A Role for Translesion Synthesis in Spontaneous Mutagenesis in *Saccharomyces cerevisiae*

By

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A ROLE FOR TRANSLESION SYNTHESIS IN SPONTANEOUS MUTAGENESIS IN *Saccharomyces cerevisiae*

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

The *REV3* gene is believed to be involved in translesion synthesis in the yeast *Saccharomyces cerevisiae*. Inactivation of *REV3* confers an antimutator phenotype. To characterize the specificity of this antimutator, *SUP4*-o mutations arising spontaneously in a *rev3* strain were sequenced. Comparison of the resulting mutational spectrum to that for the isogenic wild-type strain revealed that the *rev3* antimutator caused substantial decreases in the rates of single base pair substitution and deletion. Smaller reductions were observed in the rates of all other mutational classes detected in both strains, suggesting an unusually broad specificity for the *rev3* antimutator. To determine the *REV3* dependency of the mutators conferred by inactivation of the DNA repair genes *RAD1*, *RAD6*, *RAD18*, and *RAD52*, spontaneous *SUP4*-o mutagenesis was characterized in strains having combinations of the wild-type and mutant *REV3* and *RAD* alleles. Analysis of the *SUP4*-o mutations revealed that for each *rad* mutator, *REV3*-dependent processing contributed to the production of each mutational class whose rate was enhanced by that mutator. However, the degree of *REV3* participation differed among the various *rad* mutators. Whereas the *rad1* and *rad6* mutators were largely *REV3*-dependent, the *rad18* mutator was mainly *REV3*-independent but the *rad52* mutator was entirely *REV3*-dependent. Similarly, the different classes of mutation promoted by the *rad6* mutator relied on *REV3* to different extents. Collectively, these results suggest that the majority of unrepaired spontaneous DNA damage is processed by the *REV3* gene product, and so point to a potentially important role for translesion synthesis in spontaneous mutagenesis in yeast. An attempt to characterize the specificity of another antimutator,

that reportedly conferred by inactivation of *DDR48*, was unsuccessful. Disruption of *DDR48* did not reduce the rates of spontaneous forward mutation at the plasmid-borne *SUP4*-o and the chromosomal *CAN1* loci, or spontaneous reversion of the chromosomal *lys2-1* allele. Further analysis revealed that, despite previous suggestions to the contrary, disruption of *DDR48* also did not alter the rate of any particular class of spontaneous mutations detected in both the *ddr48* and isogenic wild-type strains. These results question the validity of the *ddr48* antimutator effect.

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LIST OF ABBREVIATIONS

A	adenine
amp	ampicillin
bp	base-pair
BPS	base-pair substitution
C	cytosine
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
dGTP	2′-deoxyguanosine 5′-triphosphate
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
EMS	ethyl methanesulphonate
G	guanosine
g, mg, ng, μg	gram, milligram, nanogram, microgram
h(rs)	hour(s)
kb	kilobase-pair
l,ml,μl	litre, millilitre, microlitre
M, mM, μM	molar, millimolar, micromolar
m, cm, nm	meter, centimetre, nanometre

min	minute
NaOH	sodium hydroxide
Na_2EDTA	disodium ethylenediaminetetraacetic acid
PEG	polyethylene glycol
pol	polymerase
pur	purine
pyr	pyrimidine
RNA	ribonucleic acid
s	second
SDS	sodium dodecyl sulfate
T	thymine
TEMED	N,N,N′,N′-tetramethylethylenediamine
tRNA	transfer RNA
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

1 INTRODUCTION

The primacy of spontaneous mutagenesis in the science of mutagenesis stems from its purported role in aging (Kirkwood 1989; Mullaart et al. 1990; Sohal and Allen 1990; Ames and Gold 1991), evolution (Arber 1990, 1991; Drake 1991a; Smith 1992) and carcinogenesis (Totter 1980; Loeb 1989, 1991; Rudiger 1990; Ames and Gold 1991). Spontaneous mutations are thought to originate as a consequence of intracellular events including the formation of DNA lesions, the occurrence of errors during DNA replication, repair and recombination, and the movement of transposable elements (Sargentini and Smith 1985; Smith and Sargentini 1985; Ramel 1989; Loeb and Cheng 1990; Drake 1991b; Kunkel 1992; Smith 1992; Amariglio and Rechavi 1993; Ames et al. 1993; Lindahl 1993). This review focuses on the production of spontaneous mutations with emphasis on DNA damage, DNA repair, and translesion synthesis in bacteria and yeast, and the roles of mutators and antimutators in regulating spontaneous mutation rates in yeast.

1.1 Spontaneous DNA Damage and Mutation

Processing of spontaneous DNA damage can lead to spontaneous mutations. Spontaneous DNA damage is produced via endogenous mechanisms. For example, compounds generated during the metabolism of amino acids and sugars have been proven to be mutagenic (Ames 1983; Quinones and Piechocki, 1985; Sargentini and Smith 1986; Glatt 1990; Lee and Cerami 1990; Ames et al. 1990, 1993). Furthermore, the intrinsic instability of DNA, which results in, e.g., cytosine deamination, and depurination, contributes significantly to spontaneous mutagenesis (Lindahl 1993). In addition, DNA can be damaged by reaction with endogenous methylating

agents and reactive oxygen species (Cilento 1980; Simic et al. 1989; Breimer 1990, and references therein; Smith 1992).

1.1.1 Damage by Deamination

When DNA bases are modified spontaneously they often result in miscoding lesions, leading to fixation of a mutation following two rounds of DNA replication (Smith 1992). Hydrolytic deamination of adenine in DNA under physiological conditions produces hypoxanthine, which is potentially mutagenic (Karran and Lindahl 1980; Lindahl 1993). It can pair both with thymine and cytosine resulting in A•T → G•C transitions (Hill-Perkins et al. 1986).

Deamination of cytosine or 5-methylcytosine in DNA generates the bases uracil or thymine, respectively, which following replication produce G•C → A•T transitions (Duncan and Miller 1980). Protonation at the N^3 position of cytosine, which imparts one or more positive charges to the pyrimidine ring, is known to result in deamination, and can be enhanced by loss of a base-pairing partner (Frederico et al. 1990). Zhang and Mathews (1994) have determined that methylation at C-5 increases the rate of cytosine deamination more than 21-fold. Methylation distorts the structure of DNA (Hodges-Garcia and Hagerman 1992) thereby altering H-bonding interactions and increasing the protonation of cytosine (Zhang and Mathews 1994). Frederico et al. (1993) have also shown that deamination of cytosine increases 1- to 2-fold in mismatched base pairs, presumably because the mismatch improves accessibility for protonation. Similarly, the rate of deamination of single-stranded DNA is much higher than double-stranded DNA, and the difference is believed to be due to the greater accessibility of cytosine to protons and hydroxyl ions (for review see

Billen 1990). A higher deamination rate for single-stranded DNA may account for the estimation that the rate of spontaneous deamination of DNA cytosine residues *in vivo* is 40-fold higher in the yeast *Saccharomyces cerevisiae* than in *Escherichia coli* (Impellizeri et al. 1991). It has been suggested that the slower rate of eukaryotic transcription might be responsible for keeping DNA locally in a single-stranded form for a longer period during which it is rendered susceptible to damage (Impellizeri et al. 1991).

Studies of the *lacI* gene of *E. coli* demonstrated that spontaneous base substitution hotspots were due to deamination of 5-methylcytosine, and subsequent replication (Coulondre et al. 1978). Duncan and Miller (1980) later showed that G•C → A•T transitions occurred at a high rate in a strain deficient for uracil-DNA glycosylase (*ung*-) and that 5-methylcytosine residues did not remain hotspots relative to other cytosine residues. Thus, under these circumstances, deamination of cytosine also served as a significant source of spontaneous mutation. Uracil-DNA glycosylase-deficient yeast strains also exhibited increased G•C → A•T transition rates (Impellizeri et al. 1991) and so, collectively, the data for bacteria and yeast indicate that cytosine deamination can be a potent mutagenic process.

1.1.2 DNA Damage by Methylation

Methylation at the N^1, N^3, and N^7 positions of adenine, the N^3, N^7 and O^6 positions of guanine, and the O^4 position of thymine is potentially mutagenic both in prokaryotic and eukaryotic cells (Lindahl et al. 1988). This is because either base pairing is directly affected by alkylation, or removal of the alkylated base by specific glycosylases is followed by

error-prone repair (Singer and Kusmierek 1982). For example, O^6-methylguanine (O^6-MeG) mispairs with thymine and O^4-methylthymine (O^4-MeT) mispairs with guanine giving rise to G•C → A•T and A•T → G•C transitions, respectively (Loechler et al. 1984; Preston et al. 1986; Singer and Dosanjh 1990).

S-adenosylmethionine is a source of methyl groups for various biosynthetic pathways in cells and the methylation of adenine and cytosine in DNA. Alone, it is known to be a weak alkylating agent *in vitro* (Naslund et al. 1983) and *in vivo* and has been shown to methylate proteins and DNA (Drake 1991b). Consequently, it is believed to be the major cellular metabolite causing endogenous DNA alkylation (Lindahl 1993). Reactive methylating compounds are also obtained as end-products of lipid peroxidation (Vaca et al. 1988) and endogenous nitrosation of amines (Calmels et al. 1987; Tsimis and Yarosh 1990).

E. coli cells that are deficient in repairing O^6-MeG and O^4-MeT display enhanced spontaneous mutation rates (Rebeck and Samson 1991). The results of studies conducted by Mackay et al. (1994) showed that all types of base pair substitution, except the G•C → T•A transversion, occurred at higher rates in O^6-MeG methyltransferase-deficient cells than in wild-type cells. This result was distinctly different from the predominance of G•C → A•T transitions observed in earlier studies of alkylation-induced mutagenesis (Horsfall et al. 1990). It was concluded that endogenously produced O^6-MeG and O^4-MeT lesions, which are substrates for the methyltransferase (see section 1.1.5.1), were responsible for the high mutation rate observed at G•C and A•T base pairs, respectively (Mackay et al. 1994). It was also suggested that a fraction of the O^4-MeT lesions could pair with C or T resulting in A•T → C•G and A•T → T•A transversions,

respectively, and some of the O^6-MeG lesions could pair with G to generate G•C → C•G transversions (Mackay et al. 1994). Alkylation repair-deficient yeast cells have also been shown to exhibit increased rates of spontaneous mutations in response to endogenous alkylation damage (Xiao and Samson 1993).

1.1.3 Oxidative DNA Damage

Oxidative DNA damage is generated by hydroxyl ions, superoxide anion, and the active species of hydrogen peroxide, the by-products of normal aerobic metabolism (Ames et al. 1993). These active oxygen species are potent oxidants of DNA (Lindahl 1993). For example, the hydroxyl radical is capable of producing a variety of DNA lesions including single-strand breaks, damage to the deoxyribose moiety, and base damage (Teoule et al. 1987). The major base lesion caused by hydroxyl radicals, however, is 7,8-dihydro-8-oxoguanine (8-oxoG) caused by attack on guanine. This lesion base-pairs with adenine as well as cytosine, and thus generates G•C → T•A transversions after replication (Kasai and Nishimura 1984; Shibutani et al. 1991). Another oxidative base lesion is thymine glycol, a major stable form of thymine modification both *in vivo* and *in vitro* (Cathcart et al. 1984; Kow and Wallace 1985; Hayes and LeClerc 1986; Clark and Beardsley 1987; Teoule et al. 1987; Hayes et al. 1988; Wallace 1988). Thymine glycol generally inhibits DNA synthesis *in vitro* (Ide et al. 1985; Clark and Beardsley 1986; Hayes and LeClerc 1986), although some sequence contexts are known to allow bypass of the lesion by DNA polymerases (Hayes and LeClerc 1986). Studies of site-specific mutagenesis using a phage M13mp19 derivative replicated in *E. coli* demonstrated that thymine glycol caused exclusively A•T → G•C transitions.

In *Escherichia coli* the *mutM*, *mutY* and *mutT* genes are involved in preventing mutation induction by 8-oxoG (see section 1.1.5.2 and below). Inactivation of any of these three genes results in a mutator phenotype (Michaels et al. 1992; Michaels and Miller 1992). Defects in the *mutM* (Cabrera et al. 1988) and *mutY* genes (Michaels 1990) increase the rate of spontaneous G•C → T•A transversions, whereas inactivation of *mutT* results in a specific increase in A•T → C•G transversions (Cox 1976). *In vitro*, DNA polymerases preferentially incorporate A opposite 8-oxoG (Moriya et al. 1991; Wood et al. 1992). Similarly, transfection of 8-oxoG-containing viral DNA templates into *E. coli* resulted in G•C → T•A transversions (Cheng et al. 1992). The *mutM* and *mutY* genes encode glycosylases that remove 8-oxoG or misincorporated adenine from A•8-oxoG pairs, respectively (Michaels and Miller 1992; Michaels et al. 1992). Thus, the specificity of the *mutM* and *mutY* mutators reflects the mispairing properties of 8-oxoG in template DNA. Interestingly, *in vitro* studies showed that the enhanced production of A•T → C•G transversions in the *mutT* strains also were mediated by G•A mispair formation (Schaaper and Dunn 1987). However, this is now thought to be due to the use of 8-oxo-dGTP as a substrate during DNA replication. The MutT protein removes 8-oxo-dGTP from the nucleotide pool (Maki and Sekiguchi 1992). Thus, the mutator phenotype in *mutT* strains likely is caused by misincorporation of 8-oxo-dGTP opposite template A, giving rise to the A•T → C•G transversions.

1.1.4 Abasic Sites

Cleavage of the N-glycosyl bond that connects a DNA base to the deoxyribose sugar leaves the DNA sugar-phosphate backbone intact resulting in the formation of an apurinic/apyrimidinic (AP) site. These lesions are

known to be mutagenic (Schaaper and Loeb 1981; Loeb and Preston 1986), and are subject to repair processes (Lindahl 1990). Depurination is the most frequent DNA alteration occurring spontaneously under physiological conditions (Friedberg 1985; Loeb and Cheng 1990). Failure to repair AP sites prior to DNA replication results in the replication complex encountering a template without a base, i.e., a noncoding lesion (Loeb and Cheng 1990). This can result in misinsertion of nucleotides by DNA polymerases that attempt to replicate past these lesions and so lead to mutation (Shearman and Loeb 1979; Loeb and Cheng 1990). In addition to being spontaneously produced by depurination or depyrimidination, AP sites are induced by spontaneous alkylation at the N^3 or N^7 positions of purines, or the O^2 position of the pyrimidines, which labilizes N-glycosyl bonds (for reviews see Lindahl 1982; Duncan 1985; Strauss 1985). Also, several types of DNA glycosylase cleave the N-glycosyl bonds of damaged nucleotides, liberating the bases from the DNA backbone to produce AP sites. DNA glycosylases are also capable of releasing uracil and hypoxanthine which may be produced by spontaneous deamination of cytosine and adenine residues, respectively (see section 1.1.3) (Lindahl 1974; Friedberg 1985).

In vitro studies with *E. coli* DNA polymerase (pol) III holoenzyme suggested that the intrinsic properties of the polymerase were responsible for the specificity of AP-site mutations (Hevroni and Livneh 1988; Livneh et al. 1993). Both *in vitro* and *E. coli* transfection studies have shown that DNA polymerases preferentially incorporate dAMP opposite the abasic site (Sagher and Strauss 1982: Schaaper et al. 1983; Takeshita et al. 1987; Hevroni and Livneh 1988; Lawrence et al. 1990). In *S. cerevisiae*, both spontaneous oxidation and alkylation contribute to the formation of

AP sites during aerobic growth (Ramotar et al. 1991; Xiao and Samson 1993; Kunz et al. 1994). However, deletion of the *APN1* gene, which encodes the major AP endonuclease in yeast, results in a mutator phenotype that causes the largest rate increase for A•T → C•G transversions (Kunz et al. 1994). Thus, the apparent preference for DNA polymerases to insert adenine opposite induced or manufactured AP sites might not hold for naturally-occurring abasic sites in eukaryotic cells.

1.1.5 Repair of Spontaneous DNA Damage

Left unrepaired, spontaneous DNA lesions result either in mutations or cell death. Thus, repair of spontaneous damage contributes to maintaining spontaneous mutation rates at acceptably low levels both in prokaryotes and eukaryotes (Rebeck and Samson 1991; Xiao and Samson 1993). In this section the various repair pathways that are responsible for removal or repair of damage will be discussed.

1.1.5.1 Reversal of Damage

Direct reversal of DNA damage, which is the simplest form of repair, is exemplified by the removal of the methyl groups from O^6-MeG and O^4-MeT by the O^6-alkylguanine and O^4-alkylthymine DNA alkyltransferases, respectively (Olsson and Lindahl 1980; Lindahl 1982; Lindahl et al. 1988). Reversal in *E. coli* is performed by the *Ada* and *Ogt* DNA methyltransferases (Mackay et al. 1994), and in *S. cerevisiae* by the *MGT1* O^6-methylguanine DNA methyltransferase (Xiao and Samson 1993). In yeast, the *MGT1* gene product is thought to directly transfer the methyl group from O^6-MeG and probably O^4-MeT to a cysteine residue in the Mgt1 protein, as was originally elucidated for the *E. coli* alkyltransferase, thereby eliminating the possibility of mispairing with the damaged bases during replication

(Sassanfar et al. 1991; Xiao et al. 1991).

1.1.5.2 Base Excision Repair

DNA glycosylases remove specific types of damaged base and usually there are specific glycosylases for different types of damage, although some glycosylases may have more than one substrate (Friedberg 1985; Wallace 1988, and references therein). However, as far as can be determined, these enzymes all function in the same way. They cleave the N-glycosyl bond linking the DNA base to the deoxyribose, release the free base and leave an abasic site in the DNA backbone (Friedberg 1985; Wallace 1988). With respect to the types of damage mentioned above, glycosylases that remove uracil, hypoxanthine, N^3-methyladenine, N^7-methylguanine, 8-oxoG and thymine glycol have been detected in *E. coli*, *S. cerevisiae* or mammalian cells (Wallace 1988; Michaels and Miller 1992; Xiao and Samson 1993; Saparbaev and Laval 1994). The AP sites generated by glycosylase activity (and other causes) are recognized by different types of AP endonucleases which are ubiquitous enzymes in prokaryotes and eukaryotes. These enzymes cleave the phosphodiester bond on the 3′ or 5′ side of the AP site and a cellular exonuclease then excises the 3′- or 5′-deoxyribose phosphate (Bailly and Verly 1984). The single-stranded gap thus formed is filled by DNA polymerase and sealed by DNA ligase (Loeb and Preston 1986). A variety of proteins with AP endonuclease activity have been isolated from *E. coli* (endonuclease III, endonuclease IV, endonuclease VII, exonuclease III) and yeast (Apn1 and others) (Melamede et al. 1987; Wallace 1988; Demple and Levin 1991).

1.1.5.3 Nucleotide Excision Repair

The major and general repair pathway is nucleotide excision repair

which is able to recognize a wide range of damage, unlike other pathways. It consists of five steps: detection of damage; incision; excision; synthesis of the missing DNA; and ligation (Hoeijmakers 1991; Grossman and Thiagalingam 1993). The initiation of incision is carried out by a damage-specific endonuclease (Lindahl 1982).

In *E. coli* the *uvr* system controls excision repair and consists of six structural genes, *uvrA*, *uvrB*, *uvrC*, *uvrD*, *polA*, and *lig* and two regulatory genes, *recA* and *lexA* (Yeung et al. 1983; Sancar and Sancar 1988; Van Houten 1990). The UvrA, UvrB, and UvrC proteins initiate the process of nucleotide excision repair by incising DNA on both sides of the damaged nucleotide (Sancar and Rupp 1983). The first incision is made at the 8th phosphodiester bond 5′ to the damaged nucleotide and the second at the 4th or 5th phosphodiester bond 3′ to the lesion (Lin and Sancar 1992). Helicase II, the *uvrD* gene product, together with DNA pol I, the product of *polA*, releases the damaged 12-13 base oligonucleotide. The gap is then filled by DNA pol I and sealed by the *lig* gene product DNA ligase (Sancar and Sancar 1988; Selby and Sancar 1990).

Oxidative lesions are shown to be repaired both by base excision repair and nucleotide excision repair in bacteria. UvrABC nuclease-mediated nucleotide excision plays a role in the repair of thymine glycol, an oxidative form of DNA damage (Kow et al. 1990). This system is also known to remove AP sites (Lin and Sancar 1989) and recognizes O^6-alkylguanine lesions (Van Houten and Sancar 1987; Voigt et al. 1989).

In eukaryotes, no homologs of the UvrA, UvrB, or UvrC proteins have been identified. In *S. cerevisiae*, at least ten genetic loci are involved in nucleotide excision repair, and they are all members of the *RAD3* epistasis group (Friedberg 1988). They include *RAD1*, *RAD2*, *RAD3*, *RAD4*,

RAD7, *RAD10*, *RAD14*, *RAD16*, *RAD23* and *RAD25* (Friedberg 1991; Prakash et al. 1993). Phenotypic and biochemical characterization revealed that *RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10* are absolutely essential for damage-specific recognition and incision of DNA (Friedberg 1991). Recent evidence has shown that *RAD14* and *RAD25* could also be involved in the same process (Prakash et al. 1993). The *RAD1* and *RAD10* gene products interact to form a protein complex that exhibits an endonucleolytic activity *in vitro* (Tomkinson et al. 1993) and *in vivo* (Seide et al. 1993). It has been suggested that this activity functions in nucleotide excision repair by initiating incision of damaged DNA (Tomkinson et a. 1993). The *RAD3* gene encodes a DNA helicase which initiates unwinding of duplex regions of partially duplex DNA (Sung et al. 1987a, b). For the unwinding to occur, single-stranded regions of 4-20 nucleotides are required (Sung et al. 1987b). The enzyme shows a strict $5' \rightarrow 3'$ polarity with respect to the single strand DNA to which it binds (Sung et al. 1987a, b). The Rad3 protein is capable of unwinding very long regions of duplex DNA, up to several hundreds of base pairs long (Sung et al. 1987a). It has been speculated that the helicase activity could enhance removal of damage-containing DNA oligonucleotide fragments after incision of DNA on either side of damaged nucleotides (Friedberg 1991). Alternatively, unwinding may generate a conformation conducive to the binding of other proteins involved in nucleotide excision repair around the sites of damage. Also, DNA helicases might aid the translocation of proteins required for excision repair along the genome from non-specific to specific, stable sites of binding (Friedberg 1991). The Rad3 protein also functions as a transcription factor (Feaver et al. 1993). It has been suggested that, at sites of DNA damage, the Rad3 helicase might unwind the region ahead of a

stalled RNA polymerase, exposing the damaged site for repair. This would then allow the polymerase to pass the damaged site upon resumption of transcription (Feaver et al. 1993).

1.1.6 Tolerance of Spontaneous DNA Damage

Any process that increases cell survival by allowing DNA replication in spite of persisting DNA damage is termed damage tolerance. In order to enhance their potential for survival, *E. coli* cells adopt one of two mechanisms to resume DNA synthesis on templates containing replication blocks (Livneh et al. 1993, and references therein). The principal means of damage tolerance involves a *recA*-dependent recombinational bypass mechanism (Rupp and Howard-Flanders 1968). The presence of, e.g., a pyrimidine dimer on the template strand causes DNA replication to halt temporarily at the lesion and then continue at a point further downstream of the dimer. This generates a gap or discontinuity in the daughter strand (Friedberg 1985) which is filled post-replicatively in an error-free manner by recombination (Dasgupta et al. 1981). Alternatively, damage tolerance can also result from error-prone synthesis past replication-blocking lesions. These two processes are known as daughter-strand gap repair and translesion synthesis, respectively. For the purpose of this review, the following sections focus on translesion synthesis.

1.1.6.1 Translesion Synthesis in *Escherichia coli*

In *E. coli*, translesion synthesis has been associated with an increase in the processivity of DNA pol III (Echols and Goodman 1990), and a decrease in its error-editing activity (Woodgate et al. 1987; Shwartz et al. 1988). Enhanced processivity allows the polymerase to remain attached to the template DNA strand and replicate past blocking lesions. Decreased

ability to edit errors favours continuation of DNA synthesis after insertion of nucleotides opposite noncoding or miscoding lesions. Although translesion synthesis allows the cell to replicate its DNA in the presence of lesions, it is potentially mutagenic. There is evidence that translesion synthesis might generate a significant fraction of spontaneous mutations in bacteria (Loeb and Preston 1986).

Translesion synthesis requires the induction of the SOS system, which processes replication blocking lesions, and is dependent on the *umuDC* and *recA* gene products (Echols and Goodman 1990). Self-cleavage of UmuD is stimulated by RecA in the presence of DNA damage and results in the formation of a shorter protein, UmuD′ which is the active species required for ultraviolet (UV) mutagenesis (Burckhardt et al. 1988; Nohmi et al. 1988; Shinagawa et al. 1988). UmuC and UmuD′ interact and RecA is postulated to target the UmuD′C complex to replication-blocking lesions, thereby inhibiting the activity of 3′→ 5′ exonuclease activity of DNA pol III, and so promoting translesion synthesis (Shwartz et al. 1988; Echols and Goodman 1990). Evidence has been recently presented which indicates that the UmuD′, UmuC, and RecA proteins assist DNA pol III in the replicative bypass of an abasic site, supporting the role predicted for UmuD′C and RecA stimulated bypass synthesis by DNA pol III holoenzyme (Rajagopalan et al. 1992).

It has been shown in studies of the *E. coli lacI* gene that, in the absence of any mutagenic treatment, the activation of the SOS system stimulates primarily G•C → T•A transversion and A•T → T•A transversion less predominantly (Miller and Low 1984). It was suggested that these mutations might have been due to the spontaneous formation of AP sites. Apurinic sites are mutagenic *in vivo* when the SOS system is induced

(Schaaper and Loeb 1981), and preferential incorporation of adenine opposite the depurinated base apparently leads to transversions as the predominant type of mutation observed (Schaaper et al. 1983; Loeb and Cheng 1990). Since depurination of guanine is known to occur at a higher rate than for adenine (Lindahl 1977), this might have accounted for the increased rate of transversions observed at G•C base pairs (Miller and Low 1984).

1.1.6.2 Translesion Synthesis in Yeast

Processes for the tolerance of unrepaired DNA damage in eukaryotes remain poorly understood (Friedberg et al. 1991), although there is some evidence for translesion synthesis in UV-irradiated yeast and mammalian cells (Madzak and Sarasin 1991; Carty et al. 1993; Gibbs et al. 1993). A general-acting error-prone repair activity analogous to the SOS system of *E. coli* has not yet been demonstrated in yeast, but available evidence does suggest that mutagenesis in yeast after treatment with DNA-damaging agents involves inducible functions (Seide and Eckardt 1984). It is evident that mutagenesis in yeast induced by DNA damaging agents is under genetic control. Mutants have been isolated which show enhanced mutagen sensitivity and reduced mutability which is characteristic of mutants defective in error-prone processes (Seide and Eckardt 1984) (see section 1.2).

The *RAD6* pathway of yeast is partially responsible for a considerable degree of resistance to DNA damage and primarily responsible for induced mutagenesis, suggesting that this pathway actually involves at least two recovery processes, one being error-free and the other error-prone (Lawrence 1994). The error-prone recovery process likely entails

translesion or bypass synthesis which promotes chain elongation past sites of template damage (Lawrence 1994). The *REV3* and *REV1* genes are known to be involved in the error-prone recovery process. The Rev3 protein exhibits significant sequence homology to DNA polymerases but is not essential for chromosomal replication (Morrison et al. 1989). The *REV1* protein shares some sequence homology with the UmuC protein of *E. coli* (Larimer et al. 1989), which is known to facilitate translesion synthesis (see section 1.1.6.1).

It has been suggested that in UV-irradiated nucleotide excision repair-proficient yeast cells, translesion synthesis might occur before replicative DNA synthesis in order to fill a minor fraction of excision-repair gaps in the parental strands, and, in the absence of nucleotide excision repair, translesion synthesis might fill daughter-strand gaps (James and Kilbey 1977; Kilbey and James 1979). These possibilities are supported by the findings that UV-induced mutations originate before S-phase in excision repair-proficient strains and afterwards in the repair-deficient strains (James and Kilbey 1977; Kilbey and James 1979). Presumably, this also might be the case for spontaneous lesions that are substrates for nucleotide excision repair. The putative *REV3* polymerase is thought to be responsible for translesion synthesis in yeast (Morrison et al. 1989). It has been proposed that this enzyme may be more capable of accommodating distortions of the template structure and extracting coding information from lesions (Lawrence 1994).

1.2 Regulation of Spontaneous Mutation Rates in Yeast

To fully understand the nature of spontaneous mutagenesis, it is imperative to study mutators and antimutators which increase and decrease,

respectively, the rate of mutation. Antimutators can yield insights into the sources of spontaneous mutation, since the genes involved could be the ones responsible for the generation of mutations. Analysis of mutators could reveal mechanisms for the suppression of spontaneous mutations (Loeb and Cheng 1990).

1.2.1 Mutators

Enhanced spontaneous mutation rates in repair-deficient strains may be due to the processing of spontaneous lesions by mutagenic repair pathways (Hastings et al. 1976). Repair defects might also indirectly affect spontaneous mutagenesis by reducing either the fidelity of DNA replication (Sargentini and Smith 1985) or the efficiency of correcting replication errors (Lawrence 1982). Alternatively, the products of some repair genes might play a regulatory role (Chanet et al. 1988; Jones et al. 1988), and, by affecting processes other than DNA repair or replication fidelity, result in mutator phenotypes. In the following sections the properties of four yeast DNA repair genes which confer mutator phenotypes when inactivated are considered.

1.2.1.1 *RAD1*

RAD1 contains an open reading frame of 3,300 nucleotides (Higgins et al. 1983; Yang and Friedberg 1984) and is a weakly expressed gene. Disruption or deletion of the *RAD1* gene is not lethal to haploid yeast cells, and so *RAD1* is not essential (Higgins et al. 1983). The product of the *RAD1* gene is known to function at the incision step of nucleotide excision repair. The Rad1 and Rad10 proteins form a complex which exhibits endonuclease activity (Sung et al. 1990b; Tomkinson et al. 1993). This activity might be essential for removal of non-homologous regions of

single-stranded DNA during mitotic recombination (Fishman-Lobell and Haber 1992), and for the incision of damaged DNA during nucleotide excision repair (Tomkinson et al. 1993). Substrates for the Rad1-Rad10 endonuclease include bulky DNA adducts (Friedberg 1988), interstrand crosslinks (Miller et al. 1982) and methylated adenine or cytosine residues (Hoekstra and Malone 1986; Féher et al. 1989). Thus, defects in this gene increase the sensitivity to several DNA-damaging agents including UV radiation, 4-nitroquinoline-1-oxide (4-NQO), mono- and bifunctional alkylating agents and photoactivated psoralens (Chanet et al. 1976; Prakash 1976; Prakash and Prakash 1977; Zuk et al. 1979; Cooper and Waters 1987; Friedberg 1988).

In addition to sensitizing cells to the lethal effects of certain genotoxic agents, *RAD1*-deficiencies confer a mutator phenotype (Moustacchi 1969; von Borstel and Hastings 1980; Sargentini and Smith 1985; Kunz et al. 1990a). Although in earlier studies the rates of reversion of *his1*, *leu2* and *ade1* to prototrophy and forward mutation to canavanine resistance (*CAN1*), were found to be enhanced by the mutator effect, the specificity of this effect was not elucidated (Moustacchi 1969; von Borstel and Hastings 1980). The *rad1* mutator phenotype was subsequently shown to be associated with increased frequencies of single base pair substitution, single base pair deletion and insertion of the yeast retrotransposable element Ty (Kunz et al. 1990a). An increase in the frequencies of all substitutions was noted, with G•C → T•A and G•C → C•G transversions and G•C → A•T transitions showing the largest increases. On the basis of these and other findings it was suggested that error-prone translesion synthesis past unrepaired spontaneous DNA damage, and activation of Ty transposition by such damage, might account for the mutator phenotype (Kunz et al.

1990a). *rad1* strains do not exhibit increased post meiotic segregation (DiCaprio and Hastings 1976), and *RAD1* is not essential for mismatch correction on heteroduplex plasmids in yeast (Kang and Kunz 1992). This discounts the suggestion (Montelone et al. 1988) that the mutator phenotype observed in excision repair-deficient strains results from inefficient mismatch correction.

1.2.1.2 *RAD6*

The *RAD6* gene of *Saccharomyces cerevisiae* has been cloned and found to encode a 19 kDa ubiquitin-conjugating enzyme (E2) (Jentsch et al. 1987; Koken et al. 1991). Conjugation of ubiquitin to short-lived intracellular proteins targets these proteins for degradation via an ATP-dependent protease specific to ubiquitin (Prakash et al. 1993). Ligation of ubiquitin to proteins is a multistep process initiated by ATP-dependent binding of ubiquitin to the ubiquitin-activating enzyme E1. Ubiquitin is then transferred to the ubiquitin-conjugating E2 enzymes (Prakash et al. 1993). Ten E2-encoding genes have so far been identified in yeast (Jentsch 1992). Ubiquitin transfer to protein substrates is facilitated by E2 catalyzing the formation of an isopeptide bond between ubiquitin and target proteins (Sung et al. 1988). The E2 enzymes are postulated to either recognize the protein substrates directly, or they may require an intermediary enzyme, ubiquitin-protein-ligase (E3) which functions in substrate recognition (Sung et al. 1988).

Inactivation of *RAD6* confers a highly pleiotropic phenotype consistent with an important role for ubiquitin conjugation in DNA metabolism. Reported properties of *rad6* mutants include extreme sensitivity to a variety of DNA-damaging agents, increased spontaneous and

induced mitotic recombination, enhanced spontaneous mutation rates, and deficiencies in post-replication repair, meiotic recombination, sporulation, and most types of damage-induced mutagenesis (for reviews see Haynes and Kunz 1981; Kunz and Haynes 1981; Lawrence 1982; Game 1983; Friedberg 1988). That these features are due to loss of the ubiquitin-conjugating activity has been demonstrated by the finding that directed base-pair substitutions which eliminate the capacity of the RAD6 E2 to bind ubiquitin cause the same defects as deletion of *RAD6* (Sung et al. 1990a).

The exact mechanism of *RAD6* function in DNA repair and mutagenesis is not known. However, it has been suggested that *RAD6*-mediated conjugation of ubiquitin to proteins is directly involved in post-replication repair and mutagenesis, perhaps by activating these proteins or altering their properties (Prakash et al. 1993). It has been speculated that *RAD6*-mediated ubiquitination could also effect the dissociation of the DNA replication machinery stalled at the site of damage in DNA, allowing for the entry of the DNA repair complex at the damaged site (Prakash et al. 1993). Another possible role of *RAD6* could be in the degradation of specific subunits of the DNA replication complex, which might then enable the complex to associate with DNA repair proteins (Prakash et al. 1993). Further, *RAD6*-dependent ubiquitination and degradation of histone and nonhistone chromosomal proteins could confer altered chromatin structure and so increase accessibility of damaged sites to DNA repair proteins (for review see Prakash et al. 1993). Bailly et al. (1994) have demonstrated that the *RAD18* and *RAD6* gene products form a specific complex *in vivo* and the RAD18 protein confers single-stranded DNA binding activity to the complex. This might aid in targeting *RAD6* to

damage-containing DNA regions (Koken et al. 1991; Bailly et al. 1994), thereby allowing *RAD6* to initiate DNA repair.

rad6 mutations were shown to markedly enhance the frequency of insertion of the yeast Ty element into three different loci (Picologlou et al. 1990; Kang et al. 1992). It has been suggested that the increased rate of Ty transposition in *rad6* mutants might be caused either by failure to ubiquitinate proteins that repress transposition, or altered chromatin structure that enhances accessibility of transposition complexes to DNA (Picologlou et al. 1990). However, Ty transposition alone did not account for the entire increase in the spontaneous mutation frequency attributed to the *rad6* mutator. Hastings et al. (1976) showed that *rad6-1* mutants exhibited an increased rate of reversion of the *lys1-1* ochre allele. It was later established that defects in *RAD6* confer a mutator phenotype largely due to increases in the rates of both base pair transitions and of G•C → T•A transversions (Kang et al. 1992). These results led to the conclusion that there is more than one mechanism to account for *RAD6* activity in limiting spontaneous mutation rates.

1.2.1.3 *RAD18*

RAD18 is predicted to encode a 55 kDa protein (Jones et al. 1988). Sequencing of the *RAD18* gene led to the suggestion that its product has amino acid sequences homologous to those presumed responsible for the ability of some proteins to bind DNA or bind and hydrolyze nucleotides (Chanet et al. 1988; Jones et al. 1988). In particular, the *RAD18* protein has three characteristic DNA-binding motifs, known as zinc fingers suggesting that the *RAD18* gene product is a nucleic-acid-binding protein. Indeed, Bailly et al. (1994) have determined that the *RAD18* gene product

might have a role in assisting binding of the Rad6-Rad18 complex to damaged single-stranded DNA. The structure of the *RAD18* protein also resembles that of the regulatory proteins encoded by the *GAL4* and *GCN4* genes of *S. cerevisiae* (Ma and Ptashne 1987; Hope and Struhl 1986), strongly suggesting that it might also have a regulatory role (Chanet et al. 1988; Jones et al. 1988).

Defects in *RAD18* confer a mutator phenotype (von Borstel et al. 1971; Hastings et al. 1976; Quah et al. 1980; Kunz et al. 1991). It was determined that the rates of reversion of a missense allele (*his1-7*) and forward mutation to suppression, but not locus reversion, of an ochre allele (*lys2-1*), were enhanced in *rad18* strains (von Borstel et al. 1971; Quah et al. 1980). In a later study, the mutator phenotype was shown to specifically increase only G•C → T•A transversions (Kunz et al. 1991). This specificity was not due to failure to correct base mismatches that could rise to the transversion. Thus, it was suggested (Kunz et al. 1991) that the specificity of the *rad18* mutator might point to a role for the *RAD18* gene product in maintaining the fidelity of DNA replication or editing of replication errors. It also is possible that *RAD18* might be involved in repair of 8-oxoG, akin to the activities seen for the *mutT* and *mutY* genes of *E. coli* (Akiyama et al. 1989; Au et al. 1989). Defects in the *REV3* gene, which is believed to encode a non-essential DNA polymerase required for translesion synthesis (Morrison et al. 1989), reduces the *rad18* mutator effect (Quah et al. 1980). This suggests that translesion synthesis might contribute to the *rad18* mutator.

rad18 mutants also exhibit increased spontaneous, UV and gamma-ray-induced mitotic recombination (Boram and Roman 1976; Saeki et al. 1980). *RAD18* has been found to be semidominant for UV and trimethoprim

sensitivity and for enhanced spontaneous and UV-induced mitotic recombination (Mayer and Goin 1984), but not for methyl methanesulfonate sensitivity or the mutator phenotype (von Borstel et al. 1971; Boram and Roman 1976). Although it has often been reported that UV mutagenesis is normal in *rad18* strains, recent studies indicate that deletion of *RAD18* prevents most UV-induced mutation (Cassier-Chauvat and Fabre 1991; Armstrong et al. 1994).

In spite of being sensitive to radiation, *rad18* strains are capable of excising UV-induced pyrimidine dimers and appear to repair gamma-ray-induced DNA single- and double-strand breaks (Reynolds and Friedberg 1981; Mowat et al. 1983). *RAD18* gene has been shown to be involved in the filling of gaps after UV radiation in excision repair-deficient backgrounds (di Caprio and Cox 1981; Prakash 1981). In addition to being involved in this post-replication repair, it has been suggested that *RAD18* also functions in prereplicative repair, specifically the filling of single-stranded DNA regions containing a dimer, generated by an excision event on one strand (Cassier-Chauvat and Fabre 1990). Consistent with a possible role for gap filling, it also has been suggested that a deficiency in base excision repair subsequent to endonuclease action might account for the cross-sensitivity of *rad18* mutants to various DNA damaging agents (Mowat et al. 1983).

1.2.1.4 *RAD52*

The *RAD52* gene of *Saccharomyces cerevisiae* contains an open reading frame of 1,512 nucleotides which is predicted to encode a 60 kDa protein of undetermined function (Adzuma et al. 1984). The *rad52-1* mutant originally identified displayed extreme sensitivity to X-rays and slight

sensitivity to UV (Resnick 1969). *RAD52* appears to function in DNA double-strand break repair (Resnick and Martin 1976) and postreplication repair in excision repair-deficient backgrounds (Prakash 1981). Mutant strains also exhibit partial or complete defects in meiosis, sporulation (Game and Mortimer 1974; Strike 1978; Game et al. 1980; Prakash et al. 1980) and mitotic gene conversion (Strike 1978; Game et al. 1980; Prakash et al. 1980; Saeki et al. 1981; Jackson and Fink 1981). Recombination events may be required for postreplicative repair of UV radiation damage explaining the sensitivity of some *RAD52* group mutants to UV radiation (Kiefer 1987).

Defects in the *RAD52* gene confer a mutator phenotype (von Borstel et al. 1971; Kunz et al. 1989). It was determined earlier that *rad52-1* increased the rate of suppression of the ochre allele *lys2-1* and reversion of the putative frameshift alleles *ura4-11* and *thr3-10*, while marginally affecting locus reversion of *lys1-1* (von Borstel et al. 1971). Kunz et al. (1989) determined that the *RAD52* mutator promoted primarily events at G•C pairs, mainly G•C → C•G and G•C → T•A transversions. It has been suggested that *RAD52* could be involved in the repair of DNA damage which, if left unrepaired might be processed by translesion synthesis (Kunz et al. 1989). Spontaneously occurring AP sites, which are probably the most frequently occurring DNA alterations, are known to be mutagenic (Loeb and Preston 1986). Preferential insertion of adenine (Strauss 1991) opposite such sites during bypass synthesis would result primarily in G•C → T•A and A•T → T•A transversions. The increase in the latter events was minor in a *rad52* mutant strain suggesting that the mutator effect was not due to a failure to repair depurinated sites (Kunz et al. 1989). *RAD52* might, however, play a role in transposition of the Ty element since Ty

transpositions were not observed in the mutant strain (Kunz et al. 1989). Assuming that DNA double-strand breaks and perhaps other X-ray-induced DNA lesions are repaired via a process involving recombination (Haynes and Kunz 1981), the mutator phenotype exhibited by *rad52-1* strains might be accounted for by their defect in recombination (Kunz et al. 1989).

1.2.2 Antimutators

Antimutator strains are studied to identify processes responsible for mutagenesis (Hastings et al. 1976; Quah et al. 1980). However, antimutators isolated so far have a narrow specificity, reducing mutation rates only along certain pathways (Drake 1993). Thus, it has been suggested that general antimutators which concurrently reduce the rates of most or all types of spontaneous mutation without associated deleterious effects are improbable (Drake 1993). In the following sections the properties of two yeast antimutators, *REV3* and *DDR48*, will be discussed.

1.2.2.1 *REV3*

REV3 was initially identified by isolating strains which showed reduced frequencies of UV mutagenesis (Lemontt 1971). The nucleotide sequence of *REV3* encodes a predicted protein of 172 kDa (Morrison et al. 1989). There is significant sequence homology between *REV3* and Epstein-Barr virus DNA polymerase, mammalian DNA pol α and yeast DNA pol I (Morrison et al. 1989). Haploids with *REV3* deleted are viable, and so it has been proposed that induced mutagenesis in *S. cerevisiae* depends on the nonessential DNA polymerase encoded by *REV3* (Morrison et al. 1989).

Defects in *REV3* were shown to reduce the rates of reversion of the missense allele *his1-7* as well as locus reversion and suppression of the ochre alleles *lys1-1* and *arg4-17* (Cassier et al. 1980; Quah et al. 1980).

Hence, *REV3* appears to participate in the production of at least some spontaneous base-pair substitutions in yeast. In addition, *REV3* might also be needed for spontaneous deletion, as well as other events, because UV-induced single base-pair frameshifts and forward mutations to auxotrophy were significantly reduced in *rev3* backgrounds (Lemontt 1972; Lawrence and Christensen 1979; Lawrence et al. 1984). On the basis of these findings, it was suggested that *REV3* might function only in translesion synthesis (Morrison et al. 1989). This hypothesis is supported by the demonstration that unlike many genes required for replication of chromosomal DNA (McIntosh 1993), transcription of *REV3* does not exhibit cell cycle regulation (Singhal et al. 1992). Thus, the antimutator phenotype due to the inactivation of *REV3* might point to translesion synthesis having a major role in spontaneous mutagenesis in yeast. Consistent with this possibility, the *rev3* antimutator was shown to offset the mutators associated with defects in yeast genes required for excision (*RAD3*), postreplication (*RAD18*) or recombinational (*RAD51*) repair of UV-induced DNA damage (Quah et al. 1980).

1.2.2.2 *DDR48*

Expression of the *DDR48* gene sequence is DNA-damage-responsive and also can be induced by heat shock (Treger and McEntee 1990). The gene sequence contains two overlapping open reading frames, each capable of encoding a protein of molecular mass of approximately 45 kDa, but only one of them is expressed in yeast cells (Treger and McEntee 1990). A recent study in which the Ddr48 protein was purified and characterized revealed that the protein had an approximate molecular mass of 65 kDa (Sheng and Schuster 1993). The predicted Ddr48 protein is extremely hydrophilic and

is punctuated with multiple copies of an octapeptide sequence. Both heat shock and treatment with 4-NQO increased transcript production by 10- to 15-fold and three major and two minor transcriptional start sites were detected (Treger and McEntee 1990).

The function of the *DDR48* gene product is unknown, although viability studies of *ddr48* diploid yeast cells implicate the protein in spontaneous mutagenesis and recovery from induced DNA damage (Sheng and Schuster 1993). Disruption of the gene was associated with an antimutator phenotype, affecting only certain classes of mutation (Treger and McEntee 1990). The rates of forward mutation at *CAN1* and reversion of *his4-713*, a frameshift allele, were lowered ca. 3-fold and 6 to 14-fold, respectively, but the type(s) of mutational change influenced by the *ddr48* antimutator was not characterized (Treger and McEntee 1990).

1.3 Purpose of this Study

This study had three aims: 1. to ascertain the specificity of the *rev3* antimutator; 2. to determine the role of *REV3* in the mutators conferred by inactivation of *RAD1*, *RAD6*, *RAD18* and *RAD52*; and 3. to identify the specific sequence alterations promoted by *DDR48*. The general approach taken was to employ DNA sequencing to characterize spontaneous *SUP4*-o mutations in wild-type or single and double mutant strains. The resulting mutational spectra were then compared in an attempt to meet the goals outlined above.

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 Yeast and Bacterial Strains

The complete genotypes of all strains used in this study are given in Table 1. All haploid yeast strains are isogenic derivatives of the repair-proficient strain MKP-o. Construction of MKP-o, KAM1 (*rad1Δ::LEU2)*, RDG6 (*rad6Δ ::LEU2)*, XK15 (*rad18Δ::LEU2)* and SB-52 (*rad52::TRP1)* has been described (Pierce et al. 1987; Kunz et al. 1989, 1990a, 1991; Kang et al. 1992). The latter strains are known to be defective in nucleotide excision repair, postreplication repair or recombinational repair, respectively.

REV3-deleted (*rev3Δ)* derivatives of MKP-o (RDG3) and KAM1 (RDG31) were constructed for this study by R.D. Gietz as follows. The 10 kb pJA6 (Morrison et al. 1989) *Kpn*I fragment that carries *REV3* was first cloned into the *Kpn*I site of a pUC19 (Yanisch-Perron et al. 1985) derivative, from which the *Xba*I site had been eliminated, giving rise to pDG344. pDG344 was then digested with *Xba*I to delete the entire *REV3* coding sequence (4,512 bp) plus 111 bp and 5 bp of the immediate 5′ and 3′ flanking sequences, respectively. Next, the gel-purified 8 kb pDG344 *Xba*I fragment containing the *REV3*-flanking regions was blunt-end-ligated to the 3.8 kb pDG82 *Bam*HI fragment carrying *URA3* to generate pDG347. {pDG82 was

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)
KAM1	as for MKP-o but *rad1Δ::LEU2*	Kunz et al. (1990a)
XK15	as for MKP-o but *rad18Δ::LEU2*	Kunz et al. (1991)
SB-52	as for MKP-o but *rad52::TRP1*	Kunz et al. (1989)
RDG6	as for MKP-o but *rad6Δ::LEU2*	Kang et al. (1992)
RDG3	as for MKP-o but *rev3Δ*	R.D. Gietz
JMT48A	as for MKP-o but *ddr48::LEU2*	K. McEntee
KRMC	as for MKP-o but with *CAN1*	This study
KRJC	as for MKP-o but with *CAN1*	This study
RDG31	as for RDG3 but *rad1Δ::LEU2*	R.D. Gietz
HR36	as for RDG3 but *rad6Δ::LEU2*	This study
FY318	as for RDG3 but *rad18Δ::LEU2*	F. Yadao, this study
FY352	as for RDG3 but *rad52::TRP1*	F. Yadao, this study
JF1754	*Δlac, gal, metB, leuB, hisB436, hsdR*	Pierce et al. (1987)

constructed by blunt-end-ligation of the 3.8 kb pNKY51 (Alani et al. 1987) *Bam*HI-*Bgl*I *hisG-URA3-hisG* cassette into the *Xba*I site of pUC18 (Yanisch-Perron et al. 1985). This cassette can be excised from pDG82 with *Bam*HI because the upstream site adjacent to *Xba*I in pUC18 is *Bam*HI and the cassette was inserted into pUC18 in the 3′ → 5′ orientation *(Bam*HI-*Xba*I/*Bgl*II-*Bam*HI/*Xba*I) to reconstitute a *Bam*HI site at the *Bam*HI/*Xba*I junction.) pDG347 was digested with *Kpn*I to release the 9.2 kb fragment carrying the *hisG-URA3-hisG* cassette sandwiched between the *REV3*-flanking sequences and the digest was transformed (Ito et al. 1983; Shiestl and Gietz 1989; Gietz et al. 1992) into MKP-o and KAM1. Ura^+ transformants that emerged were propagated in uracil omission medium and plated on appropriately supplemented minimal medium containing 5-fluoro-orotic acid (Sigma) to select for loss of the *URA3* gene via crossing-over between the *hisG* repeats (Alani et al. 1987). The resulting Ura^- isolates were then assayed for UV-induced reversion of *lys2-1*. Deletion of *REV3* in reversion-deficient derivatives (RDG3, RDG31) was confirmed by DNA hybridization analysis.

YCpMP2 was transformed (Ito et al. 1983; Shiestl and Gietz 1989; Gietz et al. 1992) into MKP-o, RDG3, KAM1 and RDG31 to make MKP-op, RDG3-p, KAM1-p and RDG31-p, respectively.

FY318, carrying a *rad18Δ::LEU2* insertion, and FY352, carrying a *rad52::TRP1* insertion were constructed for this study by F. Yadao, either by deleting *RAD18* in RDG3 (Kunz et al. 1991) or disrupting *RAD52* in RDG3 with *TRP1* (Kunz et al. 1989). These constructs were confirmed by DNA hybridization analysis as described below. HR36, carries a *rad6Δ::LEU2* insertion and was constructed by deleting *RAD6* in RDG3. JMT48A carrying a *LEU2* disruption, *(ddr48A::LEU2)* was constructed by J. Treger as described

(Treger an McEntee 1990) and was kindly provided by K. McEntee. The corresponding yeast strains carrying the plasmid YCpMP2 (see section 2.3) are designated FY318-p, FY352-p, and HR36-p and JMT48A-p.

2.2.1 Construction of a *rev3 rad6* Strain

To determine whether translesion synthesis is required for the *rad6*Δ mutator, it was necessary to construct a *rev3*Δ *rad6*Δ strain. Figure 1 shows that HR36, carrying a *rad6*Δ*::LEU2* insertion, was derived by transforming RDG3 (*rev3*Δ) with the 2.6 kb *Bam*HI-*Hind*III DNA fragment from plasmid pDG315 (Kang et al. 1992). This fragment has the *LEU2* gene surrounded by sequences that flank *RAD6* and so should integrate at the chromosomal *RAD6* locus via recombination between the homologous fragment and chromosomal DNA sequences (Rothstein 1991). Since RDG3 requires leucine due to a defective *LEU2* allele (*leu2-3,112*), the *LEU2* gene in the 2.6 kb *Bam*HI-*Hind*III fragment allows integration to be detected by selection for growth on medium lacking leucine. Colonies that emerged on leucine omission medium were tested for sensitivity to UV light because *rad6* mutations decrease UV resistance.

Replacement of the *RAD6* gene with the 2.6-kb *Bam*HI-*Hind*III DNA fragment in UV-sensitive, Leu^+ transformants was confirmed by DNA hybridization analysis (for detailed methodology, see section 2.11). Total chromosomal DNA was isolated from MKP-o and the putative *rev3*Δ *rad6*Δ strains, digested with *Hind*III, 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 the 1.6-kb *Bam*HI-*Hind*III pB7 fragment containing the *RAD6* gene (Figure 1A). From Figure 1C, it can be seen that the probe should detect the 4.2 kb *Hind*III fragment when hybridized to MKP-o DNA.

Figure 1. Inactivation of the *RAD6* gene. The figure shows replacement of the *RAD6* gene (C) with the 2.6 kb *Bam*HI-*Hind*III fragment obtained from pDG315 (B). Replacement occurs via homologous recombination and deletes the chromosomal *RAD6* gene as shown in D. The 1.6 kb *Bam*HI-*Hind*III pB7 fragment used as a probe is shown in (A). The dashed line indicates a stretch of yeast chromosomal DNA that is not drawn to scale. B* denotes a *Bam*HI recognition site that was created at the boundary between the yeast chromosomal DNA and the vector DNA in pB7. This *Bam*HI site is not present in the genome. D: *Hind*III recognition site; G: *Bgl*II; R: *Eco*RI; T: *Taq*I.

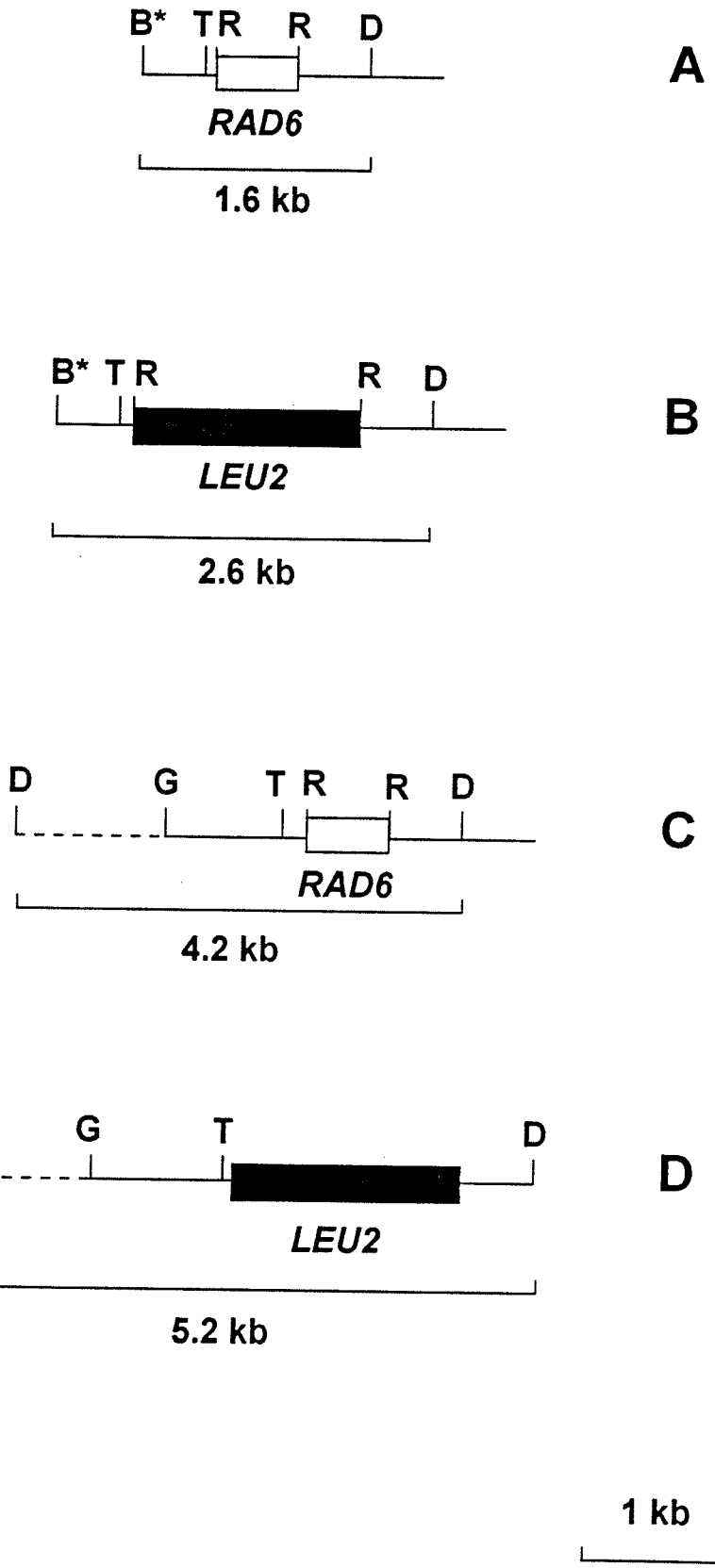

B* T R R D
RAD6
1.6 kb
A
B* T R R D
LEU2
2.6 kb
B
D G T R R D
RAD6
4.2 kb
C
D G T D
LEU2
5.2 kb
D
1 kb

Figure 2 shows that MKP-o had one band at 4.2 kb. Integration of the 2.6 kb *Bam*HI-*Hind*III pDG315 fragment at the chromosomal *RAD6* locus changes this pattern. The 4.2 kb band is replaced by a 5.2 kb band because of the *LEU2* gene insertion. All the transformants tested showed the expected 5.2 kb band (Figure 2). The fainter bands seen at the 4.2 kb position in all the transformants, and at the 2.3 kb position in lanes 3 and 4, might be artefacts associated with the random primer technique used to label the probe. When the same probe was labelled by nick translation, no bands were detected at 4.2 kb or 2.3 kb for hybridization of genomic DNA from *rad6*Δ strains (B.A. Kunz, personal communication). Phenotypic properties and the mutational spectrum of the *rev3*Δ *rad6*Δ construct, HR36-1, exhibited features typical of a *rad6* strain.

2.2.2 Verification of the *rev3 rad18* and *rev3 rad52* Constructs

FY318-1, carrying a *rad18::LEU2* insertion, was derived by transforming RDG3 (*rev3*Δ) with the 5.5 kb *Bam*HI-*Hpa*I p*rad18*Δ1 DNA fragment (Figure 3A), as described (Kunz et al. 1991). The transformation was performed earlier by F. Yadao. In this study, Leu^+ transformants were tested for UV-sensitivity because inactivation of *RAD18* decreases UV resistance. Replacement of the *RAD18* gene with the 5.5 kb *Bam*HI-*Hpa*I fragment in appropriate Leu^+, UV-sensitive transformants was then assessed by DNA hybridization analysis (see section 2.11 for methodology). Total chromosomal DNA from MKP-o and UV-sensitive, Leu^+ transformants was digested with *Eco*RI and the resulting fragments were separated by gel electrophoresis. Following denaturation of the DNA in the gel, the gel was dried and probed with the 2.0 kb *Xba*I p*rad18*Δ1 fragment containing *LEU2* for *rad18* and the 3′ end of *RAD18* (Figure 3A). As seen in Figure 3B, the

Figure 2. Hybridization analysis of Leu$^+$ transformants. Total DNA (2.5μg) from each yeast strain was digested with *Hind*III and separated by electrophoresis in a 0.7% agarose gel. The 1.6 kb *Bam*HI-*Hind*III fragment of plasmid pB7 (Figure 1A) was labelled with ^{32}P and used as a probe. Lane 1: MKP-o, lanes 2-6: Leu$^+$ transformants (HR36-1 through 6). The DNA size markers are in kb.

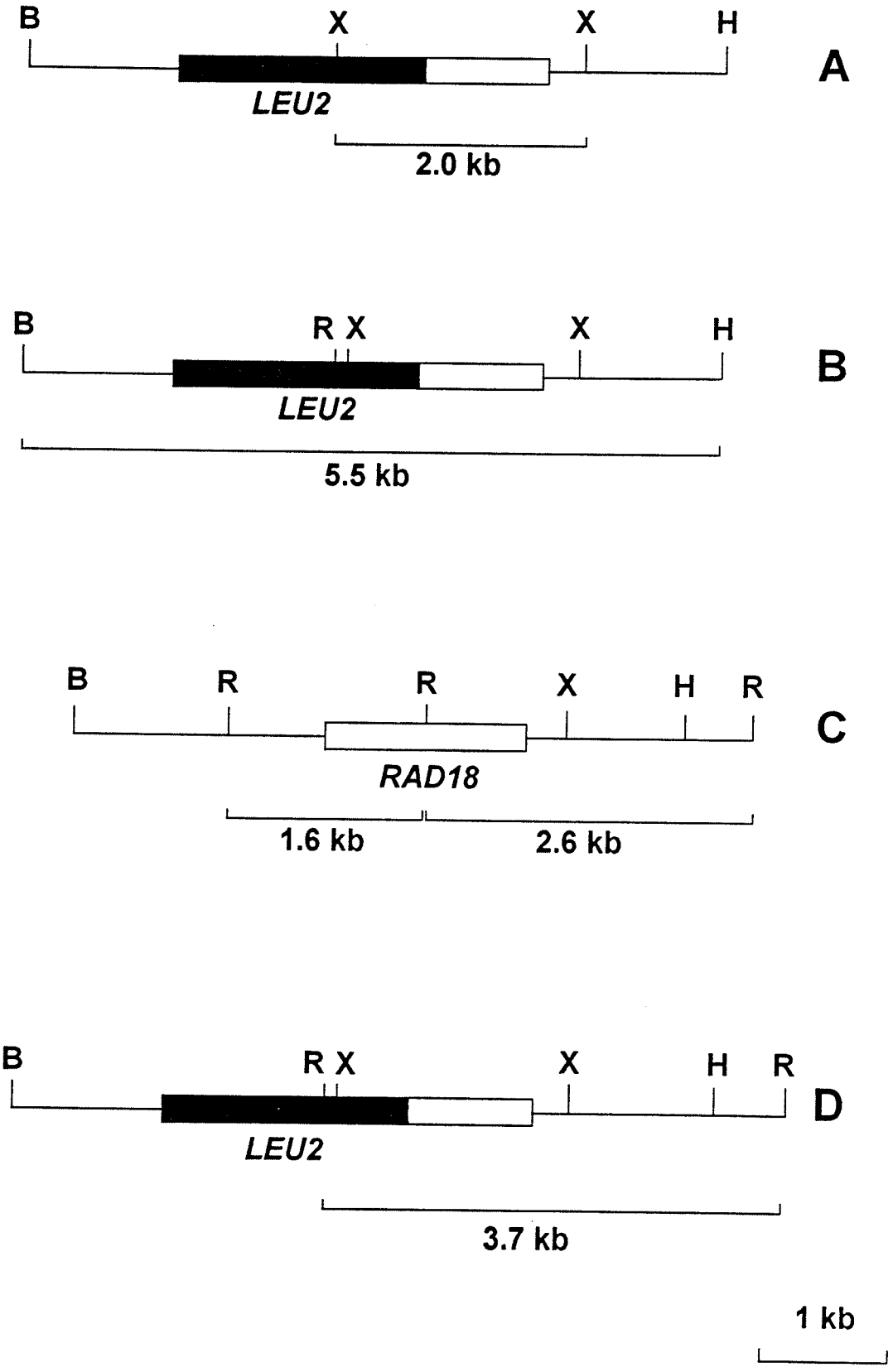
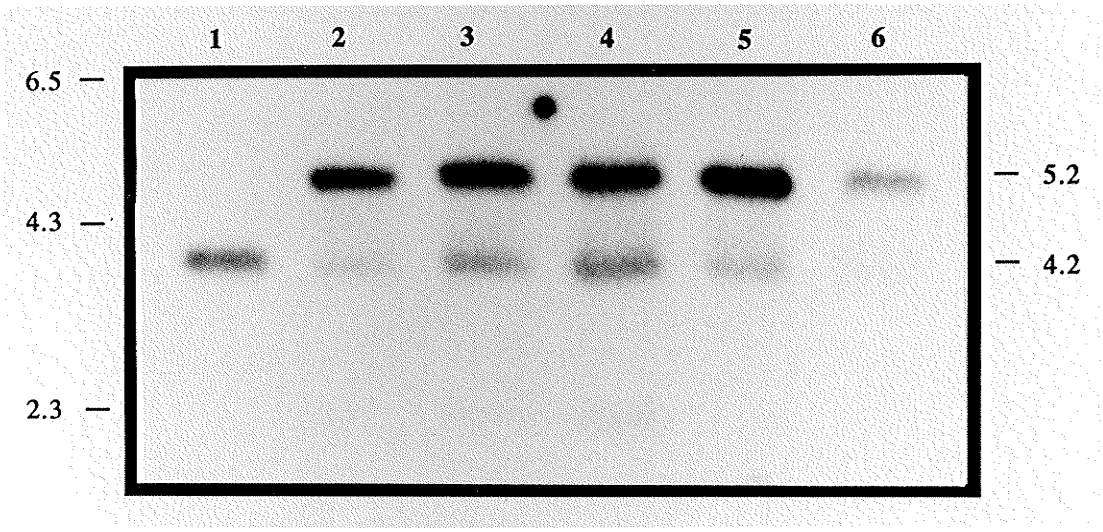

B
X
X
H
A
LEU2
2.0 kb
B
R X
X
H
B
LEU2
5.5 kb
B
R
R
X
H
R
C
RAD18
1.6 kb
2.6 kb
B
R X
X
H
R
D
LEU2
3.7 kb
1 kb

Figure 3. Inactivation of the *RAD18* gene. The figure shows replacement of the *RAD18* gene (C) with a partially deleted copy carried on the 5.5 kb *Bam*HI-*Hpa*I fragment obtained from p*rad18Δ1*(B). Replacement occurs via homologous recombination and inactivates the chromosomal *RAD18* gene as shown in D. The 2.0 kb *Xba*I p*rad18Δ1* fragment used as a probe is shown in (A). Unlabelled white boxes represent 35% of the *RAD18* gene. R: *Eco*RI recognition site; B: *Bam*HI; H: *Hpa*I; X: *Xba*I.


1
2
3
4
5
6
6.5
4.3
2.3
5.2
4.2

probe should detect the 2.6 kb *Eco*RI fragment in MKP-o DNA. The 1.6 kb *Eco*RI fragment would not be detected because the *LEU2* gene replaces this fragment in the probe (Figure 3A). Figure 4 shows that a single 2.6 kb band was detected for the MKP-o DNA. Integration of the 5.5 kb *Bam*HI-*Hpa*I fragment at the chromosomal *RAD18* locus changes the banding pattern. The 2.6 kb *Eco*RI fragment should be replaced by the 3.7 kb *Eco*RI fragment (Figure 3C). This was seen for two of the transformants, FY318-1 and FY318-2, (Figure 4, lanes 2 and 3) indicating that the chromosomal *RAD18* gene had been replaced by the *rad18Δ::LEU2* insertion. The detection of two bands in lane 5 might reflect an artefact similar to that mentioned in section 2.2.1 above, or might be caused by integration of the 5.5 kb *Bam*HI-*Hpa*I fragment at a site other than the *RAD18* locus.

FY352-5, carrying a *rad52::TRP1* insertion, was derived by transforming RDG3 (*rev3Δ*) with the 3.2 kb *Bam*HI pSM21 fragment (Figure 5B), as described (Kunz et al. 1989). The transformation was performed earlier by F. Yadao. In this study, Trp^+ transformants were tested for sensitivity to ethylmethanesulfonate (EMS) because inactivation of *RAD52* decreases EMS resistance. Replacement of the *RAD52* gene with the 3.2 kb *Bam*HI pSM21 fragment was assessed by DNA hybridization analysis (see section 2.11 for methodology). Total genomic DNA from MKP-o and Trp^+, EMS-sensitive transformants was digested with *Eco*RI and the resulting fragments were separated by gel electrophoresis. Following denaturation of the DNA in the gel, it was dried and probed with the 1.1-kb *Bam*HI-*Hpa*II pSM13 fragment carrying the 5′ portion of *RAD52* (Figure 5A). As can be seen in Figure 5C, the probe should detect a 6.2 kb fragment when hybridized to MKP-o DNA, and Figure 6 shows that this was the case. Integration of the 3.2 kb *Bam*HI pSM21 fragment at the chromosomal *RAD52* locus would alter this pattern.

Figure 4. Hybridization analysis of Leu$^+$ transformants. Total DNA (2.5 μg) from each yeast strain was digested with *Eco*RI and separated by electrophoresis in a 0.7% agarose gel. The 2.0 kb *Xba*I fragment of plasmid p*rad18Δ1* (Figure 3A) was labelled with ^{32}P and used as probe. Lane 1: MKP-o, lanes 2-7: Leu$^+$ transformants (FY318-1 through FY318-2). The DNA size markers are in kb.

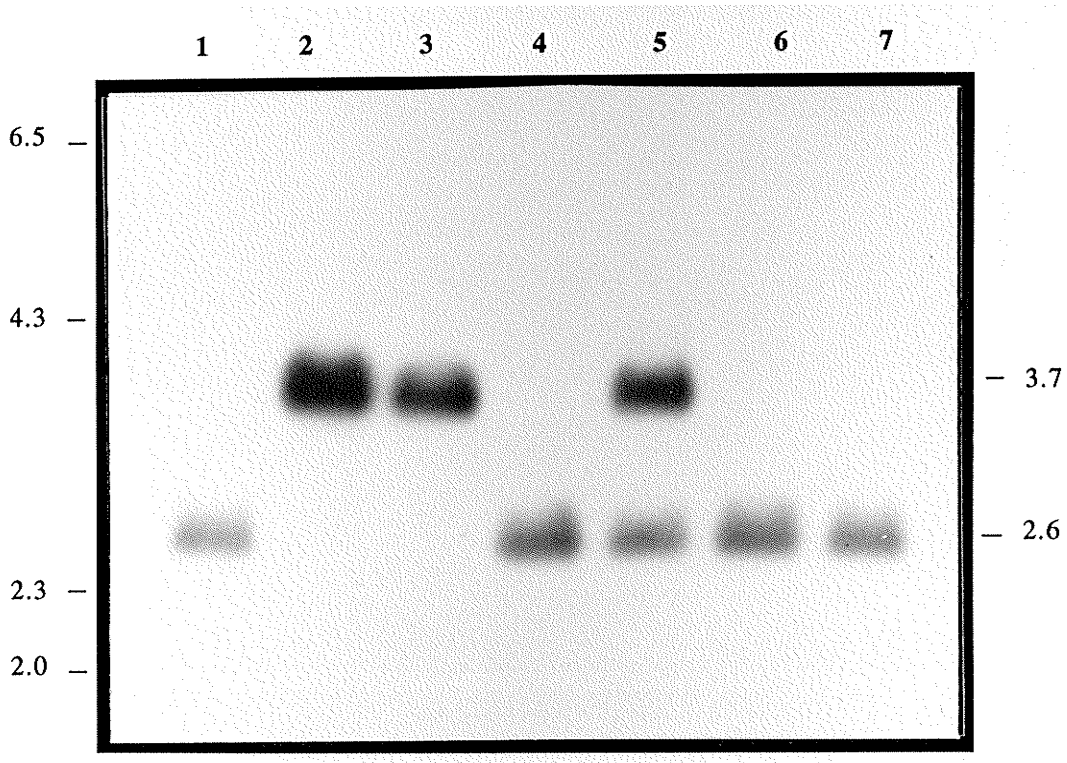

1
2
3
4
5
6
7
6.5
4.3
2.3
2.0
3.7
2.6

Figure 5. Inactivation of the *RAD52* gene. The figure shows replacement of the *RAD52* gene (C) with a disrupted copy carried on a 3.2 kb *Bam*HI fragment obtained from pSM21 (B). Replacement occurs via homologous recombination and disrupts the chromosomal *RAD52* gene as shown in D. The dashed line indicates a stretch of yeast chromosomal DNA of unknown length. The 1.1 kb *Bam*HI-*Hpa*II fragment of plasmid pSM13 used as a probe is shown in (A). Unlabelled white boxes represent portions of the *RAD52* gene. R: *Eco*RI recognition site; B: *Bam*HI site; G: *Bgl*II site; H: *Hpa*II site; S: *Sph*I site.

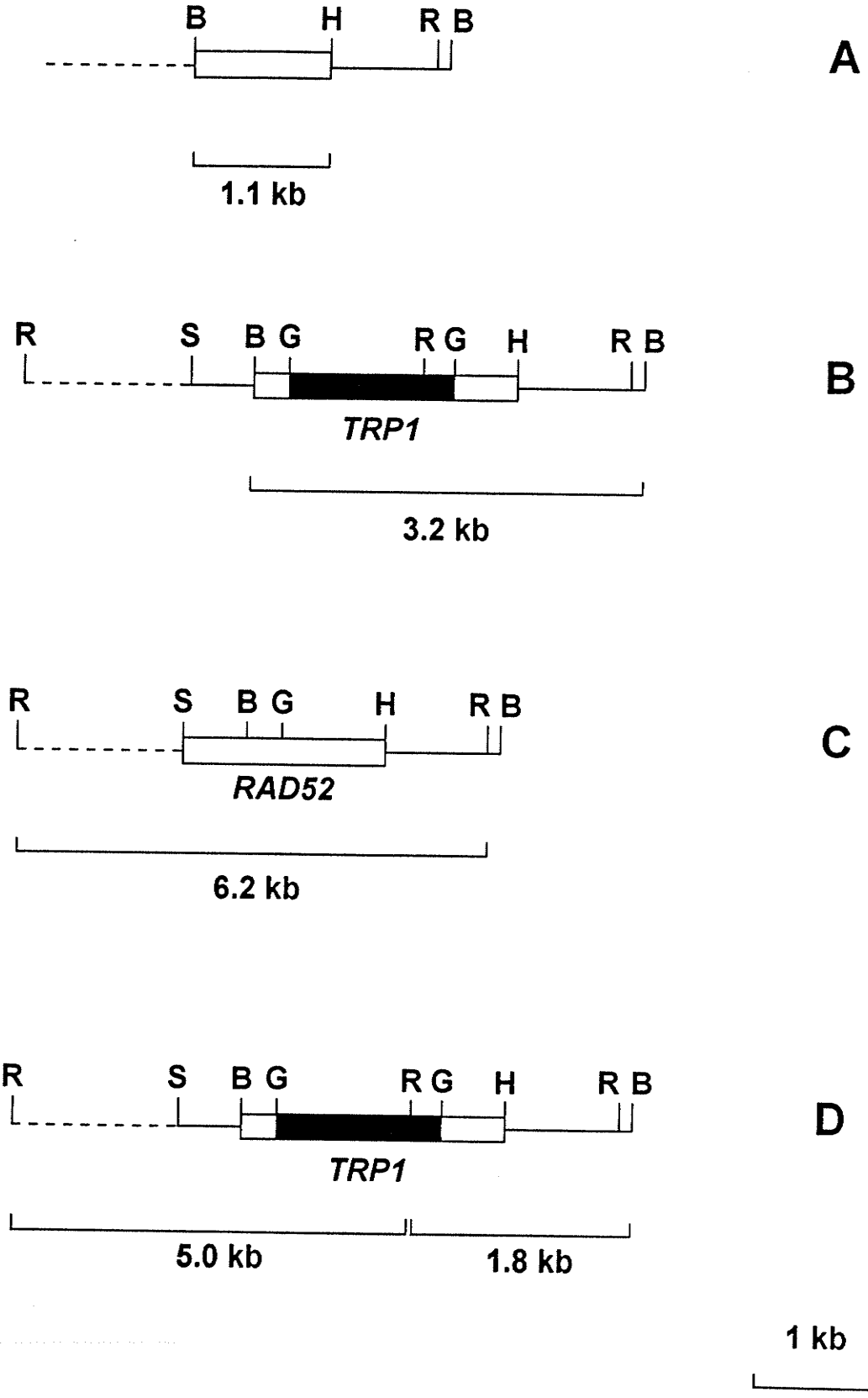

B
H
R B
A
1.1 kb
R
S
B G
R G
H
R B
B
TRP1
3.2 kb
R
S
B G
H
R B
C
RAD52
6.2 kb
R
S
B G
R G
H
R B
D
TRP1
5.0 kb
1.8 kb
1 kb

Figure 6. Hybridization analysis of Trp$^+$ transformants. Total DNA (2.5 μg) from each yeast strain was digested with *Eco*RI and separated by electrophoresis on a 0.7% agarose gel. The 1.1 kb *Bam*HI-*Hpa*II fragment of plasmid p*SM13* (Figure 5A) was labelled with ^{32}P and used as a probe. Lane 1: MKP-o, lanes 2-6: Trp$^+$ transformants (FY352-1 through FY352-5). The DNA size markers are in kb.

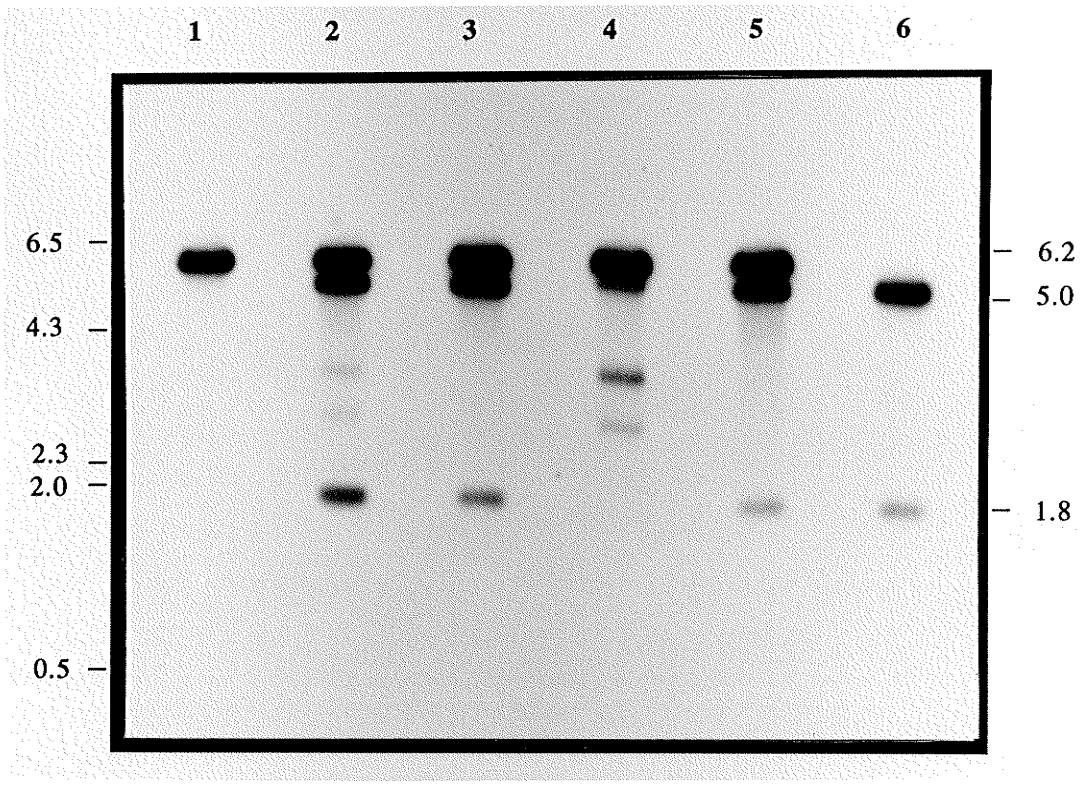
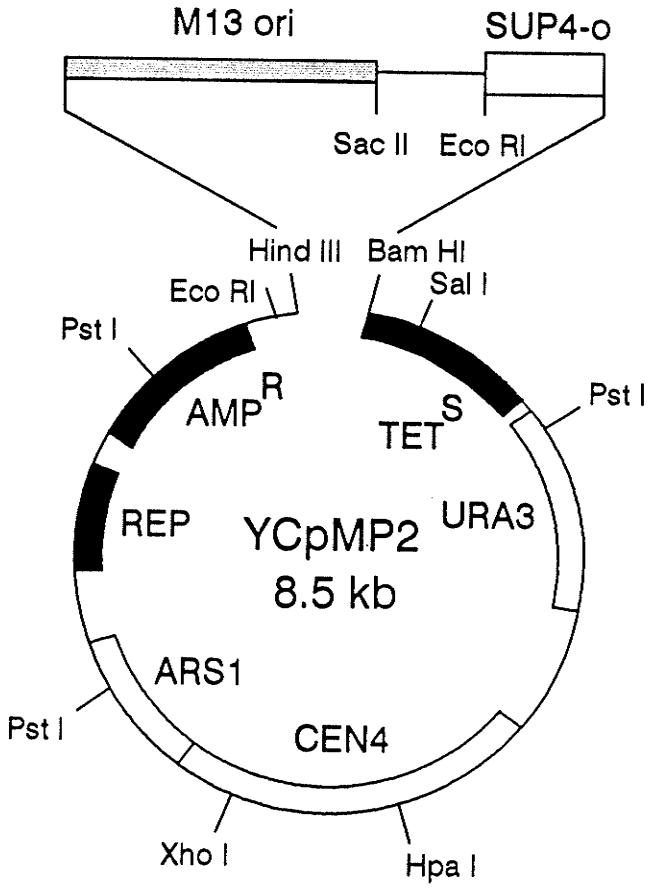

1
2
3
4
5
6
6.5
4.3
2.3
2.0
0.5
6.2
5.0
1.8

Figure 5D shows that this fragment would be replaced by a 1.8 kb *Eco*RI fragment and a 5.0 kb *Eco*RI fragment. One transformant, FY352-5 showed this banding pattern indicating that the *RAD52* gene in the chromosome was replaced with the *rad52::TRP1* insertion (Figure 6, lane 6). Additional bands seen in lanes 2 through 4 might be artefact due to the random primer labelling technique as described in section 2.11.2, or integration of the 3.2 kb *Bam*HI-*Bam*HI fragment at a location other than the *RAD52* locus.

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 7). This 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 vectors such as YCpMP2 to be maintained predominantly at a copy number of one in haploid yeast cells (Newlon 1988). The replication origin from the filamentous phage M13 is also part of this vector, but served no purpose in this study. In addition, YCpMP2 carries *SUP4*-o which is an ochre suppressor allele of a yeast tyrosine tRNA gene and is used as a mutational target.

Plasmids used for construction of yeast strains include p*rad18Δ1* (Fabre et al. 1989), pSM21 (Kunz et al. 1989), pUC18, pUC19 (Yanisch-Perron et al. 1985), pDG315 (Kang et al. 1991), pNKY51 (Alani et al. 1987), pJA6 (Morrison et al. 1989), pDG344 and pDG82 (this study).


M13 ori
SUP4-o
Sac II
Eco RI
Hind III
Bam HI
Eco RI
Sal I
Pst I
AMP R
TET S
Pst I
REP
YCpMP2
8.5 kb
URA3
ARS1
Pst I
CEN4
Xho I
Hpa I

2.3.1 Advantages of the *SUP4*-o System

There are several advantages to using the *SUP4*-o gene as a mutational target. First, simple genetic methods can be used to isolate mutations occurring in this gene (Pierce et al. 1987). Second, the transcriptional promoter is located within *SUP4*-o and only the gene itself, the first sixty 5′ and the first seven 3′ flanking base pairs are necessary for normal expression *in vivo* (Hall et al. 1982; Shaw and Olson 1984; Allison and Hall 1985). The 5′ base pairs apparently act to fine tune transcription and the 3′ base pairs constitute a transcription termination signal. The small size of *SUP4*-o (89 bp) permits sequencing of the entire gene and essential flanking regions in one operation (Pierce et al. 1987). Third, there are few limitations on the types of DNA sequence change that can occur or on the location within the gene where a mutation can be detected (Kunz et al. 1990b; Kohalmi and Kunz 1992). This is most likely due to the involvement of the various tRNA bases in: 1. pairing to maintain the tRNA secondary and tertiary structures; 2. interactions with RNA pol III, transcription factors, processing enzymes and the ribosome; and 3. codon recognition (Deutscher 1984; Sharp et al. 1985; Bjork et al. 1987). A list of all the single base pair substitutions that can be detected in *SUP4*-o is presented in Table 2. The data are the result of analyzing more than 5,000 spontaneous or induced mutants and using *in vitro* mutagenesis to make all of the substitutions not detected *in vivo* (Kohalmi and Kunz 1992). In addition to single base pair substitutions, tandem and non-tandem double substitutions, single and multiple base pair deletions and insertions, insertions of the yeast transposable element Ty, duplications, and more complex changes have also been detected in *SUP4*-o

Table 2. *SUP4*-o substitutions detected

Site	Change	Site	Change	Site	Change
1G	A C T	31T	A G	61C	T
2A	C G T	32C	A G T	62T	A C G
3G	A C T	33T	A C G	63A	
4A	T	34G	A C T	64G	A C T
5G	A C T	35A	C G T	65C	A G T
6C	A G T	36A	C G T	66C	A G T
7C	A G T	37A	C G T	67C	A G T
8A	C G T	38T	A C G	68G	C T
9T	C	39T	A C G	69C	A G T
10C	A G T	40A		70A	C G T
11G	A C T	41A		71A	C G T
12G	C T	42A		72G	A C T
13T	C*	43T	A	73C	A G
14T	A C G	44A		74T	A C G
15C	A G T	45G		75G	
16A		46T		76A	C T
17A		47G		77G	A C T
18C	A G T	48A		78C	G T
19C	A G T	49T		79G	A C T
20A		50G		80G	C T
21A		51C	A G T	81G	C T
22A		52T		82G	C T
23T	A C G	53T		83G	A C T
24T	A C	54T	C	84C	A G T
25C	A G T	55A	C T	85C	G
26C	A G T	56G	A C T	86C	A G T
27G	A C T	57A	C G T	87T	A G
28C	A	58A	C G T	88C	A G T
29G	C T	59C	A G T	89T	A C G
30T	A G	60T	G		

For simplicity, only the base at each position on the transcribed strand is given. *Identified only by *in vitro* mutagenesis (Kohalmi and Kunz 1992)

(Kunz et al. 1990b). Finally, different mutagenic conditions or treatments or conditions have been found to produce distinctly different *SUP4*-o mutational spectra, reflecting the specific types of DNA damage and/or mechanisms involved. Consequently, this system is an extremely useful tool for the study of mutational mechanisms.

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 sulfate was added per litre of YPD and the medium was designated YPDA.

C. YT (Miller 1972):

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

When required, ampicillin (100 mg/l) (Sigma) was added after autoclaving and cooling of the medium to 45°C. The medium was then designated YT+amp. Medium containing twice the concentration of the YT components was designated 2x YT and was used for bacterial transformation.

D. For solid media, 20 g/l agar was added.

2.5 Detection of *SUP4*-o Mutants, Lysine Prototrophs and Canavanine-Resistant Mutants

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 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 were not detected in the plasmid copy of *SUP4*-o from mutants isolated using less stringent selection methods (Kunz et al. 1987). Third, 178 different substitutions have been 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. 1990b; Kohalmi and Kunz 1992) (see Table 2).

Reversion of *lys2-1* in MKP-o and JMT48A 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 suppression of *lys2-1* gives rise to white, Lys^+ colonies.

Forward mutation at the *CAN1* locus in KRMC and KRJC was detected by selecting for canavanine-resistant 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 (MKP-op, RDG3-p, RDG31-p, HR36-p, FY318-p, FY352-p) were grown from low titre inocula (33 cells per ml) to stationary phase (ca. 1 X 10^7 cells/ml, as determined by a Coulter counter) in uracil omission medium at 30°C with shaking. Cell suspensions were diluted when necessary and plated on uracil omission medium to measure viability, on supplemented minimal medium to determine plasmid retention, and on uracil omission medium containing 30 mg/l canavanine sulfate (Sigma) to select canavanine-resistant colonies.

To isolate Lys^+ colonies, MKP-o or JMT48A were inoculated (33 cells per ml) in supplemented minimal medium and grown at 30°C with shaking to stationary phase. Cell suspensions (125 ml) were centrifuged (3,020 x g, 5 min, 4°C) to pellet the cells which were then resuspended in 20 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.

To isolate canavanine-resistant mutants, KRMC and KRJC were grown from low titre inocula (33 cells per ml) to stationary phase (ca. 1 x 10^8 cells per ml, as determined by a Coulter counter) in YPD medium at 30°C with shaking. Cell suspensions were diluted when necessary and plated on appropriately supplemented minimal medium, with or without 30 mg of

canavanine per litre, to select canavanine-resistant colonies or detect viable cells, respectively.

All plates were scored after 6 days incubation at 30°C. For *SUP4*-o mutation, 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.

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

A. Plasmid Retention:

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

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 1991a)

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

r = mutations in the target per round of 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)

f_m = the median mutation frequency

N_m = the median population size at the time of sampling including residual growth (3 generations for all yeast strains used in this study) on canavanine-containing medium (determined microscopically) before growth of non-resistant cells terminates

2.7 DNA Isolation

2.7.1 Large Scale Yeast DNA Preparation

SE buffer:	900 mM sorbitol 100 mM Na_2 EDTA, pH 7.5
Zymolyase:	Zymolyase 100,000 (Seikagaku Kogyo) was dissolved at a concentration of 6 mg/ml in SE buffer.
TE buffer:	50 mM Tris, pH 7.5 20 mM Na_2EDTA, pH 8.0
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:	3 M potassium acetate was adjusted to pH 4.8 with glacial acetic acid.

Total yeast DNA used for hybridization was isolated by a modification of a procedure described by Sherman et al. (1983). Yeast was grown in 50 ml YPD to stationary phase (1-2 x 10^8 cells/ml) and the cells were pelleted by centrifugation (3,020 x g, 5 min, room temperature), and washed with and resuspended in 3 ml SE buffer. Zymolyase (250 μl) was added and the cell suspension was incubated for 45 min at 37°C with gentle shaking. The resulting spheroplasts were pelleted by centrifugation

(755 x g, 5 min, room temperature) and resuspended in 5 ml TE buffer. Sodium dodecyl sulfate (SDS, 500 μl, 10%, w/v) was added, the contents were mixed gently by inversion and the tube was incubated for 30 min at 65°C. Potassium acetate, 1.5 ml, 5M, was added and the suspension was chilled on ice for 30 min. The precipitate was pelleted by centrifugation (34,800 x g, 20 min, 4°C), the supernatant was transferred to a fresh tube, and 14 ml of ice-cold ethanol (95%) was added. The precipitated nucleic acids were pelleted by centrifugation (3,020 x g, 5 min, 4°C), and the pellet was dried and dissolved in 3 ml TE buffer. RNase (150 μl) was added and the suspension was incubated for 30 min at 37°C with shaking. Then, 3 ml isopropanol (room temperature) was added, the solution was mixed by inversion and the DNA pelleted by centrifugation (1,085 x g, 4 min, 4°C). The DNA pellet was dried, dissolved in 300 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 water for 2-3 h. The water was discarded and the glass beads were dried for 2 h at 160°C.
Triton solution:	100 mM NaCl 10 mM Tris, pH 8.0 1 mM Na_2EDTA, pH 8.0 2% (v/v) Triton X-100 1% (w/v) SDS
TE buffer:	See section 2.7.1

The glass-bead technique (modified from: Hoffman and Winston 1987) was used for rapid processing of a large number of samples for bacterial transformation. Putative *SUP4*-o mutants were grown to stationary phase at 30°C with shaking in 7 ml uracil omission medium. Next, the cells were collected by centrifugation (1,850 x g, 10 min, room temperature),

resuspended in 200 μl Triton solution and transferred to a microfuge tube containing 300 mg acid-washed glass beads. Then, TE-saturated phenol and chloroform (100 μl each) was added to the tube which was mixed for 2 min on a vortex mixer and spun in a microfuge (Brinkman 5415C, 5 min, room temperature). Subsequently, 7.5 μl of the upper aqueous layer was transferred to a fresh tube, stored at -20°C and used for bacterial transformation within 4 days of isolation. During the latter half of this study, this step was slightly modified. Approximately, 200 μl of the upper aqueous phase was transferred to a fresh microfuge tube, 200 μl of 95% ethanol was added, the contents were mixed by inversion, and the precipitated nucleic acids were pelleted by centrifugation (Brinkman 5415C, 5 min, room temperature). The pellet was dried by aspiration and dissolved in 75 μl of TE buffer. This allowed samples to be stored at -20°C for longer periods of time before transformation.

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:	7.5 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 2.7.2

To isolate a large number of plasmid DNA samples for DNA sequencing, a modification of the procedure of 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 spun down (Brinkman 5415C, 30 s, room temperature), resuspended in 190 μl GTE buffer and the cell suspension was mixed gently and then transferred to ice. NaOH/SDS (400 μl) 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, 30 min, 4°C) and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated twice more but the centrifugation times were reduced to 20 min. Then, 500 μl isopropanol (room temperature) was added, the contents of the tube were mixed by inversion and the tube was held at room temperature for 10 min. Following centrifugation (Brinkman 5415C, 5 min, room temperature) the nucleic acid pellet was washed with 70% ice-cold ethanol, dried by aspiration and dissolved in 70 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_2EDTA, pH 8.0
GTE buffer:	See section 2.7.3
Lysozyme:	25 mg/ml dissolved in GTE buffer.
NaOH/SDS:	See section 2.7.3
Potassium acetate:	See section 2.7.1
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:	1.3% (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 2.7.2

RNase: See section 2.7.1

Double-stranded plasmid DNA used for probe isolation and HR36 strain construction was prepared by a scaled-up version of the alkaline procedure of Birnboim and Doly (1979). Bacterial cultures were grown overnight in 5 ml YT+amp at 37°C with shaking. Then, 2 ml of overnight culture was transferred to 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 of ice-cold STE buffer, pelleted again, and resuspended in 18 ml GTE buffer. Lysozyme (Boehringer Mannheim), (2 ml) was added and the cell suspension was incubated at room temperature for 10 min and then chilled on ice for 10 min. NaOH/SDS (40 ml) was added and the mixture was chilled on ice for 10 min. Potassium acetate (20 ml) was added and the contents were mixed by inversion and chilled on ice for 10 min. Next, 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 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 for (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 1.3% (w/v) 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, room temperature) and the upper aqueous layer was transferred to a fresh microfuge tube. Following the last extraction, one-third 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 Acids (RNA plus DNA)

STE buffer: See section 2.7.4

NaOH/SDS: See section 2.7.3

Potassium acetate: See section 2.7.4

Bacterial RNA plus DNA was prepared by modifying the latter half of the previous protocol. Nucleic acids were prepared as described in section 2.7.4 through 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. 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, 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 (Brinkman 5415C, 15 min, 4°C), washed with 1 ml ice-cold 70% ethanol, dried by aspiration, dissolved in 500 μl TE buffer, and then stored at -20°C.

2.8 Transformation Procedures

2.8.1 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

the cell suspension was transferred to a sterile microfuge tube containing ca. 7.5 μl yeast DNA that was isolated previously (see section 2.7.2). The tube was chilled on ice for 1 h, heated at 42°C for 2 min and then chilled on ice for 2 min. 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.

2.8.2 Yeast Transformation

TE buffer:	10 mM Tris, pH 7.0 1 mM Na_2EDTA, pH 8.0
PEG:	44% (w/v) polyethylene $glycol_{3350}$ (Sigma) was dissolved in 100 mM lithium acetate and filter-sterilized just prior to use.
Lithium acetate:	100 mM dissolved in TE buffer.

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 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 plastic tube (Sarstedt). The DNA (1 μg) to be transformed was added to the tube and 20 μl of bacterial nucleic acids (3.5 μg/μl) (see section 2.7.5) was added as carrier and the contents of the tube were then mixed gently by tapping. PEG (440 μl) was then added and the tube was incubated at room temperature for 30 min. Dimethyl sulfoxide (final concentration 10% v/v)

was added and the tube was heated at 42°C for 15 min. The cells were then pelleted by centrifugation (1,850 x g, 5 min, room temperature) and washed twice with, and resuspended in, 1 ml sterile water. Aliquots (0.2 ml) of the cell suspension were plated on appropriately supplemented minimal medium to select transformants and the plates were incubated at 30°C for 6 days. Individual 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.

2.9 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* (FPLC pure, Pharmacia). 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 of amberlite (Sigma) 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 -70°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. Distilled H_2O 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 brown bottles 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, 7.8 μl plasmid DNA (0.5 - 1 μg) and 2 μl *Bam*HI in a screw cap microfuge tube and incubating the tube for 30 min at 37°C. The microfuge tube was then heated for 3 min at 100°C and allowed to cool at room temperature for 5 min. Subsequently, 1 μl of RP primer was added. Then the tube was heated at 100°C for 3 min and immediately transferred to ice-water and allowed to cool for 1 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. 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 mixtures and the tubes were spun briefly (Brinkman 5415C) to mix both solutions. The microfuge tubes were immediately transferred to a 46°C waterbath and incubated for 20 min. The reactions were then 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).

2.10 Preparation of DNA Fragments for Strain Construction

2.10.1 Restriction Digests

Enzyme digest:
- 1x restriction enzyme buffer (10x buffer supplied by BRL)
- 1 μg DNA
- 50 units of enzyme per μg of DNA

Stop buffer:
50 mM Na_2EDTA
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:
400 mM Tris
200 mM sodium acetate
10 mM Na_2EDTA
Adjusted to pH 8.0 with glacial acetic acid.
Stored at room temperature.

DNA fragments used in the construction of HR36 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 (BRL) 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 distilled water.

2.10.2 Isolation of DNA Fragments

TE buffer: See section 2.8.2

DNA fragments were isolated from agarose gels by a freeze-squeeze method (Thuring et al. 1975). DNA fragments to be isolated were visualized on a UV transilluminator following ethidium bromide staining of the gels. Segments of the gels containing the desired DNA fragments were cut out, transferred to microfuge tubes (500 μl per tube), and frozen at -20°C overnight. The tubes were centrifuged (Brinkman 5415C, 1.5 h, 4°C) and the supernatant was transferred to a fresh microfuge tube. Then, 2 volumes (1

ml) 2-butanol (room temperature) were added and the contents were mixed by inversion to remove ca. 50% of the H_2O. The aqueous (bottom) layer was transferred to a fresh microfuge tube and extracted once with 200 μl TE-saturated chloroform to remove residual 2-butanol. To precipitate the DNA, 2.5 volumes ice-cold 95% ethanol were added, the contents of the tube were mixed by inversion, and the tube was held at -20°C overnight. Following centrifugation (Brinkman 5415C, 15 min, 4°C), the nucleic acid pellet was washed with ice-cold 70% ethanol, dried by aspiration and dissolved in TE buffer. DNA samples were stored at -20°C.

2.11 Hybridization Analysis

2.11.1 Agarose Gel Electrophoresis

Enzyme digest:	1x reaction buffer 3 or 1x reaction buffer 2 (10x buffer 3 or 2 supplied by BRL) 2.5 μg DNA 100 units of *Eco*RI (BRL) per μg of DNA or 100 units of *Hind*III (BRL) per μg of DNA
Stop buffer:	See section 2.10.1
λ DNA:	See section 2.10.1
Loening's buffer:	See section 2.10.1

Total yeast DNA was digested with *Eco*RI or with *Hind*III for 2.5 h, stop buffer (1/4 of the total reaction volume) was added to terminate the reaction, and the resulting DNA fragments were separated by agarose gel electrophoresis (0.7% agarose (BRL) w/v dissolved in Loening's buffer) at 1 V/cm (a λ DNA size standard was included). The gel was stained with ethidium bromide (0.5 μg/ml), destained for 20 min in distilled water and a picture was taken for later sizing of the bands. The gel was then soaked for 1 h in 0.5 M NaOH and for 1 h 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.

2.11.2 Random Primer DNA Labelling

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

Random Primer Buffer Mixture:

0.6 M	HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethane sulfonic acid])
0.17 M	Tris-HCl
17 mM	$MgCl_2$
33 mM	2-mercaptoethanol
1.33 mg/ml	bovine serum albumin
18 OD_{260}units/ml	oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8

dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA
dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA
dTTP solution: 0.5 mM dTTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA

Stop buffer: 0.2 M Na_2EDTA, pH 7.5

Reaction mix:

15 μl	Random Primer Buffer Mixture
20 μM	dCTP
20 μM	dGTP
20 μM	dTTP
50 μCi	[^{32}P]dATP

Each reaction was prepared in a final volume of 49 μl.

DNA polymerase: 5 U/μl DNA polymerase I Klenow fragment from *E.coli* (BRL). Stored at -20°C.

[^{32}P] dATP: See section 2.9.2

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

[^{32}P]-labelled probes for hybridization were prepared by using a random primer labelling kit (BRL) according to a modification of the protocol supplied by the manufacturer. 25 ng of DNA was denatured and dissolved in 5-20 μl of distilled water in a sterile screw cap microfuge tube by

heating in a boiling water bath for 5 min and then immediately cooling the tube on ice. The reaction mix was added to the tube on ice and the contents of the tube were mixed by gentle tapping. Next, 1 μl DNA polymerase was added and the tube was incubated at 25°C for 2 h. The reaction was stopped by adding 5 μl stop buffer. Successively, 5 μl yeast tRNA (20 mg/ml, Boehringer Mannheim), 7 μl $MgCl_2$ (100 mM), 7 μl sodium acetate (3 M, pH 4.8) and 200 μl ice-cold ethanol (95%) were added. The tube was then held at -60°C for 1 h, the precipitate was pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C) and the supernatant was removed with a Pasteur pipette. Then, 1 ml ice-cold ethanol (70%) was added to the tube which was held at -60°C for 1 h and centrifuged (Brinkman 5415C, 15 min, 4°C). The supernatant was removed with a Pasteur pipette and the pellet was dissolved in 100 μl sterile water. The tube was heated at 100°C for 10 min and then rapidly transferred to ice-water.

2.11.3 Hybridization Procedure

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

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 (See section 2.11.2)
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 according to a modification of the procedures of Tsao et al. (1983), and Maniatis et al. (1989). The dried gel was floated paper-side down on 2X SSC for 30 sec 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). The 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 hybridization solution (20 ml), the random-primer-labelled DNA 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 transferred to 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).

2.11 Statistical Analysis

Chi-square contingency tests (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 simulations were run on a Digital Equipment Corporation VAX/VMS version V4.5 computer located at TRIUMF (Tri-University Meson Factory, University of British Columbia). For both types of test, values of $P < 0.05$ were considered significant.

Chapter A

Specificity of the Yeast *rev3*Δ Antimutator and *REV3* Dependency of the Mutator Resulting from a Defect (*rad1*Δ) in Nucleotide Excision Repair

This work appears in published form elsewhere;

Specificity of the Yeast *rev3*Δ Antimutator and *REV3* Dependency of the Mutator Resulting from a Defect (*rad1*Δ) in Nucleotide Excision Repair.

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Genetics 137: 637-646.

ABSTRACT

The yeast *REV3* gene has been predicted to encode a DNA polymerase specializing in translesion synthesis. This polymerase likely participates in spontaneous mutagenesis, as *rev3* mutants have an antimutator phenotype. Translesion synthesis also may be necessary for the mutator caused by a *RAD1* (nucleotide excision repair) deletion (*rad1*Δ). To further examine the role of *REV3* in spontaneous mutagenesis, we characterized *SUP4*-o mutations that arose spontaneously in strains having combinations of normal or mutant *REV3* and *RAD1* alleles. The largest fraction of the *rev3*Δ-dependent mutation rate decrease was observed for single base-pair substitutions and deletions, although the rates of all mutational classes detected in the *RAD1* background were reduced by at least 30%. Interestingly, inactivation of *REV3* was associated with a doubling of the number of sites at which the retrotransposon Ty inserted. *rev3*Δ also greatly diminished the magnitude of the *rad1*Δ mutator, but not to the *rev3*Δ antimutator level, implicating *REV3*-dependent and independent processes in the *rad1*Δ mutator effect. However, the specificity of the *rev3*Δ antimutator suggested that the same *REV3*-dependent processes gave rise to the majority of spontaneous mutations in the *RAD1* and *rad1*Δ strains.

INTRODUCTION

Spontaneous mutations play a fundamental role in evolution and have been implicated in aging, carcinogenesis and human genetic disease (Harmon 1981; Kirkwood 1989; Cooper and Krawczak 1990; Arber 1991; Drake 1991a; Loeb 1991; Wintersberger 1991; Strauss 1992). They are thought to originate as a consequence of intracellular events including the formation of DNA lesions, the occurrence of errors during DNA replication, repair and recombination, and the movement of transposable elements (Sargentini and Smith 1985; Smith and Sargentini 1985; Ramel 1989; Loeb and Cheng 1990; Drake 1991b; Kunkel 1992; Smith 1992; Amariglio and Rechavi 1993; Ames et al. 1993; Lindahl 1993). Two general strategies have been used in attempts to better understand the mechanisms responsible for spontaneous mutagenesis. The first is to characterize strains having enhanced spontaneous mutation rates. The rationale for this route is that mutator phenotypes are expected to result from defects in genes whose products act to minimize genetic instability. Indeed, such studies have revealed that spontaneous mutations can arise through failure of DNA repair or processes that maintain the accuracy of DNA replication (Sargentini and Smith 1985; Kramer et al. 1989; Smith 1992). The second approach is to isolate antimutator mutants, the aim being to identify genes whose functions are required for spontaneous mutagenesis. A number of these mutants have been recovered, mainly in prokaryotic systems, and where characterized, found to have alterations primarily in genes that encode DNA polymerases (Sargentini and Smith 1985; Smith 1992; Drake 1993 and references therein; Fijalkowska et al. 1993). Evidence has been presented that such T4 bacteriophage and *Escherichia coli* antimutators are very specific and so may influence only particular pathways for error discrimination (Drake

1993; Fijalkowska et al. 1993).

Defects in the *REV3* gene (= *PSO1*, Cassier-Chauvat and Moustacchi 1988) of the yeast *Saccharomyces cerevisiae* confer an antimutator phenotype that was reported to reduce spontaneous reversion of a missense allele, as well as locus reversion and suppression of ochre alleles, by as much as 80% (Cassier et al., 1980; Quah et al. 1980). These results implicated *REV3* in the production of spontaneous base-pair substitutions in yeast. Yet, *REV3* might also be needed for spontaneous deletion, as well as other events, because UV-induced single base-pair frameshifts and forward mutations to auxotrophy were markedly reduced in *rev3* backgrounds (Lemontt 1972; Lawrence and Christensen 1979; Lawrence et al. 1984). Together, these observations suggest that the antimutator caused by *REV3* inactivation might exhibit unusually broad specificity, a feature that might reflect the function of the *REV3* protein. Cloning, sequencing and deletion of the *REV3* gene led to the prediction that it encodes a novel DNA polymerase (Morrison et al. 1989). Recently, Shimizu et al. (1993) isolated a DNA polymerase that is close in size to, and reacts with antibodies against, the *REV3* polypeptide. Since *REV3* is nonessential and distinct from the genes for the three known yeast nuclear DNA polymerases, but is required for mutation induction by a variety of DNA-damaging agents (for summaries, see Lawrence 1982; Henriques and Brendel 1990), it was argued that the putative *REV3* polymerase functions only in translesion synthesis (Morrison et al. 1989). This notion was supported by the demonstration that unlike many genes required for replication of chromosomal DNA (McIntosh 1993), transcription of *REV3* does not exhibit cell cycle regulation (Singhal et al. 1992). We caution, however, that the evidence supporting a role for the *REV3* polymerase in translesion

synthesis is compelling but not conclusive, and other mechanisms cannot be excluded at this point.

Given the proposed role of *REV3*, the antimutator phenotype due to its inactivation might point to translesion synthesis as a major source of spontaneous mutagenesis in yeast. Consistent with this possibility, the *rev3* antimutator was shown to offset the mutator phenotypes associated with defects in yeast genes required for excision (*rad3*), post-replication (*RAD18*) or recombinational (*rad51*) repair of UV-induced DNA damage (Quah et al. 1980). However, the *rev3* antimutator did not reduce the magnitudes of the mutation rates to the same extent in the *rad3*, *rad18* and *rad51* backgrounds, suggesting the existence of *REV3*-dependent and independent processes for the generation of spontaneous mutation. Previously, we demonstrated that the mutator conferred by deleting most of the yeast nucleotide excision repair gene *RAD1* enhances the rates of spontaneous single base-pair substitution and deletion, as well as transposition of the yeast retrotransposon Ty (Kunz et al. 1990). To account for these findings, we hypothesized that the increased rate of single base-pair events might result from error-prone translesion synthesis past persistent spontaneous DNA damage which normally is a substrate for excision repair, and activation of Ty transposition by such damage (Kunz et al. 1990). If so, deletion of *REV3* might be expected to decrease the rate of single base-pair mutation but not Ty movement in the *rad1*Δ background.

In this study, we have determined the specificity of the *rev3*Δ antimutator, whether it influences the *rad1*Δ mutator phenotype, and how the rates of single base-pair events and Ty transposition are affected in a *rev3*Δ *rad1*Δ strain. This was accomplished by DNA sequencing of *SUP4*-o mutations occurring spontaneously in isogenic strains bearing appropriate

combinations of wildtype and mutant *REV3* and *RAD1* alleles. The *rev3Δ* antimutator effect decreased the mutation rate in both the *RAD1* and *rad1Δ* backgrounds with the largest rate reduction in both cases seen for single base-pair events. However, the rates of all types of base-pair substitution were diminished and evidence suggestive of a role for *REV3* in Ty transposition was obtained. Preliminary reports of this work were published recently (Roche et al. 1992; 1993).

MATERIALS AND METHODS

Yeast and bacterial strains and plasmids: Construction of the haploid, repair-proficient yeast strain MKP-o (*MATα*, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3,112*, *his3-Δ200*, *trp1-Δ901*) and KAM1, an isogenic excision repair-deficient (*rad1Δ*) derivative having 70% of the *RAD1* coding sequence deleted, has been described (Pierce et al. 1987; Kunz et al. 1990). *REV3*-deleted (*rev3Δ*) derivatives of MKP-o (RDG3) and KAM1 (RDG31) were constructed by first cloning the 10-kb pJA6 (Morrison et al. 1989) KpnI fragment that carries *REV3* into the KpnI site of a pUC19 (Yanisch-Perron et al. 1985) derivative from which the XbaI site had been eliminated, giving rise to pDG344. pDG344 was then digested with XbaI to delete the entire *REV3* coding sequence (4,512 bp) plus 111 bp and 5 bp of the immediate 5′ and 3′ flanking sequences, respectively. Next, the gel-purified 8-kb pDG344 XbaI fragment containing the *REV3* flanking regions was blunt-end-ligated to the 3.8-kb pDG82 BamHI fragment carrying *URA3* to generate pDG347. [pDG82 was constructed by blunt-end ligation of the 3.8-kb pNKY51 (Alani, et al. 1987) BamHI-BglII hisG-*URA3*-hisG cassette into the XbaI site of pUC18 (Yanisch-Perron et al. 1985). This cassette can be excised from pDG82 with BamHI because the upstream site adjacent to XbaI in pUC18 is BamHI and the cassette was inserted into pUC18 in the orientation BamHI-XbaI/BglII-BamHI/XbaI to reconstitute a BamHI site at the BamHI/XbaI junction.] pDG347 was digested with KpnI to release the 9.2-kb fragment carrying the hisG-*URA3*-hisG cassette sandwiched between the *REV3*-flanking sequences and the digest was transformed (Ito et al. 1983; Schiestl and Gietz 1988; Gietz et al. 1992) into MKP-o and KAM1. Ura^+ transformants that emerged were propagated in uracil omission medium and plated on appropriately supplemented minimal medium containing 5-fluoro-

orotic acid (Sigma Chemical Company, St. Louis, Missouri) to select for loss of the *URA3* gene via crossing-over between the hisG repeats (Alani et al. 1987). The resulting Ura$^-$ isolates were then assayed for UV sensitivity and UV-induced reversion of *lys2-1*. Deletion of *REV3* in reversion-deficient derivatives (RDG3, RDG31) was confirmed by DNA hybridization analysis (data not shown). YCpMP2 was transformed (Ito et al. 1983; Schiestl and Gietz 1988; Gietz et al. 1992) into MKP-o, RDG3, KAM1 and RDG31 to make MKP-op, RDG3-p, KAM1-p and RDG31-p, respectively. The *E. coli* strain JF1754 (*Δlac*, *gal*, *metB*, *leuB*, *hisB436*, *hsdR*) (Pierce et al. 1987) was used to retrieve YCpMP2 from yeast strains. YCpMP2 (Pierce et al. 1987) which carries SUP4-o, an ochre suppressor allele of a yeast tyrosine tRNA gene, is a yeast centromere-containing vector having components that enable it to replicate autonomously in yeast and bacterial cells. In yeast, such plasmids mimic chromosome behaviour, replicating once per cell cycle in S phase and residing predominantly as single copies in haploid cells (Newlon 1988). YCpMP2 also bears the yeast *URA3* gene which permits selection for the plasmid via complementation of the *ura3-52* allele present in MKP-o, KAM1, RDG3 and RDG31.

Media: The media used with yeast or bacteria were prepared as described (Pierce et al. 1987; Boeke et al. 1984; Kunz et al. 1991).

Detection of *SUP4*-o mutations: Forward mutations in *SUP4*-o were detected via reduced suppression of ochre alleles which confer resistance to the arginine analog canavanine (*can1-100*), red pigmentation (*ade2-1*) or lysine auxotrophy (*lys2-1*) (Pierce et al. 1987). Selection for diminished suppression of all three alleles detects at least a 30% decrease in functional suppressor tRNA (Wang and Hopper 1988) and is unlikely to bias mutant recovery significantly (Kohalmi and Kunz 1992).

Mutant isolation, plasmid retention, mutation frequency and mutation rate: Spontaneous *SUP4*-o mutations were isolated and plasmid retention and mutation frequencies determined as described (Pierce et al. 1987; Kunz et al. 1991). Mutation rates were calculated according to the formula (Drake 1991a) μ_o = (0.4343Cf) / log ($N\mu_o$); where μ_o = mutations in the target per DNA replication; 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)]; f = median mutation frequency and N = median final population size (*REV3 RAD1*: 2.62 x 10^8; *rev3Δ RAD1*: 2.34 X 10^8; *REV3 rad1Δ*: 2.28 x 10^8; *rev3Δ rad1Δ*: 2.59 X 10^8) including three generations on the selection plates (determined microscopically) before growth of canavanine-sensitive cells terminates. This formula corrects for spontaneous mutations which occur but are not detected by the system, and its use accounts for the differences in the rates calculated here and those published previously (Kunz et al. 1990). Plasmid retentions and mutation frequencies and rates were calculated from new data obtained for all four strains used in this study.

DNA isolation, bacterial transformation and DNA sequencing: DNA for bacterial transformation was released from yeast cells by disruption with glass beads (Mis and Kunz 1990). Plasmid DNA was isolated from *E. coli* by alkaline extraction (Kohalmi et al. 1991). Bacterial cells were transformed using $CaCl_2$ (Pierce et al. 1987). *SUP4*-o alleles were sequenced on double-stranded YCpMP2 molecules, using dideoxynucleotides, as described (Kunz et al. 1987).

Statistical analysis: Chi-square tests employing Yates' correction for continuity (Sokal and Rohlf 1969) were used to evaluate differences in a variety of parameters. The significance of differences in the distributions of single base-pair substitutions within the *SUP4*-o gene was assessed using the Monte Carlo estimate of the P value of the hypergeometric test (Adams and Skopek 1987) [1,500 simulations were run for each comparison]. For both tests, values of $P < 0.05$ were considered significant.

RESULTS

Plasmid retention and spontaneous mutagenesis: The yeast strains (MKP-op: *REV3 RAD1*; RDG3-p: *rev3Δ RAD1*; KAM1-p: *REV3 rad1Δ*; RDG31-p: *rev3Δ rad1Δ*) used in this study are isogenic except for the *REV3* and *RAD1* loci. Hence, any differences observed among them should be due either to the *rev3Δ* antimutator, the *rad1Δ* mutator, or the combination of the two, rather than variation in genetic background. Since *SUP4*-o is carried on the yeast centromere plasmid YCpMP2, cultures of the four strains were grown from low titre inocula to stationary phase in medium selective for the plasmid and then plated to assess the stability of YCpMP2 and isolate *SUP4*-o mutations. Plasmid retention was measured by comparing the number of colonies that formed on selective medium with the corresponding value for nonselective medium. Approximately 89% of the *rev3Δ RAD1* and *rev3Δ rad1Δ* cells carried YCpMP2 (Table 1). This value was similar to those for the wildtype (86%) and *REV3 rad1Δ* (87%) strains indicating that the *REV3* deletion, alone or together with the *rad1Δ* allele, did not alter plasmid stability. The *rev3Δ* antimutator reduced the frequency of spontaneous mutation in the repair-proficient and *rad1Δ* backgrounds by 67% and 83%, respectively (Table 1). These decreases corresponded to mutation rate reductions of 60% for *rev3Δ RAD1* compared to *REV3 RAD1* and 78% for *rev3Δ rad1Δ* relative to *REV3 rad1Δ*. Interestingly, in *rev3Δ rad1Δ* the mutation rate was not lowered to the *rev3Δ* antimutator level but was closer to that for *REV3 RAD1*.

Characterization of *SUP4*-o mutations: The specificity of the *rev3Δ* antimutator and the influence of this antimutator on the specificity of the *rad1Δ* mutator were determined by DNA sequencing. To ensure that the spontaneous mutations examined arose independently, only two mutants were

chosen from any one culture. If both members of a pair were subsequently found to have the same sequence alteration, the result for only one was counted. A total of 660 *SUP4*-o mutations were isolated in parallel from *REV3 RAD1*, *rev3*Δ *rad1*Δ and *rev3*Δ *rad1*Δ and characterized. The sequencing data for 249 *SUP4*-o mutations selected in *REV3 rad1*Δ were published previously (Kunz et al. 1990) but are presented here for ease of comparison. The numbers of mutations that were rejected as potential siblings are as follows: *REV3 RAD1*: 3/231 (each at different sites); *rev3*Δ *RAD1*: 12/213 (at 10 different sites); *REV3 rad1*Δ: 11/260 (at 9 different sites); *rev3*Δ *rad1*Δ: 14/216 (at 12 different sites). This did not result in a systematic underestimation of the mutational hotspots because only a very small fraction of the total mutations sequenced were discarded, no more than two mutations at the same site were rejected, and the majority of rejected mutations occurred at different sites.

All seven classes of mutation detected in *REV3 RAD1* also were recovered in *rev3*Δ *RAD1* and their relative fractions were similar in the two strains (Table 2). In contrast, comparison of the data for *REV3 rad1*Δ and *rev3*Δ *rad1*Δ indicated that only four of the nine mutational classes detected were common to both strains (Table 2). As well, the relative fraction of single base-pair substitutions was reduced from the value for the *rad1*Δ mutator to a value close to that for *rev3*Δ *RAD1* ($P < 0.005$). Although the proportion of insertions of the yeast retrotransposon Ty appeared to increase slightly in *rev3*Δ *RAD1* and *rev3*Δ *rad1*Δ relative to *REV3 RAD1* and *REV3 rad1*Δ, respectively, the changes were not significant ($P >$ at least 0.05 in both cases). Decreases in the rates of single base-pair substitutions (which are examined in more detail below) and deletions in the *RAD1* and *rad1*Δ backgrounds accounted for 89% of the mutation rate

reduction attributable to the *rev3Δ* antimutator. This value was increased to 99% for *rev3Δ radl1Δ* when the ca. two-thirds reduction in the rate of Ty insertions in the former strain also was taken into account (Table 2). The rates of the other types of mutation recovered (see Table 3 for descriptions) in both *REV3 RAD1* and *rev3Δ RAD1* (tandem base-pair substitutions, multiple base-pair deletions, single base-pair and Ty insertions, complex changes), or *REV3 radl1Δ* and *rev3Δ radl1Δ* (multiple base-pair deletions), also were diminished in the *rev3Δ* strains (Table 2). Yet, either the decrease was small, or relatively few mutations were detected, making the significance of the reductions alone uncertain. We note, however, that Ty inserted at two to three times as many target sites in *rev3Δ RAD1* (7) and *rev3Δ radl1Δ* (6) as in *REV3 RAD1* (3) and *REV3 radl1Δ* (2) (Table 3). In addition, the fraction of Ty insertions at the previously reported (Giroux et al. 1988) transposition hotspot 5′ (with respect to the nontranscribed strand) to position 38 was reduced from 85% to 56% in *rev3Δ RAD1* relative to *REV3 RAD1* ($P < 0.01$). A lesser decline (82% to 73%) was observed for the *radl1Δ* strains but it was not significant ($P < 0.7$).

Single base-pair substitutions: All six possible types of base-pair change were recovered in each of the four strains (Table 4). Generally, for *REV3 RAD1* compared to *rev3Δ RAD1* and *REV3 radl1Δ* compared to *rev3Δ radl1Δ*, the relative fractions of the base-pair substitutions were similar but somewhat more A•T → G•C transitions ($P < 0.001$) and fewer G•C → C•G transversions ($P < 0.005$) were detected in *rev3Δ radl1Δ* than *REV3 radl1Δ*. Other apparent fluctuations in relative proportions were not significant ($P >$ at least 0.05 in each case). On the other hand, deletion of *REV3* appeared to reduce the rates of all classes of base-pair change by

ca. 50% or more regardless of the excision repair capability of the strain. The numbers of transversions detected at A•T pairs were relatively small. However, the rate decreases for A•T → C•G transversions in *rev3ΔRAD1* and *rev3Δ radlΔ* and A•T → T•A events in the latter strain were very large (≥90%) and the A•T → T•A transversion rate in *rev3Δ RAD1* was lowered by two-thirds. The magnitudes of these reductions make it seem reasonable to think that the occurrence of transversions at A•T pairs also was affected by the *rev3Δ* antimutator. The magnitudes of the total rate decreases (expressed as percents) were similar for transitions vs. transversions and changes at G•C pairs vs. A•T pairs in the *RAD1* or *radlΔ* backgrounds.

The distributions of single base-pair substitutions within *SUP4*-o are compared in Figure 1. In total, base-pair changes were detected at 61 different sites with similar numbers of positions mutated in each strain (*REV3 RAD1*: 48; *rev3Δ RAD1*: 44; *REV3 radlΔ*: 49; *rev3Δ radlΔ*: 47). For *REV3 RAD1* vs. *rev3Δ RAD1*, 58 different sites were mutated with only 34 (59%) common to both distributions, 14 (24%) detected solely in *REV3 RAD1* and 10 (17%) just in *rev3Δ RAD1*. Only three of the six most frequently mutated positions (6, 18, 27, 51, 58, 72) in the two strains overlapped, and the pattern of substitutions at one of these positions (27) was reversed in the two strains. For *REV3 radlΔ* vs. *rev3Δ radlΔ*, a total of 56 sites were mutated with 40 (71%) common to both strains, nine (16%) recovered just in *REV3 radlΔ* and seven (12%) solely in *rev3Δ radlΔ*. Only one of the seven sites mutated most frequently in these two strains (*REV3 radlΔ*: 18, 27, 32, 65, 88; *rev3Δ radlΔ*: 18, 72, 89) overlapped. Statistical evaluation of the distributions of substitutions (Adams and Skopek 1987) for these two comparisons suggested that the probability of random sampling error being

responsible for any differences was less than 1 in 500 (the upper limit of the 90% confidence interval on the estimate of *P* was 0.002).

In the *RAD1* background, the *rev3*Δ antimutator decreased the mutation rate by 70% on average (range: 44-94%) at 26 of the 34 common sites. However, the rate was increased by about two to three-fold at two sites (6, 37) and apparently not affected (less than a 40% decrease or a two-fold increase) at six sites (7, 14, 15, 72, 80, 84). For the *rad1*Δ strains, the mutation rate was reduced by 78% on average (range: 46-95%) at 36 of the 40 common sites but did not appear to change at the remaining four (54, 69, 72, 89). The influence of the *rev3*Δ antimutator on the mutation rates, therefore, was similar in the *RAD1* and *rad1*Δ backgrounds [although the *rev3*Δ antimutator seemed to diminish the mutation rate at more sites in the *rad1*Δ strain, the difference was not significant ($P > 0.1$)]. The comparisons suggest, however, that in both cases there was considerable site-to-site variation in the magnitude of the antimutator effect. Thus, DNA sequence context might have influenced the *REV3*-dependent production of spontaneous base-pair substitutions.

DISCUSSION

The yeast *REV3* gene has been proposed to encode a DNA polymerase required for translesion synthesis (Morrison et al. 1989), and there is preliminary evidence that the *REV3* protein has DNA polymerase activity (Shimizu et al. 1993). On this basis, the fact that *rev3* defects can reduce spontaneous mutation rates to one-fifth of normal (Cassier et al., 1980; Quah et al. 1980) suggests a potential role for translesion synthesis in spontaneous mutagenesis. If so, the *rev3Δ* antimutator might be expected to affect a wider assortment of mutational classes than reported previously for other antimutators (Drake 1993 and references therein; Fijalkowska et al. 1993), since it is well-known that DNA damage can give rise to a variety of DNA sequence alterations. Consequently, one aim of this study was to characterize the specificity of the *rev3Δ* antimutator. We anticipated that identifying the mutational classes whose rate was diminished by the antimutator would allow us to pinpoint the types of mutations produced spontaneously by *REV3*-dependent processes.

Initially, we determined that deletion of *REV3* decreased the rate of spontaneous forward mutation in a plasmid-borne copy of the yeast *SUP4*-o gene by 60%. This result was consistent with those observed by other investigators (Cassier et al., 1980; Quah et al. 1980) for spontaneous reversion at chromosomal loci and so, taken collectively, these findings argue that the majority of spontaneous mutagenesis in yeast is *REV3*-dependent. Since the entire *REV3* coding sequence was removed in the *rev3Δ* strains we used, the residual mutagenesis detected at *SUP4*-o could not have been due to leakiness of this *rev3Δ* mutation. Thus, we favor the interpretation that *REV3*-independent mutational mechanisms can account for

as much as 40% of spontaneous mutagenesis in yeast.

DNA sequence analysis of spontaneous *SUP4*-o mutations arising in *REV3 RAD1* and *rev3Δ RAD1* revealed that the *rev3Δ* antimutator was associated primarily with decreases in the rates of single base-pair substitutions and deletions. We emphasize, however, that all of the other mutational classes detected in *REV3 RAD1* also were found in *rev3Δ RAD1* but at reduced rates. Whether this indicates a very broad specificity for the *rev3Δ* antimutator or not is difficult to say. Either the rate reductions for these other events were small, or few mutations were recovered. Still, there were additional clues that at least single base-pair addition (see below) and Ty transposition also might be influenced by *REV3* activity. Ty elements inserted at twice as many *SUP4*-o positions in *rev3Δ RAD1* (7) as in *REV3 RAD1* (3), and the fraction detected at the Ty insertion hotspot within *SUP4*-o was significantly reduced. How this might result from inactivation of *REV3* is not obvious but there is evidence that spontaneous DNA damage can enhance Ty transposition (Kunz et al. 1993). Perhaps lack of *REV3*-dependent processing of such damage might somehow be involved. Interestingly, alteration of Ty target site distribution also has been observed for deletion of the *RAD6* ubiquitin conjugase gene (Kang et al. 1992; Liebman and Newnam 1993). *RAD6* and *REV3* belong to the same epistasis group for UV sensitivity (Lawrence and Christensen 1976), and it has been suggested that *RAD6* might influence the function of other members of this group via ubiquitination of their gene products (Sung et al. 1990). If so, then one explanation for the effect of deleting *RAD6* on the target site specificity of Ty insertion might be that this specificity is at least partly linked to the activity of the *REV3* protein. Even so, it is clear that *RAD6* must also affect transposition of Ty in some other way because,

in contrast to the results obtained in this study, the *rad6* deletion was associated with a large (>17-fold) increase in the rate of Ty insertion into *SUP4*-o (Kang et al. 1992).

With regard to base-pair substitutions, the *rev3*Δ antimutator did not seem to exhibit any distinct specificity as the rates of all six possible types of change were lowered by at least 50%. This suggests that *REV3*-dependent processes can generate each kind of spontaneous base-pair substitution but, for each change, there also must be other mutational mechanisms at work. One strong possibility is the failure to correct replication errors. A defect in proofreading by yeast DNA polymerase δ can enhance the rates of all six substitutions in *SUP4*-o *in vivo* (Ramachandran et al. 1993). The *REV3*-dependent production of base-pair changes likely is affected by DNA sequence context as there seemed to be considerable site-specific variation in the magnitude of the *rev3*Δ antimutator. Indeed, the mutation rates appeared to remain unchanged or actually increase at some positions within *SUP4*-o. If the putative *REV3* polymerase functions in translesion synthesis, then sequence context might have exerted an influence by modulating either the occurrence of spontaneous DNA damage, its removal by error-free repair or the ability of the polymerase to synthesize past spontaneous lesions.

The *rev3*Δ antimutator also diminished the rate of single base-pair deletions. With the exception of two events in *REV3 RAD1*, and one each in *REV3 rad1*Δ and *rev3*Δ *rad1*Δ, the events detected occurred in monotonic runs of two or more base-pairs (Table 3). This observation, plus the fact that the largest number of deletions in *REV3 RAD1* and *rev3*Δ *RAD1* took place in the longest such run in *SUP4*-o (79 → 83), suggests that the deletion mechanism might have involved strand slippage during DNA replication

through the runs (Streisinger et al. 1966). If so, the *rev3*Δ-dependent rate reduction for base-pair loss (54%) in these runs would seem to implicate the *REV3* protein in the production of about half of the spontaneous single base-pair deletions resulting from such strand misalignments. Although relatively few single base-pair additions were detected, their rate was reduced (by 42%) in *rev3*Δ *RAD1* and they all occurred in base-pair runs, which points to a strand slippage mechanism generating these events as well. Together, these findings suggest that the *REV3* gene product also might participate in spontaneous base-pair addition.

Inactivation of *REV3* influenced a wider range of mutational classes than observed previously for prokaryotic antimutators. This response is consistent with a potential role for replicative bypass of DNA damage in spontaneous mutagenesis, given that the *REV3* polymerase might be responsible for translesion synthesis. Yet, whether the *REV3* gene product actually catalyzes translesion synthesis, and indeed, the precise function of *REV3* in mutagenesis, remains to be established. Thus, although one can begin to imagine how certain mutations might arise during replication past DNA damage, it would be premature to attempt to model mechanistic links between our observations and *REV3* function.

The second goal of this study was to assess the effect of the *rev3*Δ antimutator on the mutator phenotype conferred by a deletion in the *RAD1* gene. *RAD1* is required for the incision step of excision repair (Friedberg 1988) and its inactivation specifically increases the rates of spontaneous single base-pair substitution, single base-pair deletion and Ty insertion (Kunz et al. 1990). We have suggested that this specificity might reflect error-prone translesion synthesis past unrepaired spontaneous lesions, and

promotion of Ty transposition by such damage (Kunz et al. 1990). Consistent with this hypothesis, the *rev3*Δ antimutator reduced the magnitude of the *rad1*Δ mutator by 78%. Contrary to expectation, however, the rate of Ty transposition also was lowered and insertion of Ty elements were detected at more sites. Thus, the effect of the *rev3*Δ antimutator on Ty insertion in *rev3*Δ *rad1*Δ was essentially the same as in the *RAD1* background. This also was true for the base-pair changes. Again, the rates of all six types of substitution were diminished and the relative magnitudes of the decreases among the substitution rates for *rev3*Δ *rad1*Δ were comparable to those for *rev3*Δ *RAD1*. Overall, these similarities suggest that the bulk of the *rad1*Δ mutator effect was due to the same *REV3*-dependent processes that produced the majority of spontaneous *SUP4*-o mutations in *REV3 RAD1*.

The mutation rate in *rev3*Δ *rad1*Δ was not lowered to the level observed for the *rev3*Δ antimutator alone, instead it was about two-fold higher. By comparing the types of sequence alterations detected in both *rev3*Δ *RAD1* and *rev3*Δ *rad1*Δ, it can be determined that this difference was due mainly to rate increases in the three events (single base-pair substitution, single base-pair deletion, Ty insertion) associated with the *rad1*Δ mutator. This indicates that the mutator also can promote these three mutational classes, although to a lesser extent, by a *REV3*-independent mechanism(s). The mechanism(s) in question would not necessarily have to be distinct from translesion synthesis. Calf thymus DNA polymerase δ, in the presence of proliferating cell nuclear antigen, can replicate past UV-induced cyclobutane dimers *in vitro* (O'Day et al. 1992). Consequently, it might be that eukaryotic DNA polymerases responsible for replication of chromosomal DNA, including those in yeast,

also are capable of translesion synthesis past some fraction of spontaneous DNA damage. Alternatively, some damage might be processed by *REV3*-independent error-prone repair.

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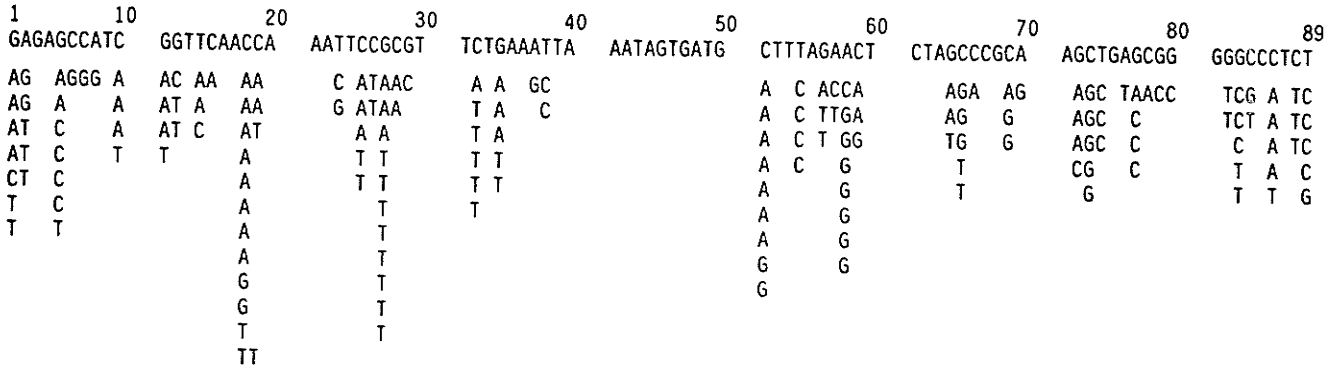
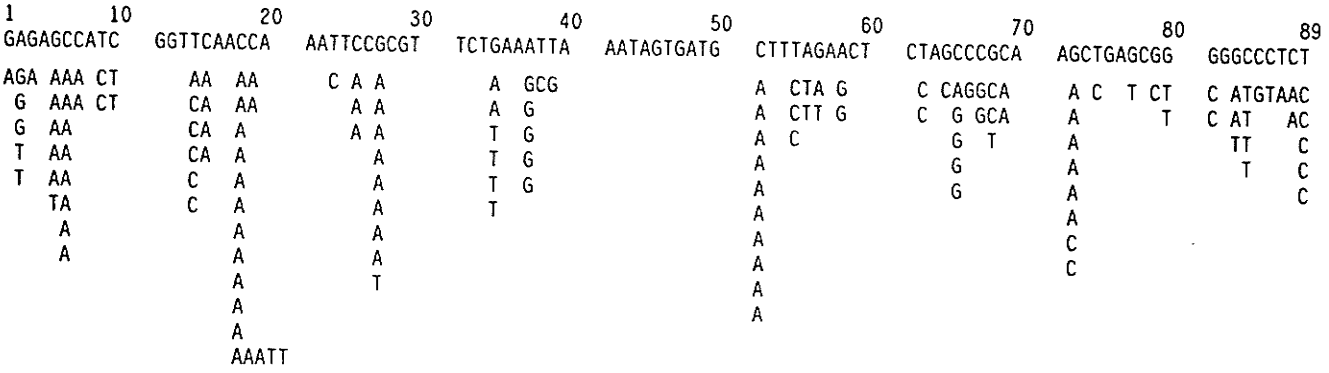
FIGURE LEGEND

FIGURE 1.-Distributions of single base pair substitutions in *SUP4*-o. For simplicity, only the region of the transcribed strand encoding the tRNA is shown. The anticodon is at 36 to 38 and the 14-base-pair intron is inferred to extend from 40 through 53 as for the sup4$^+$ allele (Knapp et al. 1978). The strains in which the mutations were isolated (*REV3 RAD1*; *rev3*Δ *RAD1*; *REV3 rad1*Δ; *rev3*Δ *rad1*Δ) are given above the transcribed strand for each distribution. Identical changes at individual sites represent independent events.

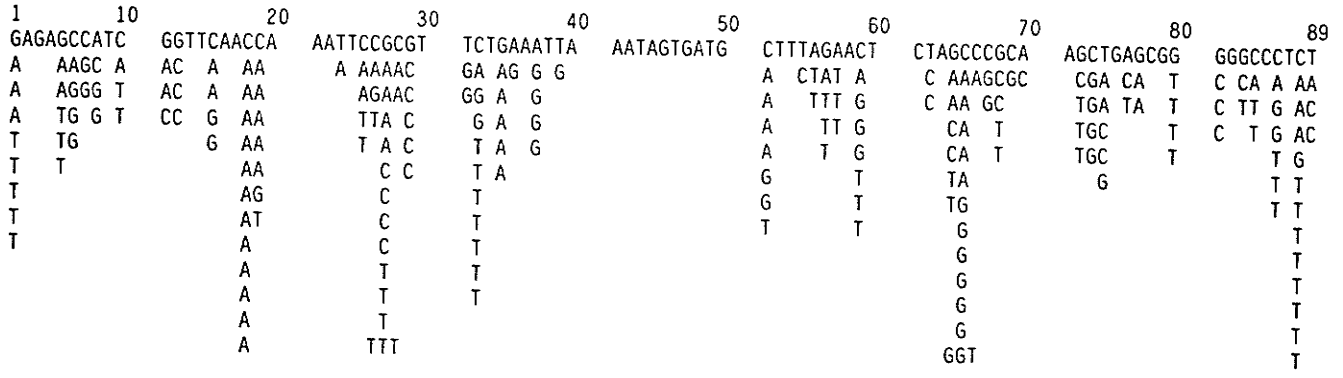
REV3 RAD1



rev3Δ RAD1



REV3 rad1Δ



rev3Δ rad1Δ

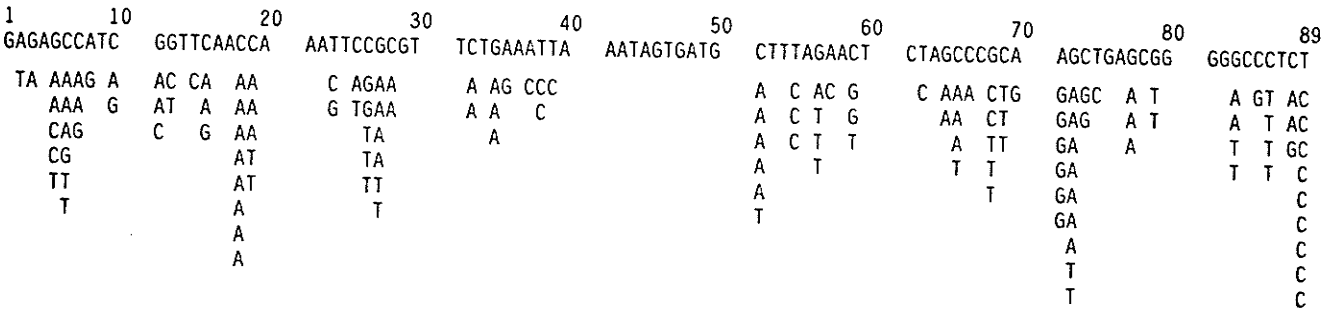


TABLE 1. Plasmid retention and mutation frequencies and rates

Strain	Colonies on selective medium	Colonies on nonselective medium	Percent plasmid retention	Mutation frequency[a] ($\times 10^{-6}$)	Mutation rate ($\times 10^{-7}$)
MKP-op (*REV3 RAD1*)	10,879	12,599	86.3	2.7	7.1
RDG3-p (*rev3Δ RAD1*)	7,743	8,748	88.5	0.9	2.9
KAM1-p (*REV3 rad1Δ*)	9,287	10,694	86.8	13.7	28.3
RDG31-p (*rev3Δ rad1Δ*)	9,678	10,799	89.6	2.3	6.2

[a] Frequencies are the medians for 30 (MKP-op), 26 (KAM1-p) or 20 (RDG3-p, RDG31-p) independent cultures.

TABLE 2. Sequence alterations in *SUP4*-o mutants

Alteration	MKP-op (*REV3 RAD1*)			RDG3-p (*rev3Δ RAD1*)			KAM1-p (*REV3 rad1Δ*)			RDG31-p (*rev3Δ rad1Δ*)		
	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})
Substitution												
Single bp	174	(76.3)[a]	54.5	147	(73.1)	21.3	211	(84.7)	239.7	146	(72.3)	45.2
Tandem bp[b]	2	(0.9)	0.6	1	(0.5)	0.1	1	(0.4)	1.1	-		
Non-tandem bp	-			-			1	(0.4)	1.1	-		
Deletion												
1 bp	25	(11.0)	7.8	23	(11.4)	3.3	16	(6.4)	18.2	19	(9.4)	5.9
>1 bp	5	(2.2)	1.6	2	(1.0)	0.3	2	(0.8)	2.3	-		
Insertion												
1 bp	4	(1.7)	1.2	5	(2.5)	0.7	-			5	(2.5)	1.5
>1 bp	-			-			1	(0.4)	1.1	1	(0.5)	0.3
Ty element	13	(5.7)	4.1	18	(9.0)	2.6	17	(6.9)	19.3	26	(12.8)	8.0
Complex change	5	(2.2)	1.6	5	(2.5)	0.7	-			5	(2.5)	1.5
Total	228		71.4	201		29.0	249		282.8	202		62.4

[a]Percent of total events.

[b]One tandem event in the *REV3 RAD1* strain featured a triple substitution.

TABLE 3. Multiple mutations, deletions and insertions

Sites	Change[a]	Number detected			
		MKP-op	RDG3-p	KAM1-p	RDG31-p
		REV3 RAD1	*rev3Δ RAD1*	*REV3 rad1Δ*	*rev3Δ rad1Δ*
-6 ↔ -5[b]	Ty	-	-	-	1
-2, 18	A → T, C → A	-	-	1	-
5, 6 → 7	G → A, -1	2	-	-	-
6 → 7	-1	1	-	-	-
6 ↔ 7	Ty	1	2	3	2
7 ↔ 8	Ty	-	2	-	-
17 ↔ 18	Ty	1	-	-	1
24	-1	1	-	-	-
24 → -144[c]	-168	-	1	-	-
25 → 26	-1	-	-	1	-
26, 27	C → T, G → T	-	-	1	-
27, 40 → 42	G → A, +1	-	-	-	1
28 ↔ 29	Ty	-	1	-	-
29	-1	-	-	1	-
31 ↔ 32	Ty	-	1	-	-
35, 36	A → G, A → G	1	-	-	-
35 → 37	+1	-	2	-	-
37 ↔ 38	Ty	11	10	14	19
38 ↔ 39	Ty	-	1	-	-
45 <-> 46	Ty	-	-	-	2
46 → -106[d]	-152	2	-	-	-
50 ↔ 52	Ty	-	-	-	1
51 ↔ 52	Ty	-	1	-	-

TABLE 3 (continued)

Sites	Change[a]	Number detected			
		MKP-op	RDG3-p	KAM1-p	RDG31-p
		REV3 RAD1	*rev3Δ RAD1*	*REV3 rad1Δ*	*rev3Δ rad1Δ*
51, 52, 53	C→A, T→G, T→C	1	-	-	-
55 → -559[e]	-614	1	-	-	-
58 → 64	-7, +GGGCC	3	5	-	4
62 ↔ 63	+2	-	-	-	1
64 ↔ 65	+2	-	-	1	-
65 → 67	-1	-	-	4	-
69	-1	-	-	-	1
74	-1	1	-	-	-
74 → -536[f]	-613	-	1	-	-
77 → 79[g]	-3	-	-	2	-
79, 80	G → T, G → A	-	1	-	-
79 → 83	-1	22	22	8	17
79 → 83	+1	4	3	-	5
84 → 86	-1	-	1	2	1
88 → 96[g]	-9	2	-	-	-

[a]Changes are given for the transcribed strand (see Fig. 1).

[b]The symbol ↔ indicates that Ty is presumed to have inserted between the two sites.

[c-g] For multiple base-pair deletions the sites are estimates because repeats (c: 5 bp; d: 6 bp - imperfect; e: 6 bp; f: 3 bp; g: 1 bp) are present at the deletion termini.

TABLE 4. Single base-pair substitutions

Substitution	MKP-op (*REV3 RAD1*)		RDG3-p (*rev3Δ RAD1*)		KAM1-p (*REV3 rad1Δ*)		RDG31-p (*rev3Δ rad1Δ*)	
	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)
Transitions								
G·C → A·T	44 (25.3)[a]	13.8	35 (23.8)	5.1	52 (24.7)	59.1	44 (30.1)	13.6
A·T → G·C	27 (15.5)	8.4	30 (20.4)	4.3	14 (6.6)	15.9	28 (19.2)	8.7
Total	71 (40.8)	22.2	65 (44.2)	9.4	66 (31.3)	75.0	72 (49.3)	22.3
Transversions								
G·C → T·A	58 (33.4)	18.2	60 (40.8)	8.8	72 (34.1)	81.8	52 (35.6)	16.1
G·C → C·G	34 (19.5)	10.6	16 (10.9)	2.3	56 (26.6)	63.6	18 (12.3)	5.6
A·T → C·G	4 (2.3)	1.3	1 (0.7)	0.1	7 (3.3)	7.9	3 (2.1)	0.9
A·T → T·A	7 (4.0)	2.2	5 (3.4)	0.7	10 (4.7)	11.4	1 (0.7)	0.3
Total	103 (59.2)	32.3	82 (55.8)	11.9	145 (68.7)	164.7	74 (50.7)	22.9

[a]Percent of total substitutions.

Chapter B

Mutator Phenotypes Conferred by Inactivation of the *Saccharomyces cerevisiae* *RAD6*, *RAD18* or *RAD52* Genes are Largely Dependent on the Function of the *REV3* Gene Product, a Putative DNA Polymerase

This manuscript is prepared for publication in the form outlined for the Journal of Bacteriology:

Mutator Phenotypes Conferred by Inactivation of the *Saccharomyces cerevisiae* *RAD6*, *RAD18* or *RAD52* Genes are Largely Dependent on the Function of the *REV3* Gene Product, a Putative DNA Polymerase

Hazeline Roche, R. Daniel Gietz, and Bernard A. Kunz

ABSTRACT

rad6, *rad18*, and *rad52* mutants of the yeast *Saccharomyces cerevisiae* are defective in the repair of induced DNA damage and have distinct mutator phenotypes. All three mutators can be offset by inactivation of the *REV3* gene, which appears to encode a DNA polymerase predicted to specialize in translesion synthesis. Thus, DNA replication past unrepaired spontaneous damage may contribute to the *rad6*, *rad18*, and *rad52* mutators. In order to assess the extent of the *REV3* dependence of the rad mutator phenotypes, we have characterized spontaneous *SUP4*-o mutation in isogenic strains having combinations of normal or mutant *REV3* and *RAD6*, *RAD18*, or *RAD52* alleles. At the level of total *SUP4*-o mutation rates, the *rad6* and *rad18* mutators relied largely, but not exclusively, on *REV3* whereas the *rad52* mutator was entirely *REV3*-dependent. A more detailed analysis revealed that the individual contributions of *REV3*-dependent and *REV3*-independent components to the specificity of the *rad6* mutator differed markedly depending on the mutational class examined. The influence of *rev3* on the production of G•C → T•A transversions by the *rad18* mutator, which induces only these substitutions, was similar to that for G•C → T•A transversion in the *rad6* background. This result is consistent with a role for the Rad6-Rad18 protein complex in the control of spontaneous mutagenesis. Taken collectively, the available data suggest that the *REV3* gene product may be involved in processing the majority of spontaneous DNA lesions left unrepaired by error-free systems.

INTRODUCTION

DNA lesions can arise naturally as a consequence of intracellular metabolism and the instability of DNA. For example, there is evidence consistent with spontaneous alkylation, deamination, and elimination of cellular DNA bases, as well as their modification by reactive oxygen species (Loeb and Preston 1986; Ames et al. 1993; Lindahl 1993; Marnett and Burcham 1993). The observation that defects in genes required for various modes of DNA repair can confer mutator phenotypes (Haynes and Kunz 1981; Sargentini and Smith 1985; Ramotar et al. 1991; Rebeck and Samson 1991; Michaels and Miller 1992; Smith 1992; Xiao and Samson 1993; Kunz et al. 1994; Mackay et al. 1994) points to a significant role for cellular responses to DNA damage in the control of spontaneous mutation rates. Regulation of these rates is important because spontaneous mutagenesis plays a fundamental part in evolution, and has been implicated in aging, carcinogenesis, and human genetic disease (Harmon 1981; Kirkwood 1989; Cooper and Krawczak 1990; Arber 1991; Drake 1991; Loeb 1991; Wintersberger 1991; Caskey et al. 1992; Strauss 1992). Thus, the relationships between the mechanisms that either repair naturally-occurring DNA lesions or process them to mutations are of considerable interest. Since a substantial fraction of endogenous DNA damage may involve the alteration or loss of nucleotide bases, many spontaneous mutations may be generated during attempted replication past miscoding or noninstructional lesions. If so, then the rate of spontaneous mutation attributable to DNA damage in mutator strains might depend in large measure on translesion synthesis.

Genes designated *RAD* control resistance to ultraviolet (UV) and/or ionizing radiation in the yeast *Saccharomyces cerevisiae*. Mutant *rad*

alleles have been assigned to three epistasis groups on the basis of radiosensitivity in multiply mutant *rad* strains (Haynes and Kunz 1981; Game 1983; Friedberg et al. 1991; Prakash et al. 1993). These groups are named for a prominent locus in each, and individual members function in one or more aspects of nucleic acid metabolism including nucleotide excision repair and transcription (*RAD3* group), postreplication repair and mutation (*RAD6* group), and DNA double-strand break repair and recombination (*RAD52* group). Despite the heuristic value of this classification, it should be noted that the epistasis groups are not mutually exclusive. There are some loci which appear to belong simultaneously to two epistasis groups, and members of two or more groups can have various properties in common (Haynes and Kunz 1981). Interestingly, all three groups contain mutants that are mutators (Zakharov et al. 1968; von Borstel et al. 1968, 1971; Hastings et al. 1976), several of which have been characterized in detail. Inactivation of the nucleotide excision repair gene *RAD1* increases spontaneous single base-pair substitution and deletion, and insertion of the yeast retrotransposon Ty (Kunz et al. 1990). Deletion of *RAD18*, which is required for post-replication repair in the absence of nucleotide excision repair (DiCaprio and Cox 1981; Prakash 1981), specifically promotes G•C → T•A transversions (Kunz et al. 1991). Elimination of the *RAD6* ubiquitin-conjugating enzyme (Jentsch et al. 1987) enhances the rates of Ty insertion, single base-pair transition, and G•C → T•A transversion (Picologlou et al. 1990; Kang et al. 1992). Disruption of *RAD52* stimulates spontaneous single base-pair substitution, but mainly at G•C pairs (Kunz et al. 1989). The intriguing differences among these mutator phenotypes suggest that, collectively, the genes involved contribute to the repair of

a variety of naturally-occurring DNA damages.

It has been hypothesized that the association of mutator phenotypes with DNA repair defects is due to channelling of spontaneous lesions through mutagenic repair pathways (Hastings et al. 1976; Quah et al. 1980). Alternatively, enhanced spontaneous mutagenesis in repair-deficient strains might reflect tolerance of spontaneous DNA damage via an error-prone mechanism that does not initially involve repair, e.g. translesion synthesis. The yeast *REV3* (= *PSO1*, Cassier-Chauvat and Moustacchi 1988) gene has been predicted to encode a DNA polymerase that functions only in translesion synthesis (Morrison et al. 1989). This possibility is supported by several lines of evidence. A DNA polymerase that is close in size to, and reacts with antibodies against, the Rev3 protein has been isolated (Shimizu et al. 1993). In addition, *REV3* is nonessential and distinct from the genes for the three known yeast nuclear DNA polymerases (Morrison et al. 1989; Campbell and Newlon 1991). Furthermore, it does not exhibit cell cycle regulation of transcription (Singhal et al., 1992), like many genes required for replication of chromosomal DNA (McIntosh 1993). Finally, *REV3* is required for mutation induction by a number of DNA-damaging agents (Lawrence 1982; Henriques and Brendel 1990). Although compelling, the evidence favoring a role for the putative *REV3* polymerase in translesion synthesis is not conclusive, and other mechanisms cannot be excluded at present. Nonetheless, defects in *REV3* also confer an antimutator phenotype that reduces spontaneous mutation rates by as much as 80% (Cassier et al., 1980; Quah et al. 1980), suggesting that translesion synthesis might be a major source of spontaneous mutagenesis in yeast.

If mutators due to DNA repair deficiencies involve translesion

synthesis, then one might hypothesize that yeast rad mutator phenotypes would be *REV3*-dependent. Indeed, the *rev3* antimutator offset the magnitude of the mutator effects in rad1, rad3, *rad18* and rad51 (*RAD52* group) strains (Quah et al. 1980; Roche et al. 1994). Yet, the *rev3* antimutator did not reduce the overall mutation rates equally in all four rad backgrounds. Thus, both *REV3*-dependent and *REV3*-independent processes may have increased spontaneous mutagenesis in the rad mutants, but to different extents in the four strains. However, this interpretation must be viewed with caution. The haploid *rad* strains used were derived from different genetic backgrounds, and, except for the *rad1* deletion, the *rad* mutations used may have been leaky. Characterization of mutational specificity further suggested that the majority of the *rad1* mutator effect was due to the same *REV3*-dependent processes that produced most of the spontaneous mutations in an isogenic *RAD1* strain (Roche et al. 1994). Whether *REV3* influenced the specificities of the other *rad* mutators was not investigated.

In this study, we have extended previous observations on the role of *REV3* in spontaneous mutagenesis by determining the relative contributions of *REV3*-dependent mechanisms to the magnitudes and specificities of the yeast *rad6*, *rad18*, and *rad52* mutators. DNA sequence analysis was used to characterize spontaneous *SUP4*-o mutations arising in isogenic strains carrying appropriate combinations of wildtype and mutant *REV3* and *RAD* alleles. The *rev3* antimutator decreased the mutation rate in all three *rad* backgrounds. Whereas the *rad6* and *rad18* mutator effects were substantially reduced, the *rad52* mutator appeared to be entirely *REV3*-dependent. Evidence was obtained for different degrees of *REV3* participation in the generation of different base-pair substitutions in the *rad6* and *rad18*

strains, and for *REV3* dependency of the increased Ty insertion rate in the *rad6* background.

MATERIALS AND METHODS

Yeast and bacterial strains and plasmid. Construction of the repair-proficient, haploid strain MKP-o (*MAT*α, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3,112*, *his3-*Δ*200*, *trp1-*Δ*901*) and isogenic derivatives having *REV3* (RDG3: *rev3*Δ), *RAD6* (RDG6: *rad6*Δ), or *RAD18* (XK15: *rad18*Δ) deleted, or *RAD52* (SB52: *rad52*) disrupted, has been described (Pierce et al. 1987; Kunz et al. 1989, 1991; Kang et al. 1992; Roche et al. 1994). Isogenic derivatives of RDG3 (*rev3*Δ) having *RAD6* (HR36: *rev3*Δ *rad6*Δ), or *RAD18* (FY318: *rev3*Δ *rad18*Δ) deleted, or *RAD52* (FY352: *rev3*Δ *rad52*) disrupted, were constructed using integrative DNA transformation (Rothstein 1991), and deletion or disruption of the appropriate *RAD* genes was verified via phenotypic and DNA hybridization analyses, as described (Kunz et al. 1989, 1991; Kang et al. 1992). YCpMP2 was transformed (Ito et al. 1983; Schiestl and Gietz 1988; Gietz et al. 1992) into MKP-o, RDG3, RGD6, XK15, SB52, HR36, FY318 and FY352 to make MKP-op, RDG3-p, RDG6-p, XK15-p, SB52-p, HR36-p, FY318-p and FY352-p, respectively. *Escherichia coli* strain JF1754 (*Δlac*, *gal*, *metB*, *leuB*, *hisB436*, *hsdR*) (Pierce et al. 1987) was used to retrieve YCpMP2 from yeast strains. YCpMP2 (Pierce et al. 1987) which carries SUP4-o, an ochre suppressor allele of a yeast tyrosine tRNA gene, is a yeast centromere-containing vector having components that enable it to replicate autonomously in yeast and bacterial cells. In yeast, such plasmids have chromatin structure and mimic chromosome behaviour, replicating once per cell cycle in S phase and residing predominantly as single copies in haploid cells (Newlon 1988). YCpMP2 also bears the yeast URA3 gene which permits selection for the plasmid via complementation of the *ura3-52* allele present in all yeast strains used in this study.

Media. The media used with yeast or bacteria were prepared as described (Pierce et al. 1987; Kunz et al. 1991).

Detection of *SUP4*-o mutations. Forward mutations in *SUP4*-o were detected via reduced suppression of ochre alleles which confer resistance to the arginine analog canavanine (*can1-100*), red pigmentation (*ade2-1*) or lysine auxotrophy (*lys2-1*) (Pierce et al. 1987). Selection for diminished suppression of all three alleles detects at least a 30% decrease in functional suppressor tRNA (Wang and Hopper 1988), and is unlikely to bias mutant recovery significantly (Kohalmi and Kunz 1992).

Mutant isolation, plasmid retention, mutation frequency and mutation rate. Spontaneous *SUP4*-o mutations were isolated and plasmid retention and mutation frequencies determined as described (Pierce et al. 1987; Kunz et al. 1991). Mutation rates were calculated according to the formula (Drake 1991): $\mu_\upsilon = (0.4343Cf) / \log (N\mu_\upsilon)$; where μ_υ = mutations in the target per DNA replication; 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)]; f = median mutation frequency and N = median final population size (*REV3 RAD*: 2.62×10^8; *rev3Δ RAD*: 2.34×10^8; *REV3 rad6Δ*: 8.04×10^7; *REV3 rad18Δ*: 2.03×10^8; *REV3 rad52*: 1.32×10^8; *rev3Δ rad6Δ*: 2.11×10^8; *rev3Δ rad18Δ*: 2.92×10^8; *rev3Δ rad52*: 1.01×10^8) including three generations on the selection plates (determined microscopically for each strain) before growth of non-mutant cells terminates. This formula corrects for spontaneous mutations which occur but are not detected by the system.

DNA isolation, bacterial transformation and DNA sequencing. DNA for bacterial transformation was released from yeast cells by disruption with glass beads (Mis and Kunz 1990). Plasmid DNA was isolated from *E. coli* by alkaline extraction (Kohalmi et al. 1991). Bacterial cells were transformed using $CaCl_2$ (Pierce et al. 1987). *SUP4*-o alleles were sequenced on double-stranded YCpMP2 molecules, using dideoxynucleotides, as described (Kunz et al. 1987).

Statistical analysis. Chi-square tests employing Yates' correction for continuity (Sokal and Rohlf 1969) were used to evaluate differences in a variety of parameters. The significance of differences in the distributions of single base-pair substitutions within the *SUP4*-o gene was assessed using the Monte Carlo estimate of the *P* value of the hypergeometric test (Adams and Skopek 1987) [1,500 simulations were run for each comparison]. For both tests, values of $P < 0.05$ were considered significant.

RESULTS

Effect of the *REV3* deletion on plasmid retention and spontaneous mutagenesis. The yeast strains (*REV3 RAD*, *REV3 rad6Δ*, *REV3 rad18Δ*, *REV3 rad52*, *rev3Δ RAD*, *rev3Δ rad6Δ*, *rev3Δ rad18Δ*, *rev3Δ rad52*) used in this study are isogenic except for the *REV3* and *RAD* loci. For this reason, the *rev3Δ* antimutator, the particular rad mutator involved, or the combination of the two, but not variation in genetic background, should be responsible for any observed differences. *SUP4*-o, the mutational target in these strains, is present on the yeast centromere plasmid YCpMP2. Inactivation of the *RAD6* and *RAD52*, but not *RAD18*, genes diminishes the stability of this vector (Kunz et al. 1989, 1991; Kang et al. 1992). To determine whether deletion of *REV3* influenced either plasmid maintenance or spontaneous mutagenesis in the rad backgrounds, yeast cultures were grown from low titre inocula to stationary phase in medium selective for the plasmid, and then plated to assess the stability of YCpMP2 and isolate *SUP4*-o mutations.

Plasmid retention data were obtained by comparing the number of colonies that emerged on selective medium with the corresponding value for nonselective medium. Previously, we determined that the *rev3Δ* allele alone did not alter plasmid maintenance (Roche et al. 1994). Table 1 shows that the *REV3* deletion also did not modify the effects of the *rad6Δ* or *rad52* mutations on plasmid stability. Although there appeared to be a slight decrease in plasmid retention in the *rev3Δ rad18Δ* strain, the difference was not significant ($P > 0.05$), and the value obtained also was not significantly different from those for the *REV3 RAD* and *rev3Δ RAD* strains ($P > 0.3$).

The *rev3Δ* antimutator reduced the frequency of spontaneous mutation in the *rad6Δ*, *rad18Δ*, and *rad52* backgrounds by 62%, 68% and 88%, respectively (Table 1). These decreases corresponded to mutation rate reductions of 58% for *REV3 rad6Δ* compared to *rev3Δ rad6Δ*, 61% for *REV3 rad18Δ* compared to *rev3Δ rad18Δ*, and 80% for *REV3 rad52* compared to *rev3Δ rad52*. Deleting *REV3* clearly diminished the *rad6Δ* mutator effect, but the overall mutation rate for *rev3Δ rad6Δ* was still 3.8-fold greater than that for the *rev3Δ RAD* strain, and 1.6-fold greater than the value for the wildtype parent. For the *rad18Δ* mutator, the *rev3Δ* antimutator lowered the total *SUP4*-o mutation rate to just under the wildtype level. However, the mutation rate remained 2-fold greater than in the *rev3Δ RAD* strain. These data indicated that both *REV3*-dependent and *REV3*-independent components contributed to the *rad6Δ* and *rad18Δ* mutators. Only for the *rev3Δ rad52* strain was the mutation rate lowered to the *rev3Δ* antimutator level (60% below the rate for wildtype cells), indicating that the *rad52* mutator effect was completely *REV3*-dependent. Since the entire *REV3* coding sequence was removed by the *REV3* deletion, the residual mutagenesis detected at *SUP4*-o in the *rev3Δ* strains could not have been due to leakiness of this *rev3Δ* allele.

***REV3* dependence of *SUP4*-o mutational classes in *rad* strains.** To assess the *REV3* dependency of the *rad* mutators in greater detail, the influence of the *rev3Δ* antimutator effect on the specificities of the *rad* mutators was determined by DNA sequencing. A total of 608 *SUP4*-o mutations arising spontaneously in the *rev3Δ rad6Δ*, *rev3Δ rad18Δ*, or *rev3Δ rad52* strains was characterized. To ensure the independence of each mutation examined, only one was chosen per culture. The resulting mutational spectra were compared to those for 1,080 *SUP4*-o mutations isolated

previously in the *REV3 RAD*, *rev3Δ RAD*, *REV3 rad6Δ*, *REV3 rad18Δ* or *REV3 rad52* backgrounds. The sequencing data for these latter mutations have been published (Kunz et al. 1989; 1991; Kang et al. 1992; Roche et al. 1994), but are presented here to facilitate comparisons. Although ten different mutational classes were detected among the eight strains, no more than eight classes were recovered in any one background, and only four different kinds of change were found in the *rev3Δ rad6Δ* and *REV3 rad52* strains (Table 2, see Table 3 for descriptions of SUP4-o sequence alterations other than single base-pair substitutions).

With respect to *SUP4*-o on YCpMP2, the *rad6Δ* mutator promotes single base-pair substitution (which is examined in more detail below) and insertion of the yeast retrotransposon Ty (Kang et al. 1992). Decreases in the rates of these events accounted for the entire mutation rate reduction attributable to the *rev3Δ* antimutator in the *rev3Δ rad6Δ* strain (Table 2). Yet, the rate of single base-pair substitution was lowered by 57% and the rate of Ty insertion by 75%, leaving the rates, respectively, 3.7-fold and 6.8-fold greater than for the *rev3Δ RAD* strain, and 1.5-fold and 4.3-fold greater than for the *REV3 RAD* strain. This indicated that *REV3*-dependent processes contributed substantially to both types of change, but the participation of *REV3*-independent mechanisms also was important. The relative fraction of base-pair changes or Ty insertions was unaffected or was reduced by 42% ($P < 0.02$), respectively. In contrast to these findings, the proportion of single base-pair deletions increased 5.8-fold ($P < 0.001$), and the rate by 2.5-fold. Earlier, we found that the rate of these events was slightly greater (1.4-fold) in the *rad6Δ* strain than its isogenic *RAD6* parent (Kang et al. 1992). Our current observation suggests that the *rad6Δ* mutator might stimulate single base-pair deletions via a

REV3-independent mechanism whose effect becomes evident only when the rates of the other mutations provoked by the *rad6*Δ mutator are reduced sufficiently.

The *rad18*Δ mutator induces only single base-pair substitution (Kunz et al. 1991). The rate of this event was reduced by 61% in the *rev3*Δ *rad18*Δ strain almost to the wildtype level, accounting for 95% of the mutation rate decrease caused by inactivation of *REV3* (Table 2). This suggested that, unlike the situation for the *rad6*Δ mutator, the bulk of the *rad18*Δ mutator effect was *REV3*-dependent. However, the substitution rate was still 2.4-fold greater than the corresponding value for the *rev3*Δ *RAD* strain, again pointing to a *REV3*-independent component of mutagenesis. The remaining 5% of the overall mutation rate reduction was attributable to rate decreases for multiple base-pair deletions and Ty insertion. Deletion of *REV3* also lowered the rates of these events in the wildtype background (Roche et al. 1994, Table 2). Thus, the influence of the *rev3*Δ antimutator on the rates of mutations other than single base-pair changes probably involved mechanisms independent of the *rad18*Δ mutator.

Disruption of *RAD52* also only increases the rate of single base-pair change (Kunz et al. 1989). For *rev3*Δ *rad52*, the total *SUP4*-o mutation rate decrease was due almost entirely (98%) to a decline (82%) in the rate of single base-pair substitution, with a 76% reduction in the single base-pair deletion rate accounting for the minor remnant of the decrease. Interestingly, the rates of the mutational classes detected in both *rev3*Δ *rad52* and *rev3*Δ *RAD*, except for Ty insertion, were similar (disruption of *RAD52* dramatically reduces the incidence of Ty insertion (Kunz et al. 1989), evidently to a rate lower than that for the *rev3*Δ *RAD*

strain). This further supported the possibility that *rad52* enhances the spontaneous mutation rate solely via *REV3*-dependent mechanisms.

The rates of other types of mutation (single base-pair deletion for the *rad18*Δ strains and complex change for the *rad6*Δ and *rad18*Δ backgrounds) recovered in both members of a pair of rad strains appeared similar.

***REV3* participation in production of single base-pair substitutions by rad mutators.** Deletion of *REV3* appeared to reduce the production of single base-pair changes by each of the rad mutators. However, the extent to which *rev3*Δ affected the particular types of base-pair substitution promoted by each mutator was not clear. This information was obtained by analyzing the different classes of base-pair change (Table 4). All six kinds of base-pair substitution were recovered in *REV3 RAD*, *rev3*Δ *RAD*, *REV3 rad52*, and *rev3*Δ *rad52*. No transversions were detected at A•T pairs in *REV3 rad6*Δ, no A•T → T•A events were found in *rev3*Δ *rad6*Δ, and no A•T → C•G transversions were identified in the *rad18*Δ background.

The *rad6*Δ mutator produces both base-pair transitions and the G•C → T•A transversion (Kang et al. 1992). Comparison of *rev3*Δ *rad6*Δ to *REV3 rad6*Δ (Table 4) revealed that the relative fractions of the transitions were decreased by the *rev3*Δ antimutator ($P <$ at least 0.01), whereas the fraction of G•C → T•A transversions increased ($P < 0.001$). This resulted in the ratio of transversions to transitions being 3-fold greater (1.6 vs. 5.5, $P < 0.001$), and the ratio of substitutions at G•C to A•T pairs 9-fold greater (8.5 vs. 73.5, $P < 0.005$), when *REV3* was deleted. The rate reductions for the G•C → A•T (77%) and A•T → G•C (98%) transitions were considerably larger than that for the G•C → T•A transversion (42%). However, the rates for G•C → A•T and G•C → T•A

substitutions remained 2.3-fold or 7.4-fold greater, respectively, than for the *rev3Δ RAD* strain, with the G•C → T•A transversion rate also being 3.6-fold greater than for the wildtype parent. In contrast, the A•T → G•C transition rate was substantially below the corresponding value for the *rev3Δ RAD* strain. Thus, *rad6Δ*-mediated production of G•C → A•T transitions was largely *REV3*-dependent, whereas the opposite was true for the G•C → T•A transversions. On the other hand, the increased rate of A•T → G•C transition in the *REV3 rad6Δ* background appeared to be entirely *REV3*-dependent.

Among the yeast mutators examined to date, *rad18Δ* is unique in producing only a single type of mutation, the G•C → T•A transversion (Kunz et al. 1991). The proportion of G•C → T•A transversions was slightly greater in *rev3Δ rad18Δ* than *REV3 rad18Δ* ($P < 0.02$), whereas the relative fractions of the other base-pair substitutions detected in both strains were not significantly different (Table 4). Consequently, there was no significant change in the ratio of either transversions to transitions (5.0 vs. 7.2, $P > 0.2$) or substitutions at G•C to A•T pairs (10.6 vs. 13.5, $P > 0.5$). Given that *rev3Δ* lowered the total mutation rate in the *rad18Δ* background to just below the wildtype rate, it was surprising to find that the *rev3Δ* antimutator reduced the G•C → T•A transversion rate to a lesser degree (57%) than the rates of the other substitutions. Furthermore, the former events still occurred at rates 4.9-fold or 2.4-fold greater than in the *rev3Δ RAD* or *REV3 RAD* backgrounds, respectively. These results suggested that the enhanced production of G•C → T•A transversions in the *REV3 rad18Δ* relied almost equally on *REV3*-dependent and *REV3*-independent mechanisms. The rates of the other substitutions that were detected in both the *rev3Δ rad18Δ* and *rev3Δ RAD*

strains were similar, indicating that the rate decreases for these base-pair changes were independent of the *rad18*Δ mutator, as expected.

Inactivation of *RAD52* primarily increases the rate of substitution at G•C pairs (Kunz et al. 1989). The fractions of G•C → T•A and G•C → C•G transversions were increased 2-fold or decreased by 81%, respectively ($P < 0.001$ in both cases) in *rev3*Δ *rad52* compared to *REV3 rad52* (Table 4). Other apparent fluctuations in the relative proportions of substitutions were not significant ($P >$ at least 0.05). The two changes noted did not affect the ratio of transversions to transitions (2.1 vs. 1.7, $P > 0.3$), but the ratio of substitutions at G•C to A•T pairs was reduced by 50% (7.1 vs. 3.6, $P < 0.02$), so that it was very close to the ratio (3.1) observed for the *rev3*Δ *RAD* strain ($P > 0.5$). The rates of all substitutions were diminished by at least two-thirds, with the rates of total transitions and total transversions decreasing by about 80%, and the G•C → C•G transversion rate showing the largest reduction (97%). The net result of the various modulations was a high degree of similarity between the fractions and rates of the substitutions detected in the *rev3*Δ *rad52* and *rev3*Δ *RAD* strains. This was the outcome expected for total *REV3* dependency of the *rad52* mutator effect.

Variability of *rev3*Δ antimutator effect within *SUP4*-o. The production of single base-pair substitutions by the three rad mutators exhibited different magnitudes of *REV3* dependence. We next sought to determine whether the *REV3*-dependent and *REV3*-independent (for *rad6*Δ and *rad18*Δ) components of the rad mutator effects acted uniformly across *SUP4*-o. This was accomplished by examining the distributions of single base-pair changes within *SUP4*-o (Fig. 1). Several pairwise comparisons were made including *REV3 rad6*Δ vs. *rev3*Δ *rad6*Δ, *REV3 rad18*Δ vs.

rev3Δ rad18Δ, and *REV3 rad52* vs. *rev3Δ rad52*, with the latter member of each pair also compared to the *rev3Δ RAD* strain. Base-pair changes were detected at 40, 51, 58, 51, 51 or 59 different sites for the respective pairs, with similar numbers of positions mutated in each member of a pair of strains (*REV3 rad6Δ*: 33, *rev3Δ rad6Δ*: 31; *REV3 rad18Δ*: 44, *rev3Δ rad18Δ*: 38; *REV3 rad52*: 51, *rev3Δ rad52*: 46; *rev3Δ RAD*: 43, *rev3Δ rad18Δ*: 38; *rev3Δ RAD*: 43, *rev3Δ rad52*: 46), except for *rev3Δ RAD* (43) and *rev3Δ rad6Δ* (31). No substitutions were detected in the 5′ or 3′ flanking regions and only one intronic position, site 51, was mutated. A statistical evaluation of the distributions (Adams and Skopek 1987) for each of the pairwise comparisons suggested that the probability of random sampling error being responsible for differences was less than 1 in 500 (the upper limit of the 90% confidence interval on the estimate of *P* was 0.002 in each case).

For *rev3Δ rad6Δ* vs. *REV3 rad6Δ*, 24 (60%) sites were common to both distributions, 9 (22%) were detected solely in *REV3 rad6Δ*, and 7 (18%) just in *rev3Δ rad6Δ*. Only 3 of the 9 most frequently mutated positions (6, 25, 32, 33, 51, 56, 83, 84, 86) (arbitrarily defined as having at least 8 substitutions) in the two strains overlapped. In the *rad18Δ* background, 31 (61%) sites were detected in both distributions with 13 (25%) found just in *REV3 rad18Δ*, and 7 (14%) only in *rev3Δ rad18Δ*. Five of the twelve sites (6, 15, 18, 25, 27, 32, 34, 51, 56, 65, 83, 84) mutated most frequently in the two strains overlapped, but substantially more substitutions were detected at one of these positions (18) in *rev3Δ rad18Δ*. A total of 39 (67%) sites were mutated in both the *REV3 rad52* and *rev3Δ rad52* distributions, 12 (21%) in *REV3 rad52* alone, and 7 (12%) only in *rev3Δ rad52*. Of the ten sites (6, 15, 18, 27, 29, 51, 65, 68, 73, 88)

mutated most frequently in these two strains, only one overlapped and the relative proportions of the transversions at this position (51) differed considerably. Thus, for each comparison, mutations were not recovered at more than two-thirds of the total sites where changes were detected in both strains, and there was relatively little overlap among the more frequently mutated sites.

The marked similarities between the proportions and rates of the various mutational classes detected in the *rev3Δ rad52* and *rev3Δ RAD* strains (Tables 2 and 4), suggested that the distributions of substitutions in *SUP4*-o might be very much alike for these two backgrounds. Yet, only 52% (31/59) of the sites mutated in both strains were common to each. Of the 6 sites (6, 15, 18, 27, 51, 72) mutated most frequently in both distributions, just two overlapped. Similarly, 45% (23/51) or 59% (30/51) of the sites mutated in *rev3Δ rad6Δ* and *rev3Δ RAD* or *rev3Δ rad18Δ* and *rev3Δ RAD*, respectively, were common to both members of the pair. Again, the number of overlapping sites among the most frequently mutated positions were in the minority [*rad6Δ* background: 2/8 (6, 18, 27, 51, 72, 83, 84, 86); *rad18Δ* background: 4/11 (6, 18, 27, 32, 37, 51, 56, 65, 72, 83, 84)].

The *rev3Δ* antimutator reduced the site-specific mutation rates as follows: by 15% to 92% at 20 of the sites common to *rev3Δ rad6Δ* and *REV3 rad6Δ*, with an average decrease of 55%; by 19% to 96% at 26 of the sites common to *rev3Δ rad18Δ* and *REV3 rad18Δ*, with an average reduction of 63%; and by 24% to 97% at 33 of the sites common to *rev3Δ rad52* and *REV3 rad52*, with an average decrease of 76% (the mutation rate was 2.6-fold greater at position 15 in *rev3Δ rad52*). However, mutation rates were 1.2- to 3.3-fold greater at 3 sites in *rev3Δ rad6Δ* (12, 82, 88), 4

sites in *rev3Δ rad18Δ* (34, 68, 67, 86), and 2 sites in *rev3Δ rad52* (15, 36). Similarly, the *REV3*-independent components of the rad mutator effects exhibited site-specific differences. Compared to *rev3Δ RAD*, the rates were 1.3- to 34-fold, 1.3- to 18-fold, or 1.4- to 6.4-fold greater at 22, 19 or 18 of the sites also common to *rev3Δ rad6Δ*, *rev3Δ rad18Δ*, or *rev3Δ rad52*. Relative to *REV3 RAD*, the rates were 1.2- to 16-fold greater at 20 or 10 of the sites also mutated in *rev3Δ rad6Δ* or *rev3Δ rad18Δ*, respectively, and 1.3- to 5.3-fold greater at 6 sites also common to *rev3Δ rad52*.

Collectively, these observations indicated that there was considerable site-to-site variation in the magnitude of the *rev3Δ* antimutator effect on enhanced mutagenesis in the rad strains, and the magnitudes of the *rad* mutator effects in the absence of *REV3*. Such variation suggests that DNA sequence context might have influenced both the *REV3*-dependent and *REV3*-independent production of spontaneous base-pair substitutions in the *rad6Δ*, *rad18Δ*, and *rad52* mutator backgrounds.

DISCUSSION

Defects in various *RAD* genes belonging to the three epistasis groups for repair of UV photoproducts in the yeast *S. cerevisiae* confer mutator phenotypes (Zakharov et al. 1968; von Borstel et al. 1971; Hastings et al. 1976; Quah et al. 1980; Haynes and Kunz 1981). On the basis of these and other observations, von Borstel and colleagues suggested that the *rad* mutator phenotypes result from channelling of spontaneous DNA lesions through mutagenic repair pathways (Hastings et al. 1976; Quah et al. 1980). However, inactivation of the yeast *REV3* gene cause an antimutator effect (Cassier et al., 1980; Quah et al. 1980) that offsets the *rad* mutator phenotypes in the multiply mutant strains examined so far (Quah et al. 1980; Roche et al. 1994). Analysis of the *REV3* gene led to the prediction that it encodes a DNA polymerase required for translesion synthesis (Morrison et al. 1989), and there is preliminary evidence that the Rev3 protein has DNA polymerase activity (Shimizu et al. 1993). Together, these findings suggested that translesion synthesis might have an important role in *rad* mutator phenotypes. Consistent with this possibility, the rate of *SUP4*-o mutation in a *rad1*Δ (*RAD3* epistasis group) mutator strain was reduced by the *rev3*Δ antimutator to the wildtype level (Roche et al. 1994). To further investigate the importance of *REV3* in the control of spontaneous mutagenesis in yeast, we determined the contribution of *REV3* to the magnitudes of rad mutators from the remaining two epistasis groups (*RAD6* and *RAD52*), and evaluated the role of the putative *REV3* polymerase in their specificities.

The *rad6*Δ mutator promotes Ty transposition as well as both base-pair transitions and the G•C → T•A transversion (Picologlou et al. 1990;

Kang et al. 1992). Our results indicated that the *REV3* gene product is involved in the production of all four types of change. However, comparison of the mutation rates revealed that the *REV3* requirement was not uniform for each of the mutational classes associated with the *rad6Δ* mutator. Three-quarters of the *rad6Δ*-induced increases in the rates of Ty transposition and G•C → A•T transition relied on *REV3*, but the remainder occurred via a *REV3*-independent mechanism. Even more striking was the dramatic reduction in the rate of A•T → G•C transition in *rev3Δ rad6Δ* to a level 98% lower than that for the *REV3 rad6Δ* background. Apparently, the occurrence of this substitution is almost entirely, if not wholly, *REV3*-dependent when *RAD6* is deleted. Conversely, a *REV3*-independent process(es) was responsible for about 60% of the G•C → T•A transversion rate increase in the *REV3 rad6Δ* strain.

REV3 belongs to the *RAD6* epistasis group for UV sensitivity (Haynes and Kunz 1981). It has been suggested that *RAD6* might influence the function of other members of this group via ubiquitination of their gene products (Sung et al. 1990). However, the manifestation of the *rev3Δ* antimutator phenotype in the *rad6Δ* background implies that *RAD6*-mediated ubiquitination is not necessary for the role of the putative *REV3* polymerase in spontaneous mutagenesis.

The manner in which *REV3* might enhance Ty transposition in the *rad6Δ* background is not known, but Ty insertion into *SUP4*-o can be increased by failure to repair spontaneous DNA lesions (Kunz et al. 1994). Perhaps certain types of DNA damage can provoke transposition via a mechanism involving *REV3*-dependent processing. The *REV3*-independent component of *rad6Δ*-mediated Ty transposition has been suggested to involve the failure to ubiquitinate Ty proteins or histones (Picologlou et al. 1990), thereby

interfering with the regulation of Ty transposition, or leading to alterations in chromatin structure that might increase access of transposition complexes to DNA. The *REV3* dependency of the base-pair transitions argues against our previous suggestion (Kang et al. 1992) that a reduced efficiency of mismatch correction might be the cause of the enhanced transition rates in the *REV3 rad6*Δ background. At present, the simplest explanation for the role of *REV3* in the production of base-pair changes by the rad mutators would involve translesion synthesis past unrepaired spontaneous DNA damage. DNA sequence context likely affects *REV3*-dependent processing of DNA lesions to substitutions as there was considerable site-specific variation in the magnitude of the *rev3*Δ antimutator. Indeed, for each *rev3*Δ rad combination the substitution rates appeared to remain unchanged or actually increase at some positions within *SUP4*-o. If the putative *REV3* polymerase functions in translesion synthesis, then sequence context might have influenced the occurrence of spontaneous DNA damage, its removal by error-free repair or the ability of the polymerase to synthesize past spontaneous lesions. The *REV3*-independent mechanism(s) involved in substitution production would not necessarily have to be distinct from translesion synthesis. *In vitro*, calf thymus DNA polymerase δ, aided by proliferating cell nuclear antigen, can replicate templates containing UV photoproducts (O'Day et al. 1992). This suggests that replicative DNA polymerases in eukaryotes might be capable of translesion synthesis past some forms of DNA damage. Alternatively, some lesions might be processed by *REV3*-independent error-prone repair. The identity of the lesions that give rise to the particular substitutions induced by the *rad6*Δ mutator is not known (but see below). Conceivably, the *REV3*-dependent and *REV3*-independent components might each process

different lesions that normally are substrates for *RAD6*-mediated repair. Alternatively, two different *RAD6*-dependent pathways, only one involving *REV3*-dependent processing, might compete for the same lesions.

With respect to the total *SUP4*-o mutation rate, the *rad18*Δ mutator effect seemed to be largely *REV3*-dependent with a minor *REV3*-independent component. However, the *rad18*Δ mutator specifically induces the G•C → T•A transversion (Kunz et al. 1991), and the rate of this base-pair change showed the smallest decrease of any substitution rate in *rev3*Δ *rad18*Δ, remaining more than twice as great as the corresponding value for *REV3 RAD*. The larger decline in the total *SUP4*-o mutation rate was due to reductions in the rates of the other base-pair substitutions to the *rev3*Δ levels. Evidently, almost half of the *rad18*Δ-mediated increase in the G•C → T•A transversion rate was *REV3*-independent. This result was similar to that for the *rad6*Δ mutator effect on G•C → T•A transversions, an interesting outcome given that the *RAD6* and *RAD18* gene products may interact in the repair of DNA damage. The Rad6 and Rad18 proteins form a complex possessing Rad18-dependent single-stranded DNA binding activity (Bailly et al. 1994). These findings support the earlier suggestion (Koken et al. 1991) that Rad18 might interact with and direct Rad6 to damaged DNA regions. Thus, a functional Rad6-Rad18 complex might be required to limit the production of G•C → T•A transversions by certain spontaneous DNA lesions. This possibility, plus our observations, suggests that the increased G•C → T•A transversion rates in the *REV3 rad6*Δ and *REV3 rad18*Δ strains might reflect diminished repair of the same type of naturally-occurring DNA damage. A potential candidate for the lesion involved is 7,8-dihydro-2′-deoxyguanosine, which is produced by attack of endogenously generated reactive oxygen species on DNA, and predominantly induces

G•C → T•A transversions (Shibutani et al. 1991; Cheng et al. 1992; Klein et al. 1992; Michaels and Miller 1992; Wood et al. 1992; Moriya 1993). Despite the foregoing, we caution that which spontaneous lesions actually might be responsible for particular *rad* mutator phenotypes, and what the precise role of *REV3* is in spontaneous mutagenesis remains to be established. Consequently, one may speculate about how replication past DNA damage might give rise to certain mutations, but it would be premature to attempt to model mechanistic links between our observations and *REV3* function.

The *rad52* mutator effect also was diminished by the *rev3Δ* antimutator, but in marked contrast to the results for *rev3Δ rad6Δ* and *rev3Δ rad18Δ*, the total *SUP4*-o mutation rate in the *rev3Δ rad52* strain was decreased to the *rev3Δ* antimutator level. Inactivation of *RAD52* primarily increases the rate of substitution at G•C pairs (Kunz et al. 1989). The decline in the substitution rate in the *rev3Δ rad52* strain accounted for essentially the entire mutation rate decrease, with most of this reduction being specific for events at G•C pairs. Thus, it appears that *REV3* is absolutely necessary for the enhanced production of *SUP4*-o mutations by the *rad52* mutator. This is not the case for the rad1Δ, *rad6Δ* or *rad18Δ* mutator effects (Kunz et al. 1990, this study), which feature *REV3*-independent components. We interpret these results to indicate that *RAD52* functions in the repair of spontaneous DNA damage that can give rise to mutation only by *REV3*-dependent processing. So far, the only DNA lesion unequivocally identified as being repaired by a *RAD52*-dependent process is the double-strand break (Ho 1975; Resnick and Martin 1976). However, it is difficult to envisage how processing of such a break could produce primarily substitutions at G•C pairs. Furthermore, the fact that the

increased rate of A•T → G•C transitions in the *REV3 rad6Δ* strain relied entirely on the function of *REV3* indicates that *REV3*-dependent spontaneous mutagenesis is not restricted to G•C pairs.

In this and a previous study (Kunz et al. 1990), we have shown that the specificity of mutators (*rad1Δ*, *rad6Δ*, *rad18Δ*, *rad52*) derived from each of three epistasis groups for the repair of UV-induced DNA damage in yeast exhibits considerable dependence on the *REV3* gene. The product of this gene could be a DNA polymerase required for translesion synthesis. Together with our observations, this suggests that a substantial fraction of spontaneous mutagenesis in yeast might reflect mechanisms for tolerance rather than error-prone repair of naturally-occurring DNA damage. The fact that deletion of *REV3* diminishes the rates of all the different types of mutation produced by the rad mutators examined further suggests that the *REV3* gene product may be involved in processing a variety of spontaneous DNA lesions. Although we obtained evidence for *REV3*-independent components of spontaneous mutagenesis, our results imply that certain types of endogenous damage may give rise to mutations only via *REV3*-dependent processing.

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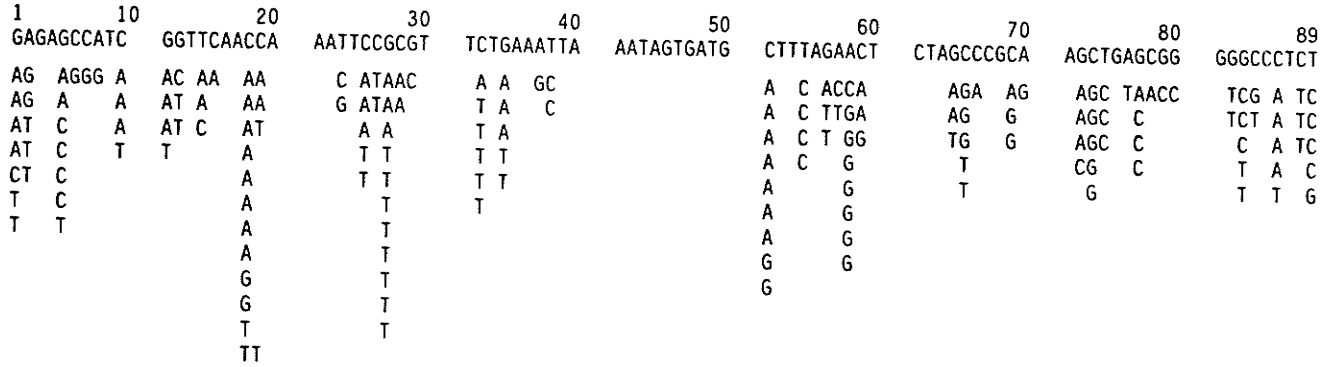
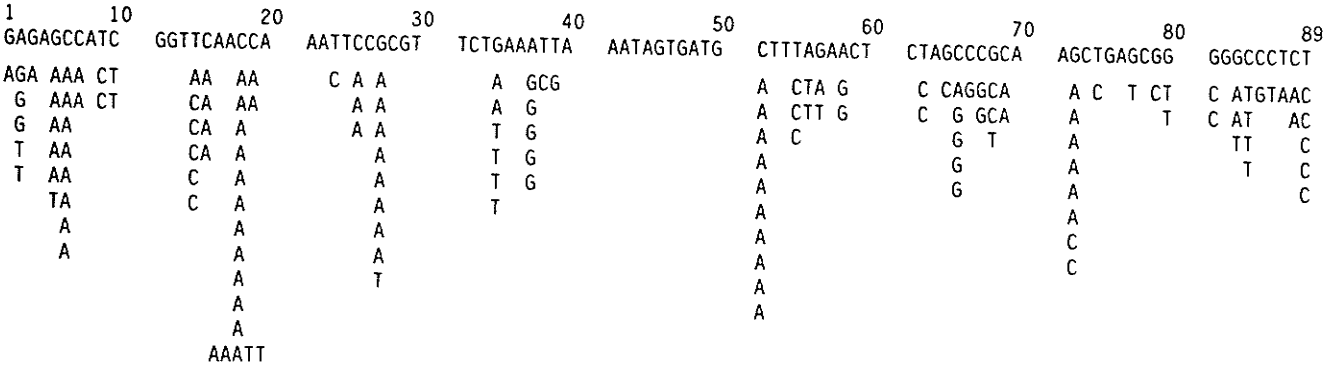
FIGURE LEGEND

FIG. 1. Distributions of single base pair substitutions in *SUP4*-o. For simplicity, only the region of the transcribed strand encoding the tRNA is shown. The anticodon is at 36 to 38 and the 14-base-pair intron is inferred to extend from 40 through 53 as for the *sup4*$^{+}$ allele (Knapp et al. 1978). The strains in which the mutations were isolated (*REV3 RAD*; *rev3*Δ *RAD*; *REV3 rad6*Δ; *rev3*Δ *rad6*Δ; *REV3 rad18*Δ; *rev3*Δ *rad18*Δ; *REV3 rad52*; *rev3*Δ *rad52*) are given above the transcribed strand for each distribution. Identical changes at individual sites represent independent events.

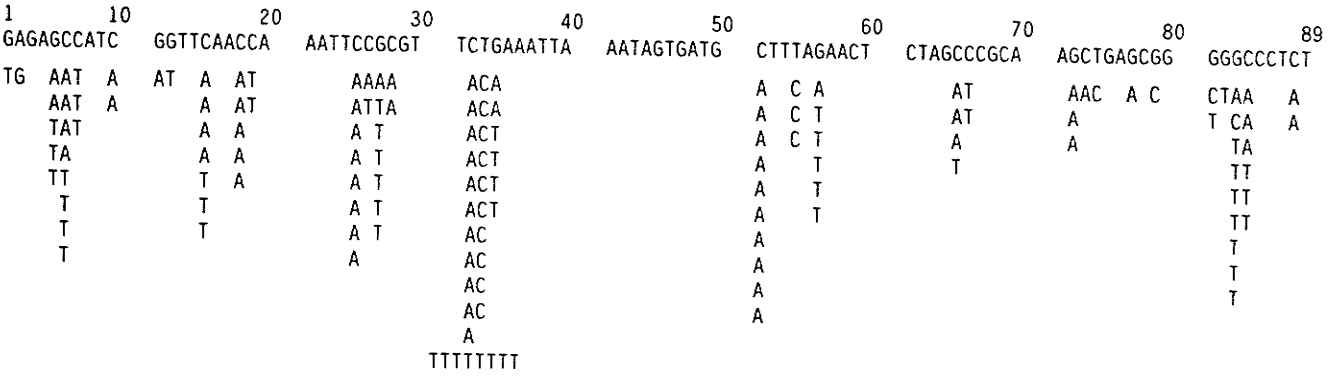
REV3 RAD



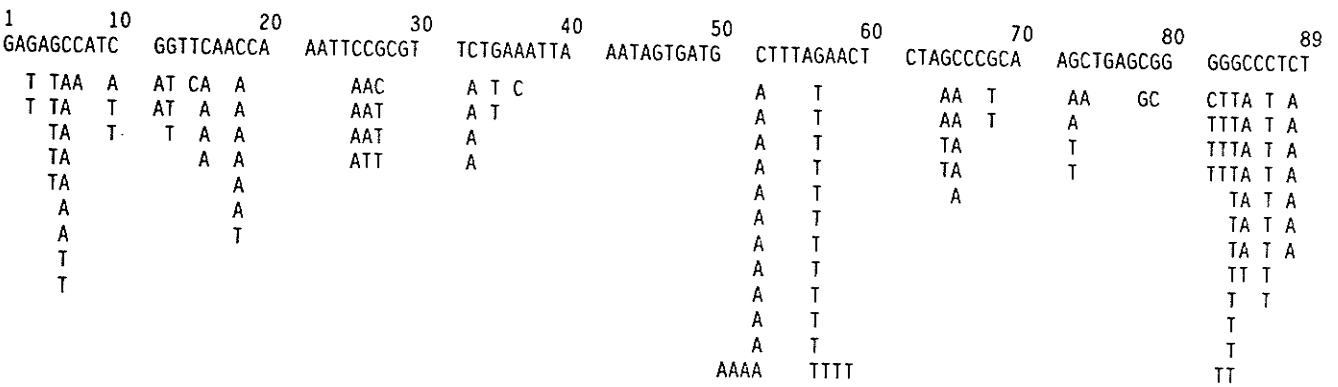
rev3Δ RAD



REV3 rad6Δ



rev3Δ rad6Δ



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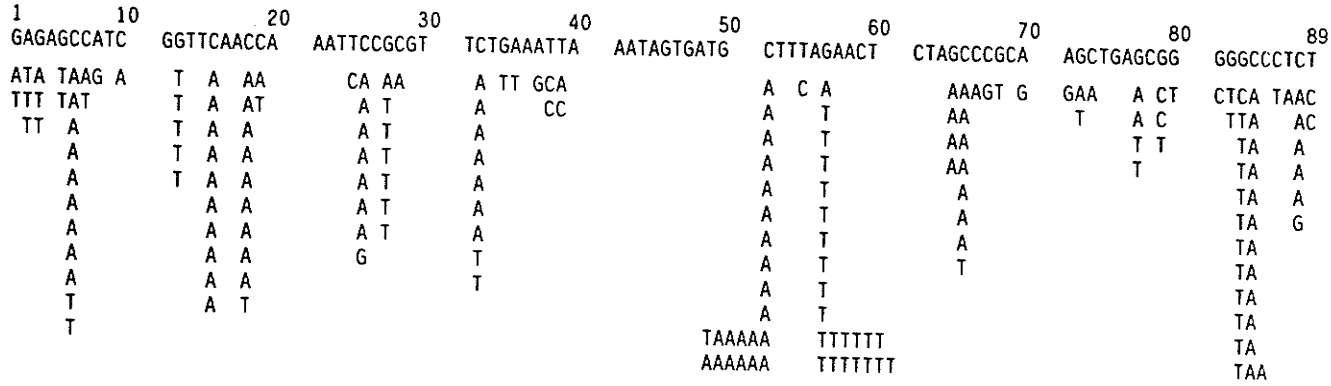
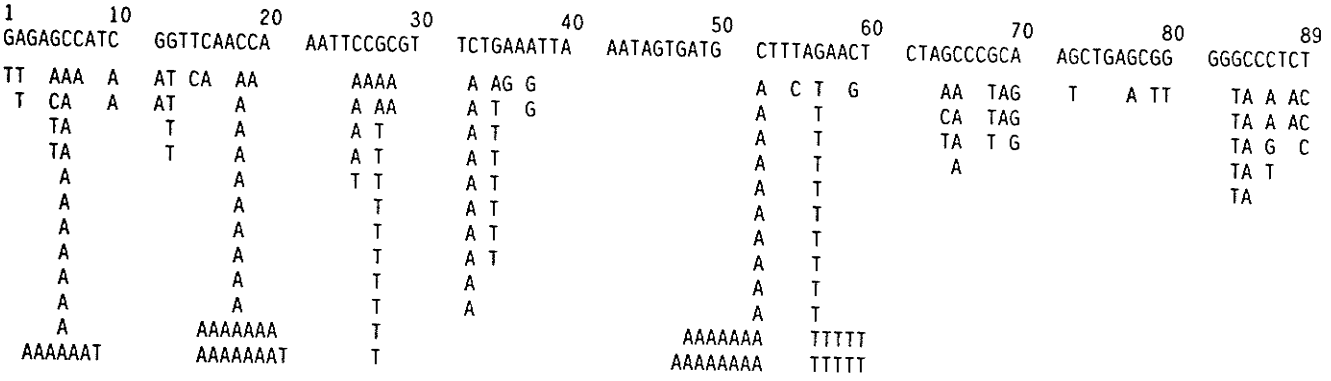
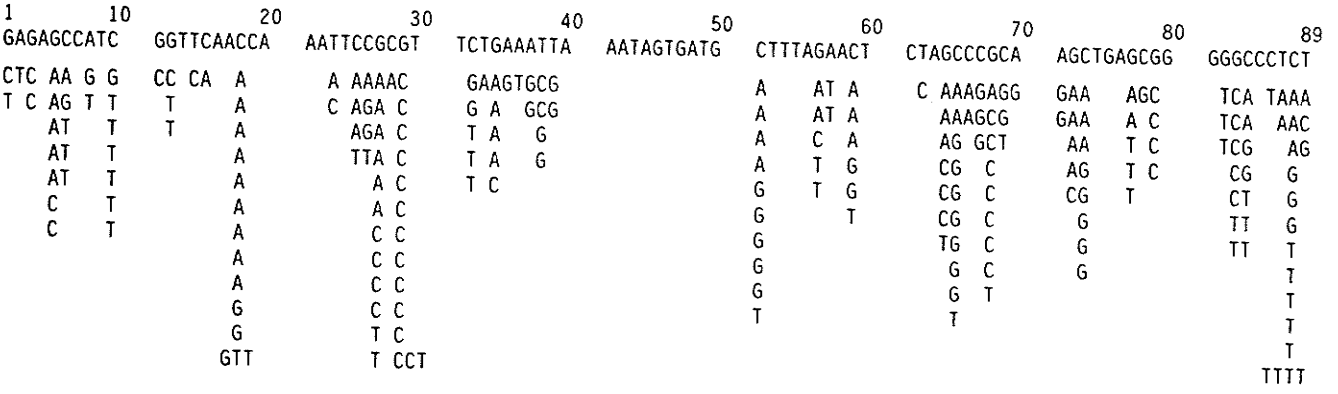


rev3Δ rad18Δ



REV3 rad52



rev3Δ rad52

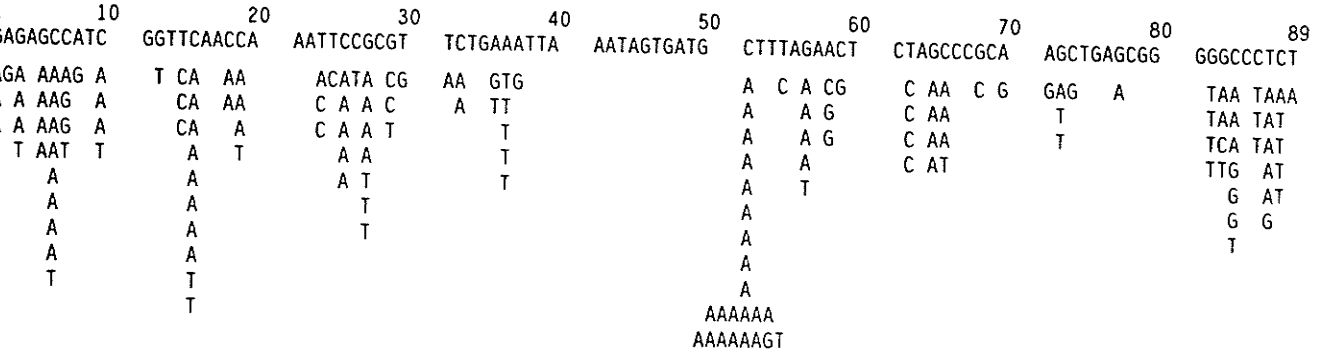


TABLE 1. Plasmid retention and mutation frequencies and rates[a]

Strain	Colonies on selective medium	Colonies on nonselective medium	Percent plasmid retention	Mutation frequency ($\times 10^{-6}$)	Mutation rate ($\times 10^{-7}$)
(*REV3 RAD*)	10,879	12,599	86.3	2.7	7.1
(*rev3Δ RAD*)	7,743	8,748	88.5	0.9	2.9
(*REV3 rad6Δ*)	11,387	17,866	63.7	11.7	26.5
(*rev3Δ rad6Δ*)	7,016	11,073	63.4	4.5	11.1
(*REV3 rad18Δ*)	8,488	9,355	90.7	6.2	14.2
(*rev3Δ rad18Δ*)	9,857	11,238	87.7	2.0	5.6
(*REV3 rad52*)	4,302	5,755	74.7	6.0	14.8
(*rev3Δ rad52*)	3,391	4,470	75.9	0.7	2.9

[a]Plasmid retention data for *REV3 RAD*, *rev3Δ RAD*, *REV3 rad6Δ*, *REV3 rad18Δ* and *REV3 rad52Δ* are taken from (Kunz et al. 1989, 1991; Kang et al. 1992; Roche et al. 1994). Frequencies are the medians for 20 (*rev3Δ RAD*, *REV3 rad18Δ*, *REV3 rad52Δ*, *rev3Δ rad6Δ*, *rev3Δ rad18Δ*, *rev3Δ rad52Δ*) or 30 (*REV3 RAD*, *REV3 rad6Δ*) independent cultures. Frequency and rate data for *REV3 RAD* and rev3 RAD are taken from (Roche et al. 1994).

TABLE 2. Sequence alterations in *SUP4*-o mutants

Alteration	*REV3 RAD*			*rev3Δ RAD*			*REV3 rad6Δ*			*rev3Δ rad6Δ*		
	Number recovered		Rate ($\times 10^{-8}$)	Number recovered		Rate ($\times 10^{-8}$)	Number recovered		Rate ($\times 10^{-8}$)	Number recovered		Rate ($\times 10^{-8}$)
Substitution												
Single bp	174	(76.3)[a]	54.5	147	(73.1)	21.3	142	(70.3)	186.0	149	(71.6)	79.6
Tandem bp[b]	2	(0.9)	0.6	1	(0.5)	0.1	-			-		
Non-tandem bp	-			-			-			-		
Deletion												
1 bp	25	(11.0)	7.8	23	(11.4)	3.3	4	(2.0)	5.2	24	(11.5)	12.8
>1 bp	5	(2.2)	1.6	2	(1.0)	0.3	1	(0.5)	1.3	-		
Insertion												
1 bp	4	(1.7)	1.2	5	(2.5)	0.7	-			-		
>1 bp	-			-			-			-		
Ty element	13	(5.7)	4.1	18	(9.0)	2.6	53	(26.2)	69.5	33	(15.9)	17.6
Duplication	-			-			1	(0.5)	1.3	-		
Complex change	5	(2.2)	1.6	5	(2.5)	0.7	1	(0.5)	1.3	2	(1.0)	1.1
Total	228		71.4	201		29.0	202		264.6	208		111.1

TABLE 2 (Continued)

Alteration	*REV3 rad18Δ*			*rev3Δ rad18Δ*			*REV3 rad52*			*rev3Δ rad52*		
	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})	Number recovered		Rate (x 10^{-8})
Substitution												
Single bp	197	(92.9)	131.7	188	(90.0)	50.9	226	(95.4)	137.3	165	(86.4)	25.1
Tandem bp	-			1	(0.5)	0.3	-			-		
Non-tandem bp	1	(0.5)	0.7	-			2	(0.8)	1.2	-		
Deletion												
1 bp	4	(1.9)	2.7	14	(6.6)	3.7	8	(3.4)	4.9	8	(4.2)	1.2
>1 bp	2	(0.9)	1.3	1	(0.5)	0.3	-			1	(0.5)	0.1
Insertion												
1 bp	-			2	(0.9)	0.5	1	(0.4)	0.6	11	(5.8)	1.7
>1 bp	-			1	(0.5)	0.3	-			-		
Ty element	6	(2.8)	4.0	1	(0.5)	0.3	-			1	(0.5)	0.1
Duplication	1	(0.5)	0.7	-			-			-		
Complex change	1	(2.2)	1.4	1	(0.5)	0.3	-			5	(2.6)	0.8
Total	212		142.5	209		56.5	237		144.0	191		29.0

[a]Per cent of total events.

[b]One tandem event in the *REV3 RAD* strain featured a triple substitution.

TABLE 3. Multiple mutations, deletions and insertions

Sites[a]	Change[b]	Strain and number detected							
		REV3 *RAD*	*rev3Δ* *RAD*	*REV3* *rad6Δ*	*rev3Δ* *rad6Δ*	*REV3* *rad18Δ*	*rev3Δ* *rad18Δ*	*REV3* *rad52*	*rev3Δ* *rad52*
-6 <-> -5	Ty	-	-	1	-	-	-	-	-
-5, 6 → 7	G → A, -1	2	-	-	-	-	-	-	-
2, 4	A → G, A → G	-	-	-	-	-	-	1	-
6 → 7	-1	1	-	-	-	-	-	-	-
6 <-> 7	Ty	1	2	3	2	-	-	-	-
7 <-> 8	Ty	-	2	-	-	-	-	-	-
8, 10	A → G, C → T	-	-	-	-	1	-	-	-
10	-1	-	-	-	-	-	-	1	-
11 <-> 12	Ty	-	-	-	-	-	1	-	-
17 <-> 18	Ty	1	-	1	1	-	-	-	-
18 <-> 19	Ty	-	-	8	2	1	-	-	-
24	-1	1	-	-	-	-	-	-	-
24 → -144	-168	-	1	-	-	-	-	-	-
25 → 26	-1	-	-	-	-	1	-	-	-

TABLE 3 (continued)

Sites[a]	Change[b]	Strain and number detected							
		REV3 *RAD*	*rev3Δ* *RAD*	*REV3* *rad6Δ*	*rev3Δ* *rad6Δ*	*REV3* *rad18Δ*	*rev3Δ* *rad18Δ*	*REV3* *rad52*	*rev3Δ* *rad52*
26 → 48	duplication of 23 bp at 3→25	-	-	-	-	1	-	-	-
27	-1	-	-	-	-	-	1	-	-
28 <-> 29	Ty	-	1	-	-	-	-	-	-
29 <-> 30	+GCTT	-	-	-	-	-	1	-	-
29 → -139	-168	-	-	1	-	1	-	-	-
31 <-> 32	Ty	-	1	-	-	-	-	-	-
35, 36	A → G, A → G	1	-	-	-	-	-	-	-
35 → 37	+1	-	2	-	-	-	-	-	-
37 <-> 38	Ty	11	10	33	28	4	-	-	1
38 <-> 39	Ty	-	1	-	-	-	-	-	-
39 <-> 40	Ty	-	-	1	-	-	-	-	-
46 → -106	-152	2	-	-	-	-	-	-	-
49 → 63	duplication of 7 bp at 57→63	-	-	1	-	-	-	-	-

TABLE 3 (continued)

Sites[a]	Change[b]	Strain and number detected							
		REV3 *RAD*	*rev3Δ* *RAD*	*REV3* *rad6Δ*	*rev3Δ* *rad6Δ*	*REV3* *rad18Δ*	*rev3Δ* *rad18Δ*	*REV3* *rad52*	*rev3Δ* *rad52*
50 <-> 51	Ty	-	-	2	-	-	-	-	-
51 <-> 52	Ty	-	1	2	-	-	-	-	-
51, 52, 53	C→A, T→G, T→C	1	-	-	-	-	-	-	-
55 → -559	-614	1	-	-	-	-	-	-	-
55, 56	A → T, G → T	-	-	-	-	-	1	-	-
56 <-> 57	Ty	-	-	-	-	1	-	-	-
58 → 64	-7, +GGGCC	3	5	1	2	1	-	-	4
59 <-> 60	Ty	-	-	2	-	-	-	-	-
62, 80	T → C, G → A	-	-	-	-	-	-	1	-
64 → -570	-634	-	-	-	-	-	1	-	1
65 → 67, 74	-1, T → G	-	-	-	-	-	-	-	1
74	-1	1	-	-	-	-	-	-	-
74 → -536	-613	-	1	-	-	-	-	-	-
78	-1	-	-	-	-	-	-	-	1

TABLE 3 (continued)

Sites[a]	Change[b]	Strain and number detected							
		REV3 *RAD*	*rev3Δ* *RAD*	*REV3* *rad6Δ*	*rev3Δ* *rad6Δ*	*REV3* *rad18Δ*	*rev3Δ* *rad18Δ*	*REV3* *rad52*	*rev3Δ* *rad52*
78, 79 → 83	C → T, -1	-	-	-	-	-	1	-	-
79 → -655	-843	-	-	-	-	1	-	-	-
79, 80	G → T, G → A	-	1	-	-	-	-	-	-
79 → 83	-1	22	22	4	23	4	13	7	6
79 → 83	+1	4	3	-	-	-	2	1	10
84 → 86	-1	-	1	-	1	-	-	-	1
84 → 86	+1	-	-	-	-	-	-	-	1
88 → 96	-9	2	-	-	-	-	-	-	-

[a] Sites for deletions of 9, 152, 168, 613, 614, 634, or 843 bp are estimates because short sequence repeats (1 to 6 bp) were 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 Fig. 1).

TABLE 4. Single base-pair substitutions

Substitution	*REV3 RAD*		*rev3Δ RAD*		*REV3 rad6Δ*		*rev3Δ rad6Δ*	
	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)
Transitions								
G·C → A·T	44 (25.3)[a]	13.8	35 (23.8)	5.1	39 (27.5)	51.2	22 (14.7)	11.8
A·T → G·C	27 (15.5)	8.4	30 (20.4)	4.3	15 (10.6)	19.6	1 (0.7)	0.5
Total	71 (40.8)	22.2	65 (44.2)	9.4	54 (38.1)	70.8	23 (15.4)	12.3
Transversions								
G·C → T·A	58 (33.4)	18.2	60 (40.8)	8.8	85 (59.8)	111.3	121 (81.2)	64.7
G·C → C·G	34 (19.5)	10.6	16 (10.9)	2.3	3 (2.1)	3.9	4 (2.7)	2.1
A·T → C·G	4 (2.3)	1.3	1 (0.7)	0.1	-		1 (0.7)	0.5
A·T → T·A	7 (4.0)	2.2	5 (3.4)	0.7	-		-	
Total	103 (59.2)	32.3	82 (55.8)	11.9	88 (61.9)	115.2	126 (84.6)	67.3

TABLE 4 (Continued)

Substitution	*REV3 rad18Δ*		*rev3Δ rad18Δ*		*REV3 rad52*		*rev3Δ rad52*	
	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)	Number recovered	Rate ($\times 10^{-8}$)
Transitions								
G·C → A·T	22 (11.2)	14.7	12 (6.4)	3.2	60 (26.5)	36.5	43 (26.1)	6.5
A·T → G·C	11 (5.6)	7.4	11 (5.8)	3.0	13 (5.8)	7.9	19 (11.5)	2.9
Total	33 (16.8)	22.1	23 (12.2)	6.2	73 (32.3)	44.4	62 (37.6)	9.4
Transversions								
G·C → T·A	151 (76.6)	100.9	159 (84.6)	43.0	53 (23.5)	32.2	74 (44.8)	11.3
G·C → C·G	7 (3.6)	4.7	4 (2.1)	1.1	85 (37.6)	51.6	12 (7.3)	1.8
A·T → C·G	-		-		5 (2.2)	3.0	3 (1.8)	0.5
A·T → T·A	6 (3.0)	4.0	2 (1.1)	0.6	10 (4.4)	6.1	14 (8.5)	2.1
Total	164 (83.2)	109.6	165 (87.8)	44.7	153 (67.7)	92.9	103 (62.4)	15.7

[a]Per cent of total substitutions.

Chapter C

Failure to Detect an Antimutator Phenotype Following Disruption of the *Saccharomyces cerevisiae DDR48* Gene

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Failure to Detect an Antimutator Phenotype Following Disruption of the *Saccharomyces cerevisiae DDR48* Gene.

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Abstract. The antimutator reportedly conferred by disruption of the *Saccharomyces cerevisiae DDR48* gene was suggested to affect only a specific spontaneous mutational pathway. We attempted to identify the types of mutation that are *DDR48*-dependent by determining the specificity of the *ddr48* antimutator. However, disruption of *DDR48* did not decrease the rates of spontaneous forward mutation in a plasmid-borne copy of the yeast *SUP4*-o gene, reversion or suppression of the *lys2-1* allele, or forward mutation at the *CAN1* locus. Interestingly, the latter gene had been reported previously to be subject to the antimutator effect. DNA sequence analysis of spontaneous *SUP4*-o mutations arising in the *DDR48* and *ddr48* backgrounds provided no evidence for a reduction in the rates of individual mutational classes. Thus, we were unable to verify that disruption of *DDR48* causes an antimutator phenotype.

Key words: antimutator - *DDR48* - *Saccharomyces cerevisiae* - spontaneous mutation

Introduction

Transcription of the *DDR48* gene of *Saccharomyces cerevisiae* is increased by cellular exposure to DNA-damaging agents or heat shock (McClanahan and McEntee 1986; Treger and McEntee 1990). Although the function of the *DDR48* gene product is unknown, disruption of the gene has been reported to confer an antimutator phenotype (Treger and McEntee 1990). The rates of *his4-713* reversion and forward mutation at *CAN1* were lowered by ca. 83%-93% and 66%-75%, respectively. The *his4-713* mutation is a single base-pair insertion (Donahue et al. 1981) that might revert by a base-pair deletion and is subject to frameshift suppression (Sherman 1982), presumably involving single base-pair insertions in chromosomal sequences for tRNA anticodons. On the other hand, *CAN1* probably can be inactivated by a variety of DNA sequence alterations. Thus, it was suggested that the lesser effect of the *ddr48* antimutator on mutagenesis at *CAN1* might implicate *DDR48* in a particular spontaneous mutational pathway in yeast, and that *his4-713* reverts by this pathway (Treger and McEntee 1990). If so, one might anticipate a lowered rate of single base-pair deletion and/or addition, in a *ddr48* strain. To assess this possibility, we examined the influence of the *DDR48* disruption devised by Treger and McEntee (1990) on the rates of spontaneous mutation for a plasmid-borne copy of *SUP4*-o, the chromosomal *CAN1* locus, or the *lys2-1* allele, and determined the specificity of spontaneous *SUP4*-o mutagenesis in the *ddr48* background. Unexpectedly, disruption of *DDR48* did not diminish the rates of total mutation in the three systems used or of the individual classes of mutation arising in the *SUP4*-o gene.

Materials and methods

Yeast and bacterial strains. Construction of the haploid, repair-proficient yeast strain MKP-o (*MATα*, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3,112*, *his3-Δ200*, *trp1-Δ901*) has been described (Pierce et al. 1987). An isogenic derivative (JMT48A) of MKP-o having the 3′ 913 bp (70%) of the *DDR48* coding region, and 1.2 kb of the 3′ flanking sequences, deleted and replaced with *LEU2* was provided by J.M. Treger and K. McEntee. The *ddr48*::*LEU2* disruption in JMT48A was also confirmed in our lab via DNA hybridization analysis as described (Treger and McEntee 1990), except that the 3.8 kb BamHI-HindIII pBR48L3-2 (provided by K. McEntee, Treger and McEntee 1990) fragment that encompasses the *DDR48* gene was used as the probe. MKP-o and JMT48A were transformed (Gietz et al. 1992) with YCpMP2 (Pierce et al. 1987) to make MKP-op and JMT48A-p, respectively. YCpMP2, which carries *SUP4*-o, an ochre suppressor allele of a yeast tyrosine tRNA gene, is a yeast centromere-containing vector having components that enable it to replicate autonomously in yeast and bacterial cells. In yeast, such plasmids mimic chromosome behaviour, replicating once per cell cycle in S phase and residing predominantly as single copies in haploid cells (Newlon 1988). YCpMP2 also bears the yeast *URA3* gene which permits selection for the plasmid via complementation of the *ura3-52* allele present in all yeast strains used in this study. Isogenic derivatives of MKP-o (KRMC) and JMT48A (KRJC) having *can1-100* replaced with *CAN1* were constructed using YIplac211-CAN (provided by R.D. Gietz) (this plasmid was assembled by cloning a 1.8 kb BamHI-SalI yeast chromosomal DNA fragment carrying the *CAN1* gene into a derivative of the *URA3*-containing plasmid YIplac211 (Gietz and Sugino 1988) lacking the EcoRI site). YIplac211-CAN

was linearized by EcoRI-catalyzed digestion within the *CAN1* gene, the digest was transformed (Gietz et al. 1992) into MKP-o and JMT48A, and Ura$^+$ colonies were selected. Digestion with *Eco*RI targets YIp*lac211-CAN* integration to the chromosomal *CAN1* locus such that *URA3* and the bacterial DNA sequences of the plasmid are flanked on one end by *CAN1* and on the other by *can1-100*. Insertion of YIp*lac211-CAN* at *CAN1* in several Ura$^+$ transformants was verified using DNA hybridization analysis (data not shown). These isolates were then propagated in appropriately supplemented minimal medium containing uracil and plated on the same medium supplemented with 5-fluoro-orotic acid (Sigma Chemical Company, St. Louis, Missouri) to select for loss of the *URA3* gene (Boeke et al. 1984) and restoration of a single *CAN1* allele via crossing-over between *CAN1* and *can1-100*. The resulting Ura$^-$ colonies were assayed for canavanine sensitivity, and loss of the YIp*lac211-CAN* insert was confirmed by DNA hybridization analysis (data not shown). *Escherichia coli* strain JF1754 (Δ*lac*, *gal*, *metB*, *leuB*, *hisB436*, *hsdR*) (Pierce et al. 1987) was used to retrieve YCpMP2 from yeast strains.

Media. The media used with yeast or bacteria were prepared as described (Boeke et al. 1984; Pierce et al. 1987; Kunz et al. 1991).

Detection of mutations. Forward mutations in *SUP4*-o were detected via reduced suppression of ochre alleles which confer resistance to the arginine analog canavanine (*can1-100*), red pigmentation (*ade2-1*) or lysine auxotrophy (*lys2-1*) (Pierce et al. 1987). Testing for diminished suppression of all three alleles detects at least a 30% decrease in functional suppressor tRNA (Wang and Hopper 1988), and is unlikely to bias mutant recovery significantly (Kohalmi and Kunz 1992). Forward mutations

at *CAN1* were isolated by screening for canavanine-resistance. 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 suppression of *lys2-1* gives rise to white, Lys^+ colonies.

Spontaneous mutagenesis. Yeast strains were inoculated (33 cells per ml) in appropriately supplemented minimal medium (MKP-op, JMT4A-p, MKP-o, JMT48A), or nutrient medium (KRMC, KRJC), and grown at 30°C with shaking to stationary phase. For detection of *SUP4*-o (MKP-op, JMT4A-p) or *CAN1* (KRMC, KRJC) mutants, cell suspensions were diluted when necessary and plated on appropriately supplemented minimal medium, without or with 30 mg of canavanine per litre, to assay viability and plasmid retention (MKP-op, JMT4A-p only) or select canavanine-resistant colonies, respectively. For selection of lysine prototrophs, suspensions (125 ml) of MKP-o or JMT48A were centrifuged to pellet the cells which were then resuspended in 20 ml sterile H_2O. 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. All plates were scored after 6 days incubation at 30°C. For *SUP4*-o mutation, red colonies that appeared on canavanine-containing medium were patched to uracil omission medium, grown overnight at 30°C, and replicated to uracil-lysine omission medium which was then incubated at 30°C for 2 to 3 days. Lys^- colonies were scored as *SUP4*-o mutants.

Mutation rate. Mutation rates were calculated according to the formula (Drake 1991): $\mu_\upsilon = (0.4343Cf_m) / \log (N\mu_\upsilon)$ where μ_υ = mutations per DNA replication, C = reciprocal of efficiency of base-pair substitution (BPS)

detection, calculated (using data from Table 2) 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 (Kohalmi and Kunz 1992)], f_m = median mutation frequency and N = median final population size (MKP-op1: 3.6×10^8; MKP-op2: 3.92×10^8; JMT48A-p: 3.13×10^8; MKP-o: 1.4×10^{10}; JMT48: 1.5×10^{10}; KRMC: 7.4×10^9; KRJC: 8.0×10^9), including three generations on canavanine-containing medium (determined microscopically) before growth of non-resistant cells terminates. There was no residual growth on lysine omission medium. The correction factor C, which accounts for mutations that occur but are not detectable by a particular system, could not be used for calculation of the *CAN1* → *can1* or *lys2-1* → Lys^+ rates. Thus, in these two cases the values given may be slight underestimates, but the degree of underestimation should be the same for the *DDR48* and *ddr48* strains.

DNA isolation, bacterial transformation and DNA sequencing for characterization of SUP4-o mutations. DNA for bacterial transformation was released from yeast cells by disruption with glass beads (Mis and Kunz 1990). Bacterial cells were transformed using $CaCl_2$ (Pierce et al. 1987). YCpMP2 was isolated from *E. coli* by alkaline extraction (Kohalmi et al. 1991). *SUP4*-o alleles were sequenced on double-stranded YCpMP2 molecules, using dideoxynucleotides, as described (Kunz et al. 1987).

Results and discussion

In order to attempt identification of *DDR48*-dependent class(es) of spontaneous mutation, we used the *SUP4*-o system (Pierce et al. 1987) which facilitates characterization of DNA sequence alterations occurring in a plasmid (YCpMP2)-borne copy of a yeast tRNA gene. Cultures of MKP-op (*DDR48*) and JT48A-p (*ddr48*) were grown from low titre inocula to stationary phase in medium selective for YCpMP2, and the cells were plated to measure plasmid retention and isolate *SUP4*-o mutants. Since the pairs of wildtype and *ddr48* strains compared in this study are isogenic except for the *DDR48* locus, any differences detected should reflect disruption of *DDR48* rather than variation in genetic background. Plasmid retention was determined by comparing the number of colonies that emerged on medium selective for the plasmid (YCpMP2 carries the *URA3* gene and the strains have the *ura3-52* allele) with the corresponding number for nonselective medium. Disruption of *DDR48* did not decrease plasmid stability (MKP-op: 10,879/12,599 = 86%; JT48A-p: 5,933/6,669 = 89%) or the rate of *SUP4*-o mutation, which for the *ddr48* strain fell within the range observed for two independent assessments made with MKP-op (Table 1). The latter result was surprising given that the *ddr48* antimutator reportedly decreased the forward mutation rate at the chromosomal *CAN1* locus by two-thirds to three-quarters (Treger and McEntee 1990).

The failure to observe an antimutator phenotype for *SUP4*-o mutation might be explained if the *DDR48* gene product functions in the mutagenesis of chromosomal genes but not those located on plasmids. This possibility, although interesting, seemed unlikely since, so far, all yeast mutators (*apn1*, *dcd1*, *pol3*, *pms1*, *rad1*, *rad3*, *rad6*, *rad18*, *rad52*, *ung1*) and the one

other antimutator (*rev3*) tested modulate the spontaneous mutation rate for *SUP4*-o on YCpMP2 in the same fashion as they do for chromosomal loci (Kunz et al. 1989; 1990; 1991; 1994; Impellizzeri et al. 1991; Kohalmi et al. 1991; Kang et al. 1992; Roche et al. 1994; Yang and Kunz 1994). Nevertheless, we also examined the effect of the *ddr48::LEU2* disruption on locus reversion and extragenic suppression of the chromosomal *lys2-1* allele, and forward mutation at the chromosomal *CAN1* locus. The only endpoint that showed a diminished rate in the *ddr48* background was intragenic reversion of *lys2-1* (Table 1). However, the 30% decrease in the rate was considerably less than the 66%-93% mutation rate reductions reported previously for disruption of *DDR48* (Treger and McEntee 1990). Furthermore, intragenic reversion of the ochre *lys2-1* allele almost certainly would involve a base-pair substitution, but it was suggested that *DDR48* might primarily influence a base-pair deletion/addition pathway for spontaneous mutagenesis (Treger and McEntee 1990). Therefore, given that the rate of extragenic suppression of *lys2-1* actually appeared to increase by 2.5-fold in the *ddr48* strain, and no change was observed for the forward mutation rate at *CAN1*, it is doubtful that the minor decrease in the *lys2-1* locus reversion rate was genuine.

Another potential explanation for the lack of an antimutator effect with regard to *SUP4*-o mutation is that single base-pair deletion/addition is responsible for a only minor fraction of the total spontaneous mutations in *SUP4*-o (Kang et al. 1992; Roche et al. 1994) (the proportion of spontaneous *can1* mutations attributable to base-pair loss or gain has not been determined). Thus, if *DDR48* is involved primarily in the production of these events, the effect of disrupting this gene on spontaneous mutagenesis might be obscured when measuring the overall rate

of *SUP4*-o mutation. In order to evaluate this suggestion, it was necessary to compare the rates of the individual classes of mutation occurring spontaneously in the *DDR48* and *ddr48* strains.

To do so, 234 spontaneous *SUP4*-o mutations arising in the *ddr48* strain were characterized by DNA sequencing. The resulting mutational spectrum was compared to two spectra obtained previously for spontaneous mutagenesis in MKP-op. Data set 1, designated MKP-op1, consists of 354 *SUP4*-o mutations (Kang et al. 1992), whereas data set 2 (MKP-op2) features 228 mutations (Roche et al. 1994). Although the difference in the mutation rates for MKP-op1 and MKP-op2 is small, both data sets are shown to give an indication of the ranges for the rates of specific types of mutation. The independence of the mutations analyzed was ensured by choosing only one mutant per culture for MKP-op1. Two mutants were chosen per culture for the *ddr48* strain and MKP-op2 but if both members of a pair were subsequently found to have the same sequence alteration, the result for just one was counted. Only a few mutations were rejected as potential siblings (MKP-op2: 3/231; JT48A-p: 6/240), so that any effect of this restriction on the relative proportions of mutational classes should have been negligible.

Table 2 shows that single and non-tandem double base-pair substitutions, single base-pair deletions and insertions, multiple base-pair deletions, insertions of the yeast retrotransposon Ty, duplications and more complex changes were detected in both strains. Both the relative fractions and rates of these events in the *ddr48* background were within, or close to, the corresponding fraction and rate ranges for MKP-op. A more detailed examination of the single base-pair substitutions did not reveal any notable difference in rates between the two strains (Table 3).

Evidently, disruption of *DDR48* did not confer an antimutator phenotype with respect to the mutational classes detected in *SUP4*-o, including base-pair deletion/addition.

In contrast to a previous report (Treger and McEntee 1990), the results of this study provided no evidence for an antimutator effect associated with disruption of *DDR48*, even when spontaneous mutagenesis at a locus (*CAN1*) previously reported to be influenced by the *ddr48* antimutator was tested. At present, we have no obvious explanation for this discrepancy. Perhaps the *ddr48* antimutator is highly sensitive to differences in genetic background. Unfortunately, we could not assess the effect of disrupting *DDR48* on reversion of *his4-713* in our strains since MKP-o does not carry this allele. Still, our results question the reproducibility of *ddr48* antimutator phenotype, and so point to the need for further investigation.

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Table 1. Spontaneous mutation in isogenic *DDR48* and *ddr48*⁻ strains

Location and type of mutation scored	Strain	Mutation frequency[a]	Mutation rate
Plasmid: *SUP4*-o → *sup4*⁻	MKP-op1 (*DDR48*)	1.7×10^{-6}	4.8×10^{-7}
	MKP-op2 (*DDR48*)	2.7×10^{-6}	7.1×10^{-7}
	JMT48A-p (*ddr48*⁻)	2.5×10^{-6}	6.5×10^{-7}
Chromosome: *lys2-1* → Lys⁺ (reversion)	MKP-o (*DDR48*)	4.7×10^{-9}	1.0×10^{-10}
	JMT48A (*ddr48*⁻)	3.3×10^{-9}	0.7×10^{-11}
Chromosome: *lys2-1* → Lys⁺ (suppression)	MKP-o (*DDR48*)	1.5×10^{-7}	2.5×10^{-8}
	JMT48A (*ddr48*⁻)	3.8×10^{-7}	5.7×10^{-8}
Chromosome: *CAN1* → *can1*	KRMC (*DDR48*)	6.2×10^{-7}	9.4×10^{-8}
	KRJC (*ddr48*⁻)	6.0×10^{-7}	9.0×10^{-8}

[a] Frequencies are the medians for 23 (MKP-op1), 30 (MKP-op2), 20 (JMT48-p), or 10 (MKP-o, JMT48A, KRMC, KRJC) independent cultures. Data for MKP-op1 and MKP-op2 are from Kang et al. (1992) and Roche et al. (1994), respectively.

Table 2. Sequence alterations in *SUP4*-o mutants[a]

DNA sequence alteration	MKP-op1 (*DDR48*) No. detected (% of total)	MKP-op1 (*DDR48*) Rate ($\times 10^{-8}$)	MKP-op2 (*DDR48*) No. detected (% of total)	MKP-op2 (*DDR48*) Rate ($\times 10^{-8}$)	JMT48A-p (*ddr48*$^-$) No. detected (% of total)	JMT48A-p (*ddr48*$^-$) Rate ($\times 10^{-8}$)
Substitution						
Single bp	290 (81.9)	39.3	174 (76.3)	54.5	184 (78.7)	51.4
Tandem bp	-		2 (0.9)	0.6	-	
Non-tandem bp	2 (0.6)	0.3	-		1 (0.4)	0.3
Deletion						
1 bp	24 (6.8)	3.3	25 (11.0)	7.8	11 (4.8)	3.1
>1 bp	7 (2.0)	1.0	5 (2.2)	1.6	2 (0.8)	0.6
Insertion						
1 bp	1 (0.3)	0.14	4 (1.7)	1.2	5 (2.1)	1.4
Ty element	26 (7.3)	3.5	13 (5.7)	4.1	27 (11.5)	7.5
Duplication	1 (0.3)	0.14	-		1 (0.4)	0.3
Complex change	3 (0.8)	0.4	5 (2.2)	1.6	3 (1.3)	0.8
Total	354	48.08	228	71.4	234	65.4

[a] Data for MKP-op1 and MKP-op2 are from Kang et al. (1992) and Roche et al. (1994), respectively.

Table 3. Single base-pair substitutions[a]

Substitution	MKP-op1 (*DDR48*) No. Detected (% of total)	MKP-op1 (*DDR48*) Rate ($\times 10^{-8}$)	MKP-op2 (*DDR48*) No. Detected (% of total)	MKP-op2 (*DDR48*) Rate ($\times 10^{-8}$)	JMT48A-p (ddr48) No. Detected (% of total)	JMT48A-p (ddr48) Rate ($\times 10^{-8}$)
Transitions						
G·C → A·T	76 (25.8)	10.2	44 (25.3)	13.8	49 (26.6)	13.7
A·T → G·C	44 (15.0)	5.9	27 (15.5)	8.4	13 (7.2)	3.6
Total	120 (40.8)	16.1	71 (40.8)	22.2	62 (33.8)	17.3
Transversions						
G·C → T·A	99 (33.7)	13.3	58 (33.4)	18.2	68 (36.9)	19.0
G·C → C·G	59 (20.1)	7.9	34 (19.5)	10.6	42 (22.8)	11.7
A·T → C·G	5 (1.7)	0.7	4 (2.3)	1.3	5 (2.7)	1.4
A·T → T·A	11 (3.7)	1.6	7 (4.0)	2.2	7 (3.8)	2.0
Total	174 (59.2)	23.5	103 (59.2)	32.3	122 (66.2)	34.1

[a] Data for MKP-op1 and MKP-op2 are from Kang et al. (1992) and Roche et al. (1994), respectively.

CONCLUDING REMARKS

Summary of Results

Chapter A: Assessment of the Specificity of the *rev3*Δ Antimutator and the Role of *REV3* in the *rad1*Δ Mutator Effect

There are several mechanisms which cells can employ in order to recover from the attack of intracellularly-generated DNA-damaging agents. Among these, damage tolerance allows the cell to survive in spite of persisting DNA lesions. One form of damage tolerance involves replication of DNA past the damage site(s), and is termed translesion synthesis. Translesion synthesis, however, is not error-free and hence can contribute to mutagenesis resulting from spontaneously-arising damage. The *REV3* gene of *Saccharomyces cerevisiae* appears to encode a DNA polymerase which has been predicted to function in translesion synthesis. *rev3* mutants exhibit an antimutator phenotype suggesting that this polymerase participates in spontaneous mutagenesis, and so pointing to an important role for translesion synthesis in the production of spontaneous mutations. Yet the types of mutational change generated by the *REV3* gene product were not identified. Translesion synthesis also was suggested to contribute to the mutator phenotype conferred by inactivation of the nucleotide excision repair gene *RAD1*, an hypothesis that also remained to be tested.

To assess the role of *REV3* in the specificity of spontaneous mutagenesis and the mutator effect caused by a *RAD1* deletion, the types and distributions of spontaneous *SUP4*-o mutations were characterized in isogenic strains having combinations of wild-type and mutant *REV3* and *RAD1* alleles. DNA sequencing was used to characterize 201, 202, 249 and 228 *SUP4*-o mutations in *REV3 RAD1*, *rev3*Δ *RAD*, *REV3 rad1*Δ and*rev3*Δ *rad1*Δ

strains, respectively. Relative to the wild-type parent, inactivation of *REV3* primarily decreased the rates of single base pair substitution and deletion, although the rates of all classes of mutations detected in both strains were reduced to some extent. Thus, the *rev3Δ* antimutator seemed to have a much broader effect than any of the prokaryotic antimutators described to date. The *rad1Δ* mutator increased the rates of single base pair substitution and deletion as well as insertion of the yeast retrotransposon Ty. The rates of all three classes of mutation were substantially reduced in the *rev3Δ rad1Δ* strain. Furthermore, comparison of the effects of *rev3Δ* in the *RAD1* and *rad1Δ* backgrounds revealed that the bulk of the *rad1Δ* mutator effect could have been due to the same *REV3*-dependent processes that produced the majority of spontaneous mutations in the wild-type strain. Taken collectively, these results were consistent with roles for translesion synthesis in spontaneous mutagenesis and the *rad1Δ* mutator effect.

Chapter B: *REV3* Dependency of the *rad6Δ*, *rad18Δ* and *rad52* Mutator Effects

There are three different epistasis groups for the repair of UV-induced DNA damage in *S. cerevisiae*. Certain *RAD* genes in each of these groups had been found to confer mutator effects when mutated. *RAD1* belongs to the group which is involved in nucleotide excision repair. As detailed above (Chapter A), evidence was obtained that the mutator associated with a *rad1* deletion was largely dependent on the function of the *REV3* gene. *rev3* mutations were reported earlier to offset certain of the *rad* mutator effects, but the influence of the *rev3* antimutator on the specificities of these mutators was not examined. Thus, it was of interest to determine the extent to which mutators caused by defects in genes belonging to the other

two epistasis groups also were *REV3*-dependent.

Another epistasis group is concerned with postreplication repair and mutation induction. Mutators due to defects in two members of this group, *RAD6* and *RAD18*, increase the rates of spontaneous base pair transition, G•C → T•A transversion and Ty insertion, or only G•C → T•A transversion, respectively. The remaining epistasis group functions in recombinational repair of DNA double-strand breaks, and inactivation of a representative of this group, *RAD52*, increases the rate of substitution at G•C pairs. Once again, wild-type amd mutant alleles of the various genes were combined in the same genetic background, *SUP4*-o mutations that arose spontaneously in these strains were characterized, and the resulting mutational spectra were compared. The *REV3* gene product was found to participate in the production of all mutational classes promoted by the three *rad* mutators, but to different extents. For example, the production of G•C → A•T transitions in the *rad6*Δ background was largely *REV3*-dependent, whereas the promotion of G•C → T•A transversions was mainly *REV3*-independent in the *rad18*Δ strain, and *REV3* was essential for the entire *rad52* mutator effect. Given the different phenotypes of the mutators examined, it would appear that the *REV3* gene product participates in processing a number of different types of spontaneous DNA lesions. Although *REV3*-independent mechanisms handle some of this damage, it seems that the failure to repair certain types of spontaneous damage can lead to mutations only via a *REV3*-dependent pathway, possibly translesion synthesis. Collectively, the results of Chapters A and B suggest that translesion synthesis might have a far more important role than error-prone repair in the processing of spontaneous DNA damage into mutations in yeast.

Chapter C: Attempt to Characterize the Specifity of the *ddr48* Antimutator

Treatment with DNA-damaging agents or heat shock stress increases the rate of transcription of the yeast *DDR48* gene. The function of this gene is unknown, although it has been reported that disruption of *DDR48* confers an antimutator phenotype. Furthermore, the antimutator was suggested to affect only a specific mutational pathway involving loss of base pairs. To investigate this possibility, spontaneous *SUP4*-o mutations were isolated in a *ddr48* strain. Unexpectedly, comparison of the *SUP4*-o mutation rates in the isogenic wild-type and *ddr48* backgrounds provided no evidence for an antimutator effect. However, *SUP4*-o is carried on a plasmid and the *ddr48* antimutator might have been specific for chromosomal genes. Yet, an antimutator phenotype could not be confirmed by measuring the rates of forward mutation at the chromosomal *CAN1* locus (which was previously reported to be diminished by the *ddr48* antimutator) or reversion of the chromosomal *lys2-1* allele. Still, these results might have been explained by a highly specific effect of the *DDR48* gene product, such as the production of only a single class of mutation. A detailed analysis of the *SUP4*-o mutations isolated in the *ddr48* strain revealed no decrease in the rate of any individual class of mutation. Aside from some differences in experimental protocols, there did not appear to be any obvious explanation for the failure to confirm the *ddr48* antimutator phenotype. The results suggested that a re-assessment of this antimutator is in order.

Future Experimentation

The results of the *REV3* studies suggest several other areas of investigation that could be explored in the future. These include

determining the dependency of other *rad* mutators on *REV3*. It would be of interest to examine whether the effect of the *rev3*Δ antimutator is similar for *rad* mutators derived from genes in the same repair pathway, e.g. *RAD2*, *RAD3*, *RAD10*, *RAD14* and *RAD25* for nucleotide excision repair, and *RAD51* and *RAD54* for DNA double-strand break repair. The *REV3* gene could be deleted in all these strains, thereby eliminating the effect of the putative *REV3* polymerase and its role in translesion synthesis. The effect of the *rev3*Δ antimutator on the specificity of the *rad* mutators in the nucleotide excision repair and strand break repair pathways could then be compared to the data obtained for *rad1*Δ and *rad52* in this study.

Another yeast antimutator, *ant1*, has been described (Quah et al. 1980) but neither its specificity, nor its affect on *rad* mutators has been determined. By construction of appropriate double mutants, the influence of *ant1* on spontaneous mutagenesis could be compared to that for *REV3*. In this way it would be possible to determine whether the *REV3*-independent components of spontaneous mutagenesis rely on the *ANT1* gene.

Finally, it would also be important to begin identifying DNA lesions whose mutagenicity depends, at least to some extent, on *REV3*. Two approaches could be taken. First, the *REV3* dependence of mutators due to defects in the repair of specific spontaneous lesions could be evaluated. For example, the *APN1* and *MGT1* genes are known to function in the repair of AP sites and O^6-alkylguanine, respectively. Construction of appropriate double mutants would permit one to assess the influence of *REV3* on mutagenesis resulting from the failure to repair these types of spontaneously-arising DNA lesions. Second, particular DNA lesions could be built at specific sites in a plasmid-borne copy of *SUP4*-o. These plasmids could then be transformed into wild-type and *rev3*Δ strains and the effect

of the *REV3* deletion on site-specific mutagenesis determined. In these ways, one could gain insights into the types of damage processed by the *REV3*-dependent pathway.

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