot G$ and $A \cdot T \to T \cdot A$ transversions would have been observed.

The foregoing line of reasoning would hold only if there also are differences in the efficiency with which <u>naturally-occurring</u> mismatches are repaired. If, instead, the efficiency of correcting different

endogenous mispairs was the same, then the magnitude of the mutation rate increases should have been constant at different sites within *SUP4*-o. However, neither the *pms1Δ* nor *msh2Δ* mutators uniformly increased mutation rates at all sites common to both the mutator and wildtype strains (Figures 11 and 12). Indeed, there were considerable differences in the rate increases for the same substitution at different locations. For example, the G·C → A·T transition rate increased by 46-fold at site 27 but by only 4-fold at site 56 in the *pms1Δ* background. This constitutes strong evidence that the efficiency of mismatch correction can vary from site to site within a gene. Thus, neighbouring DNA sequences might be one factor that can modulate site-specific correction efficiencies. Together, the mutational specificity and heteroduplex repair data suggest that the efficiency of mismatch correction in yeast is not only dependent on the nature of the mispairs but also on DNA sequence context.

The fact that yeast Pms1 and Msh2 proteins are homologous to the *E. coli* MutL and MutS proteins (Kramer *et al.* 1989b; Reenan and Kolodner 1992a), respectively, suggests that DNA mismatch repair developed before the separation of prokaryotes and eukaryotes during evolution. However, in order to deal with more complicated genomic structures in eukaryotes, the mismatch repair system(s) may have evolved to recognize and correct the more complex replication errors associated with the repetitive sequences found in eukaryotic DNA. This might explain why the specificity of mismatch repair differs in eukaryotes and prokaryotes with respect to the repair efficiency of single nucleotide loops. The fact that there are two *mutL* homologs (*PMS1* and *MLH1*) in yeast supports the possibility that the yeast mismatch repair system might be more complex than that of prokaryotes, and suggests that Pms1 and Mlh1 might have different roles in

mismatch correction.

The results of this study also indicate that the mutator phenotypes conferred by deletion of *PMS1* and *MSH2Δ* mutators are largely similar. This suggests that *PMS1* and *MSH2* may be involved in the same mismatch repair pathway in yeast. Prolla *et al.* (1994b) showed that the Pms1 and Mlh1 proteins physically associate *in vitro*, possibly forming a heterodimer, and act in concert to bind a Msh2-heteroduplex complex containing a mismatch. Therefore, they suggested that the Msh2, Mlh1 and Pms1 proteins may form a complex during the initiation of the mismatch repair in yeast. Whether all three proteins are required to be complexed throughout the mismatch correction process is not known. However, expression of the *PMS1* and *MSH2* genes increases as cells enter S phase, while that of *MLH1* does not (Morrison *et al.* 1993; B.A. Kunz, personal communication). This suggests that the Mlh1 protein may not be present *in vivo* at the same concentrations as the Pms1 and Msh2 proteins. If not, it would seem unlikely that mismatch correction requires the continued presence of a 1:1:1 complex of all three proteins.

Finally, our assessment of heteroduplex repair showed that none of the mismatches in *SUP4*-o gave rise only to sectored colonies following transformation of the heteroduplexes into the *pms1Δ* or *msh2Δ* strains (Table 7). This is consistent with the results of Bishop *et al.* (1989) and Kramer *et al.* (1989a) for different *pms1Δ* strains, and indicates that about 25% to 30% of the mismatches were repaired in cells supposedly deficient in mismatch correction. Thus, additional mismatch repair systems may exist in yeast, reminiscent of the situation in *E. coli* which possesses more than one mechanism for repairing mismatches (Grilley *et al.* 1990). The recent identification of a protein (Myh) in calf thymus and

human HeLa cells that is homologous to the *E. coli* MutY protein (McGoldrich *et al.* 1995), and the isolation of a yeast protein with properties similar to that of MutM (de Oliveira *et al.* 1994) support this possibility. However, it seems clear that the *PMS1-MSH2* system is responsible for the majority of mismatch repair in yeast, since a defect in either gene severely impairs mismatch correction.

4.2 *rad3*-1 Mutator Effect

The *RAD3* gene is required for the incision step in yeast (Reynolds and Friedberg 1981; Wilcox and Prakash 1981). Its essential role is now known to be in transcription since it encodes the 85 kDa subunit of yeast RNA polymerase II initiation factor b, a homolog of human TFIIH (Feaver *et al.* 1993). Analysis of the Rad3 protein revealed that it has DNA-dependent ATPase/helicase activity and unwinds DNA and DNA·RNA duplexes (Sung *et al.* 1987a,b; Harosh *et al.* 1989; Bailly *et al.* 1991; Naegeli *et al.* 1992). The Rad3 ATPase/DNA helicase function likely is necessary for NER (Sung *et al.* 1988), but may not be required for transcription (Feaver *et al.* 1993). Thus, Rad3 protein has some similarities to the UvrD helicase of *E. coli* which also has DNA·DNA and DNA·RNA unwinding activity (Matson 1989) but is required for mismatch correction as well as nucleotide excision repair (Lahue and Modrich 1988; Modrich 1991). Therefore, it was suggested that the *RAD3* gene might also be involved in postreplicative mismatch correction in yeast (Song *et al.* 1990).

It has been shown in this study that the efficiency of heteroduplex repair in yeast was not decreased, nor was its directionality biased, by the *rad3-1* or *rad3-25* mutations (Table 12). On the other hand, deletion of the mismatch correction gene *PMS1* or *MSH2* dramatically reduced repair of

some of the same heteroduplexes tested in the *rad3* strains (see section 3.1.4 of the Results). Furthermore, the magnitude of the *SUP4*-o mutation rate increase in the *rad3-1* background was lower than expected for a mutator due to a mismatch correction deficiency (Table 8). In addition, the relative fractions of single base-pair substitutions arising in the *rad3-1* strain differed somewhat from those observed in a *pms1Δ* or *msh2Δ* background (Tables 9-11). For instance, significantly fewer single base-pair deletions/insertions were detected the in *rad3-1* background, and the proportion of deletions in the run of five G·C pairs at 79 → 83 was considerably lower in the *rad3-1* strain. Collectively, these observations argue against *rad3-1* diminishing mismatch correction in either a general or specific manner. This also might be true for the *rad3-102* mutator. The *rad3-102* allele confers marginal UV sensitivity, and is mutated outside of the consensus helicase domains in the Rad3 protein (Montelone *et al*. 1988; Song *et al*. 1990). Unlike *rad3-1* and *rad3-25*, it confers a large (20-fold) increase in the *SUP4*-o mutation rate (Montelone *et al*. 1992), but in common with *rad3-1*, the specificity of the *rad3-102* mutator differs from that of the *pms1Δ* or *msh2Δ* mutators. In particular, it can be determined from the data of Montelone *et al*. (1992) that the ratios of single base-pair changes to deletions/insertions and the ratios of transversions to transitions are 4- and 3-fold greater, respectively, in the *rad3-102* strain than in the *pms1Δ* background. Although the results do not negate the possibility that other *rad3* alleles may interfere with mismatch correction, it now seems likely that the mutators conferred by *rad3-1* and possibly *rad3-102* are not a consequence of defective mismatch repair.

Unexpectedly, we discovered that heteroduplex repair was several-fold more efficient in *rad3-1* than its wildtype parent, implying that normal

RAD3 function might somehow limit mismatch correction. Since this phenomenon was not observed for heteroduplex correction in an isogenic *rad1Δ* strain (Kang and Kunz 1992), it is unlikely to be a consequence of NER deficiency. It might, however, be a feature of other *rad3* mutations since mismatch correction was slightly but consistently more efficient in the *rad3-25* background.

A potential explanation for the greater efficiency of mismatch correction in *rad3-1* is related to the suggestion (Montelone *et al.* 1988; Song *et al.* 1990) that the Rad3 protein might contribute to the fidelity of DNA replication. One way to maintain genetic stability when faced with an increase in replication errors would be to enhance mismatch repair. Expression of the *PMS1* and *MSH2* genes is coordinately regulated during the cell cycle with genes required for DNA replication (Johnston and Lowndes 1992; McIntosh 1993; Morrison *et al.* 1993; B.A. Kunz, personal communication). Presumably, this is to ensure a high degree of accuracy during chromosomal DNA replication in S phase. Yeast genes encoding DNA ligase (*CDC9*), DNA polymerase α (*POL1*), ribonucleotide reductase subunits (*RNR1, RNR2*) and thymidylate kinase (*CDC8*) are both cell-cycle regulated and inducible by DNA-damaging agents (Elledge *et al.* 1993; McIntosh 1993). Perhaps yeast also can respond to diminished replication fidelity by modulating the expression of genes involved in mismatch repair independently of cell-cycle control. If so, and the *rad3-1* mutator phenotype is a manifestation of an increase in DNA replication errors, then the magnitude of the mutator might reflect a competition between decreased replication accuracy elevating the mutation rate and enhanced mismatch correction acting to lower it. This would mean that the Rad3 protein might make a more significant contribution to DNA replication

fidelity than suggested by the magnitude of the *rad3-1* mutator.

Another way in which the efficiency of mismatch correction might be increased in *rad3-1* strains would be if defects in *RAD3* altered the expression of genes required for mismatch repair. It has been demonstrated that the essential role of *RAD3* is in transcription and *RAD3* encodes the 85 kDa subunit of yeast RNA polymerase II initiation factor b, a homolog of human TFIIH (Feaver *et al.* 1993). Conceivably, Rad3 might participate in the down-regulation of a gene(s) required for mismatch correction. If so, mutations in *RAD3* that offset such down-regulation might increase the expression of genes required for mismatch repair. On this basis, one might expect transcription of *MSH2*, *PMS1* and *MLH1* to be enhanced in the *rad3-1* strain since these genes probably are components of the same DNA mismatch correction pathway (Prolla *et al.* 1994a, b). However, it has been recently determined that there are no substantial differences among the amounts of the *PMS1*, *MSH2* and *MLH1* transcripts in log phase cells of the *rad3-1*, *rad3-25* and wildtype strains (B.A. Kunz, personal communication). This argues against a direct role for *RAD3* in the transcriptional control of yeast mismatch correction genes.

The *E. coli* UvrD (MutU) helicase functions in NER and mismatch correction (Modrich 1991). However, repair of heteroduplex plasmids in yeast is not diminished by the *rad3-1* mutation or by deletion of the *RAD1* NER gene (Kang and Kunz 1992). Thus, unlike the situation in *E.coli*, NER proteins may not contribute significantly to the avoidance of spontaneous mutation in nucleated cells via participation in mismatch correction. Furthermore, yeast NER might have at best a minor role in the correction of naturally-occurring mispairs. Consistent with this possibility, base mismatches are relatively poor substrates for NER in human cell extracts

(Huang *et al.* 1994), and there is no evidence that suggests NER can discriminate between the correct and incorrect bases in a mispair. Presumably, attempted NER of mismatches would generate mutations as well as prevent them, and so might actually reduce rather than enhance replicational fidelity.

4.3 The Influence of Strand Breaks on the Direction of Mismatch Correction

Strand-directed mismatch repair relies on a mechanism which allows the organism to distinguish between the parental and newly replicated DNA strand during DNA replication. This form of repair is well understood for *E. coli* (Modrich 1989; Grilley *et al.* 1990). In *E. coli*, strand breaks provide the signal for repair on the nascent strand. However, much less is known about such direction of intracellular mismatch correction in eukaryotes. Strand breaks were found to guide the strand specificity of mismatch correction in *D. melanogaster* and human cell extracts (Holmes *et al.* 1990; Thomas *et al.* 1991). Although Hare and Taylor (1985, 1988) transfected heteroduplexes of SV40 DNA into African green monkey kidney cells, they had to isolate the progeny viruses in order to assess mismatch correction via restriction analysis *in vitro*. Thus, none of the eukaryotic systems used previously allowed the direction of intracellular mismatch repair to be established in the cells where repair occurred, and the role of strand breaks in directing mismatch correction within intact eukaryotic cells remained to be determined.

In this study, I analyzed the ability of a strand break located on either side of a mismatch, and on either strand encompassing the mismatch, to direct intracellular mismatch correction. Heteroduplex plasmids having a nick or gap 3' or 5' to the mismatched base, on the transcribed or

nontranscribed strand with respect to the *SUP4*-o gene, were constructed and transformed into the wildtype strain. Inspection of the transformant colony phenotypes revealed that the strand containing the gap was preferentially repaired, and the presence of a gap appeared to increase the efficiency of mismatch correction (Table 16). At best, only minor effects were detected when the heteroduplex plasmids contained a nick rather than a gap, even if the 5' end of the nicked strand was treated with alkaline phosphatase prior to heteroduplex construction (Tables 13, 14, and 15).

Why did the gap but not the nick act to direct the strand specificity of mismatch correction? The most reasonable explanation is that when the nicked heteroduplex plasmids were transformed into yeast cells, the nick was quickly sealed by DNA ligase to restore strand integrity. In contrast, a gap on the heteroduplex plasmids might decrease the efficiency with which the intact double-stranded structure of the plasmid is re-established, and this might provide more time for the gap to function in mismatch correction. If so, the gap would act as a more persistent strand break. The same effect might be achieved in *E. coli* by the MutH protein, which may remain bound at the nick to stabilize it and/or facilitate the entry of additional repair components (Längle-Rouault *et al.* 1987; Lahue *et al.* 1989; Grilley *et al.* 1990).

The sizes of the *Bam*HI and *Eco*RI inserts used to generate the gaps during heteroduplex construction were not uniform. The 5'T and 3'T heteroduplex plasmids had gaps of 42 and 24 bp, respectively, on the transcribed strand of the *SUP4*-o gene, whereas the 5'NT and 3'NT heteroduplex plasmids had gaps of 48 or 84 bp, respectively, on the nontranscribed strand. The 42 bp gap on the 5'T heteroduplex plasmid had

the greatest influence on the direction and efficiency of mismatch correction among the four gaps tested (Table 16). Conversely, the 48 bp gap on the 5'NT heteroduplex plasmid had the least effect on mismatch correction. However, the relative size of the gap might not be a significant factor since the largest gap (84 bp on the 3'NT heteroduplex plasmid) seemed to have a slightly more pronounced effect on mismatch correction than the shorter 5'NT gap (48 bp). Instead, the results suggest that the identity of the strand harbouring the gap might be more important. Both gaps on the transcribed strand appeared to have more influence on mismatch correction than the gaps on the nontranscribed strand. The magnitudes of the increases in the ratios of red:white colonies or the efficiency of mismatch correction were greater for the 5'T and 3'T heteroduplexes than the corresponding increases for the 5'NT and 3'NT heteroduplexes (although the difference was smaller for the 3'T and 3'NT heteroduplexes).

These observations are reminiscent of the preferential repair of the transcribed strand of protein-encoding genes by NER in mammals, yeast and bacteria (Bohr et al. 1985; Mellon and Hanawalt 1989; Selby and Sancar 1993). It has been suggested that a lesion in the template strand for transcription blocks RNA polymerase, and this results in a signal that directs NER enzymes to the damaged site (Mellon and Hanawalt 1989; Selby et al. 1991). However, my results are unlikely to be related to this phenomenon for two reasons. First, preferential NER of the transcribed strand is not observed for genes transcribed by RNA polymerase III, such as SUP4-o (Armstrong and Kunz 1995). Second, the locations of the gaps are outside the actively transcribed region of SUP4-o which runs from -12 (relative to the first base of the tRNA-encoding sequence) to about +103

(Hall *et al.* 1982; Shaw and Olson 1984; Allison and Hall 1985).

Interestingly, the 5'T gap appeared to direct mismatch correction better than the 3'T gap, whereas the opposite result was observed for the gaps on the nontranscribed strand, the 3'NT gap seemed to have a greater effect than the 5'NT gap. However, the sites of the 5'T and 3'NT gaps are opposite each other on the 5' end of the *SUP4*-o gene, indicating that gaps on this end of the gene have a more pronounced effect in directing mismatch correction than gaps on the 3' end (although it is clear that the identity of the strand involved also influences the outcome).

It might be suggested that the apparent direction of mismatch correction by the gaps reflects loss of the gapped strand if it cannot be replicated. If this was the case, then there should be no sectored colonies and only either red or white colonies in the transformants. In fact, at least 11% of the colonies were sectored, and both wholly red or white colonies were detected regardless of the gapped heteroduplex used. It also seems doubtful that the results were due to random action of exonucleases which might remove nucleotides, including the mismatched base, from the gapped strand. Were such a random process responsible, I would have expected the fractions of sectored colonies and the ratios of red to white colonies to have been similar for each gapped heteroduplex used. Clearly, this was not the case.

4.4 Future Work

The results of the *pms1∆* and *msh2∆* studies suggest several other areas of research that could be explored in the future. These include characterization of the specificities of the *mlh1* (Prolla *et al.* 1994a) and *msh3* mutators (New *et al.* 1993). It would be of interest to determine

whether the *mlh1∆*, *pms1∆* and *msh2∆* mutators have similar *SUP4*-o mutational spectra, and whether they all affect correction of base mismatches and single-nucleotide loops to the same extent. There are hints that they might not. Expression of *MLH1* is not cell-cycle regulated, as is expression of *PMS1* and *MSH2*, (B.A. Kunz, personal communication), and the human *hMLH1* and *hMSH2* genes appear to have different functions in the repair of multinucleotide loop-containing heteroduplexes in human cells (Umar *et al*. 1994a). It would be important to investigate the specificity of the *msh3∆* mutator because it has been suggested that *MSH3* is involved only in the repair of nucleotide loops in yeast (New *et al*. 1993).

Another important feature of mismatch repair to investigate is the increased efficiency of heteroduplex correction in the *rad3-1* strain. A potential explanation is that the Rad3 protein contributes to the fidelity of DNA replication, and the cell can modulate mismatch correction in response to the level of mismatches. One approach to begin testing this possibility would be to transform heteroduplex plasmids into a strain having a proofreading-deficient DNA polymerase. Presumably, cells of this strain would sustain a high level of mismatches. If the efficiency of mismatch correction was enhanced in this background, it would support the hypothesis that the *rad3-1* mutation increases the level of mismatches, perhaps by decreasing the fidelity of DNA replication. Alternatively, the *rad3-1* mutation might somehow increase the expression of genes required for mismatch repair at a post-transcriptional level. One could attempt to test this possibility by using western blotting techniques to assess the amounts and stabilities of the Pms1, Msh2 and Mlh1 proteins.

Finally, it also would be informative to repeat the gapped heteroduplex experiments using plasmids that have uniformly-sized gaps. Although there

was no consistent relationship between gap size and the results I obtained, it is not certain whether gap size has any influence on the direction of mismatch correction in the system I used. Furthermore, because the size of the gaps I constructed differ, the potential effects of strand identity and gap location may have been partially obscured.

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