Characterization of Mutator Phenotypes Conferred by *rad* Genes of *Saccharomyces cerevisiae*

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ABSTRACT

The *RAD1*, *RAD6* and *RAD18* genes are required for the repair of DNA damage in the yeast *Saccharomyces cerevisiae*. *RAD1* functions in excision repair, *RAD6* encodes an ubiquitin conjugating enzyme, and *RAD18* plays a role in post-replication repair. Inactivation of any of these three genes confers a mutator phenotype. To determine the specificities of these mutator effects, collections of 249, 202 or 212 *SUP4*-o mutations arising spontaneously in *rad1*, *rad6* or *rad18* strains, respectively, were characterized by DNA sequencing. Comparisons of the resulting spectra with the spectrum of spontaneous changes for an isogenic wild-type strain revealed a number of interesting features. The *rad1* mutator phenotype was associated with increases in the frequencies of single base-pair substitution, single base-pair deletion and insertion of the yeast retrotransposon Ty. The relative fractions of the single base-pair events and their distributions within *SUP4*-o exhibited features similar to those for spontaneous mutagenesis in the isogenic wild-type background. The *rad6* mutator enhanced the frequencies of base-pair transitions and the G·C -> T·A transversion, as well as Ty insertion. Relative to the wild-type parent, Ty inserted at considerably more *SUP4*-o positions in the *rad6* strain with a significantly smaller fraction of insertions detected at a hotspot for transposition in the parent strain. The increase in the frequency of substitutions accounted for the bulk of the *rad6* mutator effect and analysis of the distribution of these events within the *SUP4*-o gene suggested that the site specificity of the substitutions was influenced by DNA sequence context. The *rad18* mutator specifically enhanced the frequency of single base-pair substitutions, and an increase

in the frequency of G·C -> T·A transversion accounted for the elevated *SUP4*-o mutation frequency in this strain thus, *rad18* is the first eukaryotic mutator found to generate only a particular base-pair substitution. The majority of G·C pairs that were not mutated in the *rad18* background were at sites where G·C -> T·A events can be detected in *SUP4*-o, indicating that DNA sequence context influences the *rad18* mutator effect. To assay the role of mismatch correction in the mutator effects, nicked heteroduplex plasmid DNAs containing defined mismatches were constructed *in vitro* and transformed into the *rad1*, *rad6*, *rad18* and wild-type strains. The results demonstrated that none of the mutators reduced the efficiency of correcting mismatches that could have given rise to the base-pair substitutions whose frequencies were increased in the relevant strains. In addition, the mutators did not cause preferential restoration of the mismatches to the incorrect base-pairs. Finally, adenine methylation at a GATC sequence in *SUP4*-o did not direct the correction of mismatches via excision repair, although excision repair functions can remove methylated adenine from yeast DNA. These results are discussed in relation to possible mechanisms by which *RAD1*, *RAD6* and *RAD18* functions might influence spontaneous mutation rates.

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1 INTRODUCTION AND LITERATURE REVIEW

Spontaneous mutations are believed to arise as a consequence of intracellular events including the occurrence of spontaneous DNA damage, insertion of transposable elements, misalignment of DNA strands and inaccurate DNA replication (von Borstel 1969; Cox 1976; Hastings et al. 1976; Calos and Miller 1980; Lawrence 1982; Loeb and Kunkel 1982; Ripley and Glickman 1983; Friedberg 1985; Golding and Glickman 1985; Sargentini and Smith 1985; Loeb and Preston 1986; Kunkel and Soni 1988). The latter may be due directly to base misinsertion by polymerases or indirectly to tautomeric base shifts, alterations in DNA precursor levels or spontaneous damage that modifies base-pairing properties (Drake et al. 1983; Friedberg 1985a; Roberts and Kunkel 1986; Kunz 1988). Thus, it seems reasonable to believe that spontaneous mutation rates are kept low, in part, through the action of a variety of biochemical mechanisms that promote the repair of damaged DNA and the fidelity of DNA replication.

1.1 Spontaneous Mutation and DNA Repair Deficiency

Defects in DNA repair are often associated with elevated levels of spontaneous mutation in both prokaryotes and eukaryotes (Sargentini and Smith 1985). In the yeast *Saccharomyces cerevisiae*, mutations in as many as 30 genes that sensitize cells to one or more DNA damaging agents confer mutator phenotypes (Haynes and Kunz 1981). According to the DNA damage-repair hypothesis (Haynes and Kunz 1981), there exist two classes of DNA repair systems: those that are involved in the production of mutations are said to be "error-prone" or mutagenic; all other repair systems that are involved in the elimination of pre-mutational lesions are said to be "error-free". To account for the association between repair defects and enhanced spontaneous mutagenesis in yeast, the repair channelling hypothesis has been invoked. It has been hypothesized that when a

particular repair pathway is eliminated, the lesions that would have been repaired by that pathway are channelled along other, normally competitive pathways (Brendel et al. 1973; Game and Cox 1973). Analogously, the association of mutator phenotypes with DNA repair defects has been attributed to channelling of spontaneous lesions through one or more error-prone, damage processing pathways (Hastings et al. 1976; Quah et al. 1980). However, the nature of these error-prone pathways has remained obscure, and generally the magnitudes of the mutator effects in yeast cells are relatively small. Consequently, it has been suggested that repair defects might have a more indirect influence on spontaneous mutation, perhaps by somehow reducing the fidelity of DNA replication (Sargentini and Smith 1985) or the efficiency of correcting replication errors (Lawrence 1982). Alternatively, the products of some putative repair genes might play a regulatory role (Chanet et al. 1988; Jones et al. 1988) so that modulation of processes other than DNA repair could be responsible for the mutator phenotypes of certain repair-deficient mutants. Here I consider the properties of *RAD1*, *RAD6* and *RAD18*, three yeast DNA repair genes that confer mutator phenotypes when defective.

1.1.1 *RAD1*

The product of the *RAD1* gene is believed to function at the incision step of excision repair of bulky DNA adducts (Friedberg 1988), interstrand crosslinks (Miller et al. 1982) and methylated adenine or cytosine (Hoekstra and Malone 1986; Féher et al. 1989). Thus, it is not surprising that defects in this gene increase sensitivity to a variety of DNA damaging agents including ultraviolet (UV) radiation, the UV-mimetic chemical 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).

Not only do *RAD1* deficiencies sensitize cells to the lethal effects of certain genotoxic agents, but also they confer a mutator phenotype (Moustacchi 1969; von Borstel and Hastings 1980; Sargentini and Smith 1985). Both enhanced locus reversion to prototrophy and forward mutation to suppression and canavanine resistance have been reported, but neither the precise mutational changes involved, nor the specificity of the mutator effect, have been elucidated. In addition, *RAD1* defects enhance UV and 4-NQO-induced mutagenesis (Lawrence and Christensen 1976; Prakash 1976) and UV-induced mitotic inter-chromosomal recombination (Snow 1968). Recent findings suggest that the *RAD1* gene product also plays a role in mitotic intra-chromosomal recombination and in integration of linear DNA molecules into homologous genomic sequences (Klein 1988; Schiestl and Prakash 1988; Aguilera and Klein 1989). In studies on transcriptionally linked genetic recombination in *S. cerevisiae*, Keil and Roeder (1984) found that a *rad1* mutation reduced transcription-dependent recombination. This led to the suggestion that the *RAD1* protein might be involved in the unfolding of the genome during transcription, thereby facilitating recombination. Also, it was reported that a defect (*pms1*) in mismatch correction enhances mitotic recombination between duplicated, but non-identical, S-adenosylmethionine synthetase genes and this increase is dependent on *RAD1* (Bailis and Rothstein 1990). To account for this role of *RAD1* in ectopic recombination, it was suggested that the *RAD1* gene product might recognize mismatches and incise adjacent to them on one strand, marking the mispairs for further processing.

The *RAD1* gene has been cloned in two laboratories (Higgins et al.

1983; Yang and Friedberg 1984; Reynolds et al. 1987). *RAD1* contains an open reading frame of 3,300 nucleotides. A *RAD1-lacZ* fusion gene expressed very low levels of ß-galactosidase activity relative to that expressed from a *HIS4-lacZ* fusion (Friedberg 1985b) and *HIS4* is a highly expressed yeast gene (Silverman et al. 1982). In addition, only low levels of *RAD1* mRNA exist in yeast cells (Friedberg 1988). These findings suggest that *RAD1* 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).

1.1.2 *RAD6*

The yeast *RAD6* gene has been cloned and found to encode an ubiquitin-conjugating enzyme (Jentsch et al. 1987). Protein modification by attachment of ubiquitin has been implicated in the control of protein degradation and non-proteolytic modulation of protein structure and activity in eukaryotic cells (for reviews, see Hershko 1988; Jentsch et al. 1990; Monia et al. 1990). Ligation of ubiquitin to target proteins is a multi-step process initiated by ATP-dependent binding of ubiquitin to the ubiquitin-activating enzyme (E_1). Next, the activated ubiquitin is transferred to ubiquitin-conjugating enzymes (E_2s). Finally, the E_2 enzymes catalyze the production of an isopeptide bond between ubiquitin and target proteins. Alternatively, the E_2s may act as ubiquitin carriers for isopeptide ligase (E_3) which can also join ubiquitin to target proteins. Consistent with an important role for ubiquitin conjugation in DNA metabolism, inactivation of *RAD6* confers a highly pleiotropic phenotype. The reported properties of *rad6* mutants include extreme sensitivity to a wide 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 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* E_2 to bind ubiquitin result in the same defects as deletion of *RAD6* (Sung et al. 1990).

In vitro, the core histones H2A, H2B and H3 are targets for the *RAD6* enzyme (Jentsch et al. 1987; Haas et al. 1991). Thus, it has been suggested that chromatin remodelling, as a direct or indirect consequence of ubiquitination of chromosomal proteins, is central to *RAD6*-dependent processes (Jentsch et al. 1987). Yet, an immunological assay failed to detect ubiquitinated histone H2A in yeast cell extracts and mutants lacking the H2A sequence ubiquitinated in higher eukaryotes did not exhibit the deficiencies typically associated with *RAD6* mutations (Swerdlow et al. 1990). Although the identities of the targets for the *RAD6* enzyme *in vivo* remain to be established, it has been speculated that the *RAD6* ubiquitin-conjugating enzyme might activate proteins involved in DNA repair, including those assigned to the *RAD6* epistasis group for resistance to UV-induced DNA damage (Sung et al. 1990).

Together, the identity of the *RAD6* gene product and the *rad6* mutator phenotype support the intriguing possibility that ubiquitination of proteins is involved in maintaining spontaneous mutagenesis at acceptably low levels. One way in which this seems to occur is through regulation of transposition. *rad6* mutations were shown to markedly enhance the frequency of insertion of the yeast Ty element into two different loci (Picologlou et al. 1990). However, it was concluded that stimulation of Ty

transposition did not account for the entire increase in the spontaneous mutation frequency attributed to the *rad6* mutator. Indeed, it had been found earlier that the rate of reversion of the ocher allele *lys1-1* was elevated by the *rad6-1* allele (Hastings et al. 1976), arguing that the occurrence of at least one other class of mutation must also be promoted in *rad6* strains. These results suggest that there is more than one mechanism whereby *RAD6*-mediated protein ubiquitination functions to limit spontaneous mutation rates.

1.1.3 *RAD18*

The *RAD18* gene of yeast is believed to function in the repair of DNA damage (Haynes and Kunz 1981). Originally isolated on the basis of increased sensitivity to the lethal effects of UV and X-rays (Resnick 1969), *rad18* mutations also sensitize cells to killing by gamma-rays (Mckee and Lawrence 1979), 4-NQO (Prakash 1976), the folate antagonist trimethoprim (Game et al. 1975), the antitumor antibiotic bleomycin (Moore 1978) and a number of mono-and bifunctional alkylating agents (Brendel et al. 1973; Lawrence et al. 1974; Prakash 1976; Cooper and Waters 1987). In addition, defects in *RAD18* confer a mutator phenotype (Quah et al. 1980; von Borstel et al. 1971), increase spontaneous, UV and gamma-ray-induced mitotic recombination (Boram and Roman 1976; Saeki et al. 1980), enhance the induction of reverse mutation by nitrous acid (Prakash 1976) and decrease UV, ethylmethanesulfonate and 4-NQO-induced reversion of certain *cyc1* alleles (Lawrence et al. 1974; Prakash 1976; Lawrence and Christensen 1979). Furthermore, *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 methylmethane-sulfonate sensitivity or the mutator phenotype (von Borstel et al. 1971;

Boram and Roman 1976).

Despite the radiation sensitivity of *rad18* strains, they 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). However, the efficiency of strand break repair is obscured by a progressive decrease in DNA size upon post gamma-irradiation incubation (Mowat et al. 1983). There is evidence that a component of post-replication repair in yeast requires the *RAD18* gene product (di Caprio and Cox 1981; Prakash 1981), and it 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).

Recently, the *RAD18* gene has been sequenced and predicted to encode a product having amino acid sequences that show homology with those presumed responsible for the ability of certain proteins to bind DNA or bind and hydrolyze nucleotides (Chanet et al. 1988; Jones et al. 1988). On this basis, it has been speculated that the *RAD18* protein might bind at sites of DNA damage and/or act as a trancriptional regulator (Chanet et al. 1988; Jones et al. 1988).

1.2 Mismatch Correction

One process that contributes to the fidelity of DNA replication is mismatch correction. Both prokaryotes and eukaryotes correct mismatches, pairing errors in which the DNA bases occur in noncomplementary opposition within the DNA helix (Holliday 1974). Such mismatches arise mainly from errors in DNA replication, hybrid DNA formation between homologous, but nonidentical DNA sequences during recombination, and spontaneous chemical modification of bases in duplex DNA (e.g. deamination of 5-methylcytosine

to form thymine) (Radman and Wagner 1986; Modrich 1987; Lahue and Modrich 1988). Since mismatch correction recognizes and eliminates mismatches generated in these ways, it functions in ensuring the accuracy of DNA replication (Nevers and Spatz 1975; Wagner and Meselson 1976), avoiding spontaneous mutation and maintaining the fidelity of genetic exchanges (Feinstein and Low 1986; Rayssiguier et al. 1989).

1.2.1 Mismatch Correction in *Esherichia coli*.

Among prokaryotes, mismatch correction is best understood in *E. coli* which is known to possess multiple mismatch correction pathways (Claverys and Lacks 1986; Radman and Wagner 1986; Modrich 1987; Radman 1987). These have been identified by *in vivo* transfection experiments and *in vitro* cell-free extract assay methods. The former experiments involved artificially constructed heteroduplex bacteriophage DNA containing allelic variation within the two DNA strands, each of which was genetically distinct. The phenotypes of the progeny phage produced following transfection allowed quantitation of mismatch repair (White and Fox 1975; Wildenberg and Meselson 1975; Wagner and Meselson 1976). In the *in vitro* method, a mismatch within the recognition sequence of a type II restriction endonuclease was placed in heteroduplex DNA. After exposure of the heteroduplex DNA to cell-free extracts or purified proteins, the DNA was treated with the appropriate restriction enzyme to assay repair of the mismatch (Lu et al. 1983; Lahue et al. 1987; Su et al. 1988).

Three major types of mismatch repair systems have been identified in *E. coli*: (i) the methyl-directed mismatch correction system, or *mutHLS*-dependent pathway, corrects a variety of mismatches with different efficiencies in a strand specific manner, with the methylation state of d(GATC) sequences directing strand discrimination in the mismatch repair

reaction; (ii) the very short patch (VSP) mismatch correction system specifically corrects G-T mismatches to G·C pairs; (iii) the *mutY*-dependent mismatch correction system specifically corrects G-A mismatches to G·C pairs.

1.2.1.1 Methyl-Directed Mismatch Correction

The existence of the methyl-directed pathway was predicted in 1976 by Wagner and Meselson. They suggested that strand-specific processing of mismatches within newly synthesized DNA could serve to eliminate DNA biosynthetic errors and thus contribute to the overall fidelity of chromosome replication. This system has been the most extensively studied (Lu et al. 1983; Kramer et al. 1984; Radman and Wagner 1986; Modrich 1987, 1989; Lahue and Modrich 1988; Meselson 1988; Su et al. 1988; Lahue et al. 1989; Grilley et al. 1990) and methyl-directed repair appears to be the primary mechanism for the correction of replication errors. Indeed, it has been estimated that over 99% of all replication errors are repaired by this system (Glickman and Radman 1980).

Methyl-directed mismatch correction is a strand specific reaction. The strand discrimination of mismatch repair is directed by the state of adenine methylation at d(GATC) sequences (Wagner and Meselson 1976; Lu et al. 1983; Pukkila et al. 1983; Kramer et al. 1984; Wagner et al. 1984; Dohet et al. 1986; Raposa and Fox 1987). The importance of these sequences was demonstrated by the finding that removal of d(GATC) sites by mutagenesis resulted in a dramatic reduction of correction both *in vivo* and *in vitro* (Lahue et al. 1987, 1989; Längle-Rouault et al. 1987). Transfection experiments with phage λ heteroduplexes showed that: (i) if one chain is highly methylated and other is not methylated, the repair reaction is confined to the unmethylated strand with the modified strand

serving as the template; (ii) when neither strand is methylated, mismatch correction may occur on either strand with little strand bias; and (iii) if the heteroduplex is methylated on both strands, it is refractory to repair (Pukkila et al. 1983; Wagner et al. 1984; Dohet et al. 1986). Although exceptions to the latter observation exist (Meselson 1988), they involve the action of alternate correction pathways which will be discussed below. Hence, repair is usually directed to the unmethylated or newly synthesized DNA strand via discrimination between the parental and nascent DNA strands based on undermethylation of d(GATC) sequences in the nascent strand.

Methylation at the N^6 position of the adenine residue in the d(GATC) sequences is carried out by the product of the *dam* gene with a delay relative to DNA synthesis (Marinus 1976; Lyons and Schendel 1984). In addition, the biological rate of d(GATC) methylation is limited by the intracellular level of the *dam* enzyme (Szyf et al. 1984; Modrich 1987). Thus, the speed with which methylation occurs following replication should determine the time the mismatch repair system has to recognize and repair replication errors and, therefore, the efficiency of mismatch repair (Radman and Wagner 1986). Both *dam* mutants, which are deficient in adenine methylation of d(GATC) sequences, and *dam* methylase overproducer strains have a mutator phenotype (Marinus and Morris 1973, 1975; Herman and Modrich 1981). The mutator phenotype of *dam* mutants can be understood in terms of the loss of strand bias for repair, while that associated with methylase *dam* overproduction can be explained by the more rapid methylation of newly synthesized DNA coupled with the reduced efficiency of correction on symmetrically modified regions.

Methyl-directed mismatch correction can recognize and repair a

variety of base-base mispairs, small insertions and deletions with different efficiencies (Kramer et al. 1984; Dohet et al. 1985, 1987; Jones et al. 1987b; Su et al. 1988; Lahue et al. 1989). The extent of repair depends on both the mismatch and neighbouring nucleotide sequence. In general, transition mismatches (G-T and A-C) are better repaired than transversion mismatches (G-A, C-T, A-A, G-G, T-T and C-C) (Kramer et al. 1984; Wagner et al. 1984; Dohet et al. 1986) and, for a given mismatch, repair efficiency increases with increasing G-C content in the neighbouring nucleotide sequence (Jones et al. 1987b). Studies using synthetic oligonucleotide heteroduplexes revealed that a low efficiency of repair was associated with an extrahelical configuration of the mismatch (Radman and Wagner 1986). Other results indicated that the three most efficiently repaired mismatches were found only in a helical form (Werntges et al. 1986; Jones et al. 1987b). However, Kolodner and his colleagues provided data conflicting with the idea that to be efficiently repaired heteroduplexes must be in a helical form. They suggested that a slight distortion of the helical structure at the site of a mismatch might be what is recognized by the mismatch repair system (Fishel and Kolodner 1983; Fishel et al. 1986).

Application of the transfection assay for heteroduplex repair led to the identification of several genetic loci (*mutH*, *mutL*, *mutS* and *mutU* (*uvrD*)) involved in methyl-directed repair (Nevers and Spatz 1975; Rydberg 1978; Bauer et al. 1981; Pukkila et al. 1983). Defects in any of these loci were found to confer mutator phenotypes with a bias for transitions over transversions (Nevers and Spatz 1975; Rydberg 1978; Bauer et al. 1981; Pukkila et al. 1983). Repair experiments using cell-free extracts showed that the reaction required single-strand DNA-binding protein (SSB)

and DNA polymerase III holoenzyme, in addition to the *mutH*, *mutL*, *mutS* and *mutU* gene products (Lu et al. 1983, 1984; Lahue et al. 1989). In a defined reaction system which mimics results obtained *in vivo* and *in vitro* with cell extracts, mismatch repair requires the MutH, MutL, MutS and MutU proteins, SSB, exonuclease I, DNA polymerase III holoenzyme, DNA ligase, ATP, dATP, Mg^{2+} and NAD^{+} (Lahue et al. 1989). The MutH, MutL and MutS proteins have been isolated in near-homogeneous and biologically active forms (Su and Modrich 1986; Welsh et al. 1987; Grilley et al. 1989).

MutS, a 97-kDa protein, binds to 8 possible mismatches with different affinities (Su and Modrich 1986; Su et al. 1988). It displays the lowest affinity for a T-C mispair, an intermediate affinity for A-C and G-A mispairs and the highest affinity for a G-T mispair. The MutS affinities for the mismatches generally correlate with the efficiencies with which the mismatches are repaired, but exceptions exist. This indicates that aspects of the repair reaction other than mismatch recognition contribute to repair efficiency (Su et al. 1988). MutS also exhibits a weak ATPase activity and mediates formation of α-shaped DNA loops stabilized at the DNA junction by bound protein (Grilley et al. 1990).

MutH, a 25-kDa protein, has latent d(GATC) endonuclease activity, undergoing activation during assembly of a mismatch repair complex (Modrich 1987; Welsh et al. 1987). It incises the unmethylated strand at d(GATC) sites flanking, or on one side of, the mismatch to generate 3′-hydroxyl and 5′-phosphoryl termini (Modrich 1989). Furthermore, it stabilizes the incision it introduces to facilitate entry of additional repair components (Grilley et al. 1990). Different d(GATC) sequences in different local environments are subject to differential recognition by

the *mutH* gene product. The efficiency of recognition of individual sites by this protein roughly correlates with their propensity to promote a repair event (Welsh et al. 1987). Fully methylated DNAs are resistant to cleavage by MutH (Welsh et al. 1987). Given the properties of this protein, it was suggested that the *mutH* gene product is involved in the strand choice stage of methyl-directed repair (Kramer et al. 1984).

MutL, a 70-kDa protein, binds to the MutS-heteroduplex complex in the presence of ATP or ATPγs (Grilley et al. 1989). Consequently, it may act to interface mismatch recognition by MutS with activation of the latent MutH endonuclease.

The *mutU* gene product, DNA helicase II, unwinds the DNA duplex in the vicinity of the strand break or at the mismatch in an ATP-dependent manner (Längle-Rouault et al. 1987). It may facilitate the displacement of the single-stranded fragment containing the incorrect base (Maples and Kushner 1982; Kumura and Sekiguchi 1984).

Following helicase action, SSB polymerizes processively along the single-stranded DNA to stabilize the strands and afford some protection against nuclease (Grilley et al. 1990). However, it enhances the sensitivity of single strands to endonuclease I, an enzyme with which it forms a molecular complex (Molineux and Gefter 1975). SSB-coated DNA is also a better template for replication by polymerase III holoenzyme than naked single-stranded DNA (Kornberg 1980; Chase and Williams 1986).

The resynthesis of incised DNA is carried out by DNA polymerase III holoenzyme (Kornberg 1980) and the remaining nick is sealed by NAD^+-dependent ligase to restore the covalently continuous form of DNA (Lahue et al. 1989).

Interestingly, a mutation in the *mutD* locus, which encodes the ϵ

subunit (Scheuerman and Echols 1984) responsible for the editing exonuclease of DNA polymerase III (Kornberg 1980), results in a defect in methyl-directed mismatch repair *in vivo* (Schaaper 1988). This defect is not structural and it was suggested that the mismatch repair deficiency of *mutD* strains results from a saturation of the *mutHLS*- dependent mismatch repair system by an excess of primary DNA replication errors due to the editing defect (Schaaper and Radman 1989).

In addition to DNA methylation, single-strand breaks can direct the strand specificity of mismatch correction (Längle-Rouault 1987; Lahue et al. 1989). Repair directed in this fashion requires the MutL and MutS proteins, DNA helicase II, SSB and DNA polymerase III holoenzyme. It occurs whether or not the molecule contains d(GATC) sequences, does not require ligase and bypasses the requirement for MutH which is involved in the strand discrimination step of methyl-directed mismatch repair. The absence of the ligase is apparently necessary to prevent the closure of the strand break prior to the initiation of mismatch repair (Grilley et al. 1990). It has been suggested that direction of strand choice by breaks may be of significance in the processing of heteroduplex regions of recombination intermediates that contain exposed DNA ends (Längle-Rouault et al. 1987; Lahue et al. 1989).

Possible mechanisms for the initiation of methyl-directed mismatch repair have been discussed (Modrich 1989, 1987; Grilley et al. 1990). In general, it is believed that three proteins (MutH, MutL and MutS) are sufficient to initiate methyl-directed mismatch correction which is provoked by the recognition of a mismatch by MutS. Formation of a MutS-mismatch complex then promotes binding of MutL and MutH to the heteroduplex complex. Following this, the complex searches for a

hemimethylated d(GATC) site and then activated MutH incises the unmethylated strand at the d(GATC) sequence. The molecular mechanisms of the excision and resynthesis reaction have not yet been defined. However, it was reported that during repair, regions of DNA as large as several kb are excised and resynthesized (Wagner and Meselson 1976; Lu et al. 1983; Su et al. 1989).

1.2.1.2 Very Short Patch (VSP) Mismatch Correction

The VSP pathway was identified by Lieb (1983, 1985, 1987). It is characterized by short excision tracts that rarely exceed 10 nucleotides in length. The system recognizes only G-T mismatches that arise in DNA through the spontaneous deamination of 5-methylcytosine in $m^5C \cdot G$ base-pairs located at the second position within the sequence d(CCA/TGG). It corrects this mismatch to a G·C base-pair (Duncan and Miller 1980; Lindahl 1982; Lieb 1985; Lieb et al. 1986; Jones et al. 1987a; Zell and Fritz 1987). The sequence d(CCA/TGG) in *E. coli* K strains, is subject to modification at the internal cytosine by the DNA cytosine methyltransferase (Dcm) (Marinus 1984).

VSP mismatch correction *in vivo* and *in vitro* is independent of *mutH* and *mutD* function, but depends on the *mutS* and *mutL* gene products (Jones et al. 1987a; Lieb 1987; Zell and Fritz 1987; Yashar and Modrich cited in Grilley et al. 1990). The *dcm* gene is not required for VSP mismatch correction *in vivo* or *in vitro* (Jones et al. 1987a; Lieb 1987; Yashar and Modrich cited in Grilley et al. 1990), but a small open-reading frame that partially overlaps the *dcm* gene (Bhagwat et al. 1988), and potentially encodes a 156 amino acid polypeptide, appears to be required for VSP repair (Grilley et al. 1990). Data from a cell-free assay indicated that *E. coli* DNA polymerase I is responsible for DNA synthesis associated with

VSP repair (Yashar and Modrich cited in Grilley et al. 1990).

1.2.1.3 *mutY*-dependent G-A to G·C Correction

The *mutY* pathway specifically repairs G-A mismatches where the mispaired adenine resides on the nascent DNA strand. The *mutY*-dependent repair pathway is distinguished from the methyl-directed pathway by several features (Au et al. 1988; Lu and Chang 1988a,b; Radicella et al. 1988; Su et al. 1988). It is independent of the methylation state of DNA or the presence of d(GATC) sequences, does not require the MutH, MutL, MutS or MutU proteins and processes G-A mismatches by short excision and resynthesis tracts. The *mutY* mutator specifically induces G·C to T·A transversions (Nghiem et al. 1988).

Strains defective in *mutY* are deficient in methylation-independent G-A to G·C mismatch correction both *in vivo* and *in vitro* (Au et al. 1988; Radicella et al. 1988). Au et al. (1989) have purified the *mutY* gene product to near homogeneity by virtue of its ability to restore G-A to G·C mismatch correction to cell-free extracts of a *mutS mutY* strain. Recently, Michaels et al. (1990a) have cloned and sequenced the *mutY* gene, which codes for a protein of 350 amino acids (M_r = 39,123). The protein has significant sequence homology to *E. coli* endonuclease III, an enzyme that has previously been shown to have glycosylase activity on damaged base-pairs. Amino acid sequence analysis suggested that, like endonuclease III, MutY is an iron-sulfur protein with a $[4Fe\text{-}4S]^{2+}$ cluster. The MutY protein renders the strand containing the mispaired adenine sensitive to cleavage by several apurinic/apyrimidinic-site endonucleases (Au et al. 1989). This suggests that MutY is a DNA glycosylase that hydrolyses the glycosyl bond linking the mispaired adenine to deoxyribose. MutY, a 5′-apurinic/apyrimidinic-site endonuclease, DNA polymerase I and DNA

ligase are sufficient to reconstitute *mutY*-dependent G-A to G·C repair *in vitro* (Au et al. 1989).

Lu and Chang (1988a) have partially purified an endonuclease that displays specificity for G-A mispairs and cleaves the first phosphodiester bond 3′ and the second phosphodiester bond 5′ to the mismatched adenine. The relationship between this activity and the MutY glycosylase is not clear.

1.2.1.4 Related Processes

Another locus, *mutM*, distinct from *mutY* but also involved in the avoidance of G·C to T·A transversions, has been identified (Cabrera et al. 1988). The *mutM* mutant specifically generated G·C to T·A transversions and the distribution of these events in the *lacI* gene was similar to that for a *mutY* strain. Thus, it was suggested that the two loci are involved in the same pathway (Cabrera et al. 1988).

mutT, a strong mutator (100- to 10,000-fold) (Yanofsky et al. 1966; Cox 1973), specifically induces A·T to C·G transversions both *in vivo* and *in vitro* (Cox 1976; Schaaper and Dunn 1987). On this basis, it was suggested that *mutT* functions in excluding the formation of G-A mispairs in which the guanine base of the mismatch resides on the newly synthesized DNA strand (Schaaper and Dunn 1987). In direct measurements of mismatch repair by transfection of bacteriophage M13mp2 heteroduplex DNA, Schaaper et al. (1989) found that *mutT*-induced G-A replication errors are not recognized by the *mutHLS* system. They suggested that G-A mispairs may exist in several different conformations, some of which are recognized by the *mutHLS* system, and that the G-A mispairs normally dealt with the *mutT* function may be present in a structurally distinct form. This is consistent with the suggestion that G-A can adopt at least three distinct

conformations [G (*anti*) - A (*syn*), G (*anti*) - A (*anti*) and G (*syn*) - A (*anti*)] (Patel et al. 1984; Brown et al. 1986; Modrich 1987; Gao and Patel 1988). It might be that these different G-A conformations can be recognized and processed by at least three different pathways (methyl-directed mismatch correction, *mutY*, *mutT*).

The 15-kDa MutT protein has been purified to near homogeneity (Bhatnagar and Bessman 1988; Akiyama et al. 1989). It displays a nucleoside triphosphatase activity with specificity for dGTP and so may prevent dGMP misincorporation by degrading a specific form of dGTP which can pair with deoxyadenosine. Au et al. (1988) have suggested that MutY and MutT provide complementary functions that serve to exclude G-A biosynthetic errors. MutT would act in affiliation with the replication complex (Cox 1973) to prevent the formation of G-A mispairs where the G resides on the nascent strand. G-A mispairs escaping this system by virtue of containing A on the newly synthesized strand would be subject to processing by the *mutY* pathway to yield G·C base-pairs.

Recently, two new mutators, *mutA* and *mutC*, have been identified in *E. coli* (Michaels et al. 1990b). Mapping of *mutA* and *mutC* on the *E. coli* chromosome, and the mutational specifities of the mutators, indicated that they are distinct from *mutY*, *mutT* and *mutM*. The mutational specificities of the *mutA* and *mutC* mutant strains are identical, each having higher levels of A·T to T·A and G·C to T·A transversions and, to a lesser degree, A·T to C·G transversions. It has been suggested that MutA and MutC may be components of the same error-avoidance system.

1.2.2 Mismatch Correction in *Saccharomyces cerevisiae*

Evidence from gene conversion studies and experiments involving heteroduplexes transformed into mitotic cells or exposed to cell-free

extracts have indicated that cells of the yeast *Saccharomyces cerevisiae* possess a mismatch repair system(s) with some similarities to one or more of those identified in *E. coli*.

1.2.2.1 Evidence for Mismatch Repair in Yeast

The first evidence for mismatch repair in yeast came from studies of meiotic recombination, specifically gene conversion (Leblon and Rossignol 1973; Fogel et al. 1979; Hastings 1984, 1987, 1988). For a pair of allelic markers, the expected Mendelian segregation ratios is 4:4. Non-Mendelian segregation ratios due to gene conversion (6:2) or post-meiotic segregation (PMS) (5:3) are associated with meiotic recombination (Radding 1978; White et al. 1985; Hastings 1988). Gene conversion, the phenomenon of non-reciprocal transfer of genetic information, has been postulated to reflect mismatch repair of a heteroduplex DNA recombination intermediate, i.e., a region of hybrid DNA containing at least one mismatch (Holliday 1964, 1974; Meselson and Radding 1975; Radding 1978). PMS has been attributed to the failure to correct the mismatch resulting in the persistence of a heteroduplex region containing both parental genotypes (Radding 1978; White et al. 1985; Hastings 1988). The frequency of PMS per aberrant segregation was found to depend to a large extent on the type of mismatch that could be formed. Heterozygous alleles that could give rise to C-C mismatches during meiotic recombination exhibited high PMS frequencies while other allelic combinations showed lower PMS frequencies (Fogel et al. 1981; White et al. 1985). Recently, more direct studies of PMS frequencies using various mutant alleles of the *HIS4* gene have confirmed that correction of C-C mismatches during meiosis is inefficient compared to correction of A-A, A-C, G-G, G-A, T-T, T-C and T-G mismatches which are repaired with approximately equivalent efficiencies (Detloff et

al. 1991).

Mismatch correction in mitotic cells has been studied using cell extracts and transformation of heteroduplexes into intact cells. Exposure of heteroduplexes containing defined mismatches to extracts from mitotic cells revealed that the repair process required Mg^{2+}, ATP and the four DNA precursors and that less than 20 nucleotides were incorporated at the site of repair (Muster-Nassal and Kolodner 1986). In addition, the A-C and G-T transition mismatches, and multiple base-pair insertion/deletion mismatches, were corrected efficiently whereas the other six base mismatches tested were repaired poorly. Transformation of heteroduplex plasmids into mitotic cells demonstrated that defined mismatches can be recognized and corrected *in vivo* (Bishop and Kolodner 1986; Bishop et al. 1989; Kramer et al. 1989a). In particular, all 8 base mismatches used as well as single base deletion mismatches and multiple base deletion/insertion mismatches, were corrected with the hierarchy of correction efficiency depending on the nature of the mismatches. With regard to the base mismatches, the A-C and G-T transition mismatches were corrected more efficiently than most transversion mismatches with the C-C mismatch being the poorest substrate for correction. This substrate specificity is similar to that observed for methylation-directed mismatch repair in *E. coli*.

1.2.2.2 *PMS* Genes

Three genes, designated *PMS1*, *PMS2* and *PMS3*, are believed to function in mismatch repair activity in yeast (Kramer et al. 1989a). This conclusion is based on several observations (Williamson et al. 1985; Bishop et al. 1989; Kramer et al. 1989a,b): (i) mutants defective in *PMS1*, *PMS2* or *PMS3* have a mitotic mutator phenotype; (ii) homozygous mutant

diploids display elevated PMS frequencies and reduced spore viability when induced to undergo meiosis; (iii) the repair of mismatches that were corrected efficiently in wild-type mitotic cells was dramatically reduced in the *pms1*, *pms2* and *pms3* mutant strains; (iv) the *PMS1* gene product is homologous to the bacterial MutL protein. Taken collectively, these findings suggest that the *PMS* genes may encode components of a yeast mismatch repair complex.

1.2.2.3 Direction of Mismatch Repair in Yeast

The mechanism of mismatch correction in yeast has not yet been determined. It is unlikely that discrimination between the template and newly synthesized DNA strand in yeast involves methylation of DNA (Radman and Wagner 1986; Kramer et al. 1989a), because yeast DNA is not detectably methylated (Proffitt et al. 1984). Instead, it has been suggested that mismatch repair might be directed by transient single-strand nicks such as those found during DNA replication at the end of Okazaki fragments (Kramer et al. 1989a).

1.2.3 Mismatch Repair in Higher Eukaryotic Cells

Relative to bacteria, mismatch correction in higher eukaryotic cells also is not well understood. Nevertheless, systems using transfection or cell-free extracts have been developed for studies of mismatch repair in higher eukaryotes. The available information has clearly demonstrated that mismatch processing does occur and has suggested that higher organisms may possess mismatch correction systems analogous to those that have been identified in bacteria.

1.2.3.1 Evidence for Mismatch Correction in Higher Eukaryotic Cells

Direct evidence for mismatch repair in higher eukaryotic cells has been provided by transfection experiments that used artificially

constructed heteroduplex-containing viral or plasmid vectors. In such experiments the fate of heterozygotic markers, and hence the corresponding mismatches, was determined by analysis of the phenotypes of virus particles or genes carried on plasmid vectors emerging from single transfectants. The results demonstrated that mismatches in heteroduplex DNA were repaired before DNA replication in mouse and monkey cells (Miller et al. 1976; Abastado et al. 1984). In monkey kidney cells, all 8 possible base/base mismatches carried on SV40 vectors were repaired with different efficiencies and specificities, with the repair efficiencies depending on the nature of the mismatches (Brown and Jiricny 1987, 1988).

The evidence for mismatch repair from *in vitro* experiments is primarily based on the demonstrations that cell-free extracts from *Xenopus* eggs, human Hela cells and *Drosophila melanogaster* Kc cells processed different mismatches with different efficiencies, in the latter two cases in a strand specific manner (Brooks et al. 1989; Holmes et al. 1990; Varlet et al. 1990; Thomas et al. 1991). In addition, mismatch correction was accompanied by mismatch-provoked DNA synthesis localized to the mismatch-containing region (Brooks et al. 1989; Holmes et al. 1990; Varlet et al. 1990; Thomas et al. 1991), with different mismatches having different extents of mismatch-provoked DNA repair synthesis (Varlet et al. 1990; Thomas et al. 1991).

1.2.3.2 Types of Mismatch Repair Systems

The *in vivo* and *in vitro* mismatch repair studies suggested that, in mammalian cells, specific mismatch repair systems which respond to different mismatches may exist. Similar to the situation in *E. coli*, three potential systems have been suggested: a general mismatch repair system (Brown and Jiricny 1988; Holmes et al. 1990; Thomas et al. 1991); a G-T

specific mismatch repair system (Brown and Jiricny 1987, 1988; Wiebauer and Jiricny 1989, 1990); and a G-A specific mismatch repair system (Yeh et al. 1991).

Data from experiments using simian cell transfection, *Xenopus* egg extracts or *Drosophila* or Hela cell extracts support the existence of a general mismatch repair system (Brown and Jiricny 1988; Holmes et al 1990; Varlet et al. 1990; Thomas et al. 1991). This system appears able to process a wide variety of mispairs with different efficiencies. A protein which specifically binds to A-C, T-C and T-T mispairs has been detected in human cell extracts and it was suggested that this protein might be involved (Stephenson and Karran 1989; Wiebauer and Jiricny 1990). The mismatch repair reaction can be directed by a single-strand break and is highly biased to the incised DNA strand. Similar to the methyl-directed mismatch repair system in *E. coli*, the reaction is accompanied by mismatch-provoked DNA synthesis which is localized to the mismatch region and can extend about 1,000 bp. Experimental evidence suggested that a replicative DNA polymerase, DNA polymerase α, is involved in the mismatch-provoked DNA repair synthesis (Holmes et al. 1990; Thomas et al. 1991).

G-T mispairs, incorporated into the SV40 genome and transfected into monkey (Brown and Jiricny 1987, 1988) or human cells (Brown et al. 1989) were corrected with high efficiency and yielded mainly G·C pairs. On this basis, it was suggested that a repair system which acts only on G-T mismatches and specifically restores G·C pairs, might exist. Subsequently, a specific G-T mismatch repair activity was confirmed using human cell extracts (Wiebauer and Jiricny 1989). This system seems analogous to the very short patch pathway in *E. coli*, and probably developed to protect the cells from the deleterious effects of the spontaneous hydrolytic

deamination of 5-methylcytosine. Recently, a DNA glycosylase, which removes the mispaired thymine to generate an AP site opposite the guanine, was identified in nuclear extracts from human cells, (Wiebauer and Jiricny 1990). Removal of the AP site was found to involve a class I AP endonuclease and leaves a one-nucleotide gap which is filled by DNA polymerase ß (Wiebauer and Jiricny 1989).

It was supposed that since thymine is a natural DNA base, any enzyme responsible for its removal from G-T mispairs would have to be inactive on single-stranded and matched double-stranded substrates. Therefore, it seemed that the thymine glycosylase might act in conjunction with another enzyme. Two possibilities were suggested (Wiebauer and Jiricny 1990). First, the glycosylase might be guided to the mispairs by a G-T mismatch binding protein, analogous to the MutS protein of *E. coli*. Second, a G-T mismatch modifying enzyme might convert the mispaired T to a thymine derivative, such as thymine glycol or hydroxymethyluracil, which would then be removed by the glycosylase. In support of the first possibility, a 200-kDa protein which specifically binds to G-T mismatches was identified in cell-free extracts of human Hela cells (Jiricny et al. 1988). Stephenson and Karran (1989) also isolated two DNA binding proteins from human cells, one of which is specific for G-T mismatches and resembles the activity reported by Jiricny et al. (1988). However, involvement of these proteins in the G-T to G·C repair pathway has not been established. Consequently, it is not yet clear whether these proteins fulfil the "guide" role suggested above.

A G-A mismatch specific nicking enzyme system has been identified in extracts of Hela cells (Yeh et al. 1991). This activity binds to G-A mismatches and makes incisions at the first phosphodiester bonds 5′ and 3′

to the mispaired adenine but not the guanine base. Solely on this basis, it has been suggested that higher eukaryotic cells possess a G-A specific mismatch repair system similar to the *mutY*-dependent pathway in *E. coli* (Yeh et al. 1991). However, it is not clear whether a DNA glycosylase and AP endonuclease are involved in the postulated G-A to G·C repair.

1.2.3.3 Direction of Mismatch Repair in Higher Eukaryotic Cells

The methylation state of d(GATC) sequences determines the strand specificity of mismatch correction by the *E. coli* methylation-directed pathway. It has also been shown that a persistent strand break can bypass the requirements for both the d(GATC) sequences and MutH, the protein that recognizes such sites (Längle-Rouault et al. 1987; Welsh et al.1987; Lauhue et al. 1989). In CV-1 cells of the African green monkey, hemi-methylation at cytosine or adenine residues was reported to direct strand selection during mismatch repair so as to correct the base on the unmethylated strand (Hare and Taylor 1985). However, the presence of a strand break was found to be a stronger determinant of strand bias than methylation, with the strand containing the nick farthest from the mismatch serving as the template strand for repair (Hare and Taylor 1985, 1988). In other experiments using the extracts from *Drosophila* or Hela cells, it was demonstrated that for general mismatch repair activity, strand discrimination was directed by strand breaks which could be as far 808 bp from the mismatch. The specificity was such that repair was directed to the nick-containing strand (Holmes et al. 1990; Thomas et al. 1991).

1.3 Experimental Goals

In this study, I have sought to probe the nature of the *rad1*, *rad6* and *rad18* mutators and assess the role of mismatch repair in the mutator

phenotypes. To date, studies of the links between DNA repair defects and mutator activity in yeast have relied solely on genetic techniques (Moustacchi 1969; von Borstel et al. 1971; Lemontt 1972; Hastings et al. 1976; Brychcy and von Borstel 1977; Kern and Zimmermann 1978; Foury and Goffeau 1979; Morrison and Hastings 1979; Maloney and Fogel 1980; Monteleone et al. 1981). Although this approach has been informative, it has precluded a thorough investigation of the various sites of mutation and types of DNA sequence change occurring in a single gene. Yet, such information would prove valuable in characterizing mutator phenotypes in depth and could yield important clues about the mechanism of enhanced spontaneous mutagenesis in repair-deficient strains and the functions of repair gene products in DNA metabolism (Kunz et al. 1989).

To obtain detailed spontaneous mutational specificity data and assay mismatch correction, I have used a system (Pierce et al. 1987) which facilitates: (i) rapid DNA sequence analysis of mutations occurring in a yeast suppressor tRNA gene (*SUP4*-o); and (ii) construction of defined mismatches in this gene and analysis of their repair. Specifically, I have examined spontaneous *SUP4*-o mutations arising in *rad1*, *rad6* or *rad18* backgrounds and have compared the resulting spectra to that obtained in an isogenic wild-type strain. Also, different heteroduplex DNA plasmids containing defined mismatches were constructed *in vitro* and transformed into the same four strains. A number of interesting findings emerged. The *rad1* mutator phenotype was associated with enhanced frequencies of single base-pair substitution, single base-pair deletion and insertion of the yeast retrotransposable element Ty. The presence of a functional *RAD1* gene was not required for the correction of transition mismatches in yeast and adenine methylation of the heteroduplex DNA did not direct mismatch

correction via excision repair. The *rad6* mutator enhanced Ty transposition, the G·C -> T·A transversion and both types of base-pair transition. The *rad18* mutator specifically enhanced the frequency of G·C -> T·A transversions. The effects to the *rad6* and *rad18* mutators on base substitutions did not reflect an inability of mismatch repair enzymes to correct the mismatches that could have given rise to the substitutions, or preferential restoration of the mismatches to incorrect base-pairs. Possible mechanisms that might enhance single base-pair events and Ty transposition in the *rad1* strain, link the *RAD6* gene product to the control of spontaneous mutagenesis and account for the specific induction of G·C to T-A transversions by the *rad18* mutator are considered.

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. Media components such as yeast extract, tryptone, peptone, yeast nitrogen base w/o amino acids and agar were purchased from Difco Laboratories.

2.2 Bacterial and Yeast Strains

All haploid strains of the yeast *Saccharomyces cerevisiae* used throughout this experiment are isogenic derivatives of the wild-type strain MKP-o which has been described (Pierce et al. 1987). XK15, carrying a *rad18::LEU2* insertion, was constructed for this study. KAM1, carrying a *rad1::LEU2* insertion was derived by transforming MKP-o with a 6.96-kb *Sal*I DNA fragment encompassing the *RAD1* gene and flanking regions and having the internal 2.1-kb *Stu*I-*Cla*I fragment of *RAD1* (70% of the *RAD1* coding sequence) replaced with a 3.2-kb *Bgl*II fragment containing the *LEU2* gene. KAM1 was constructed by K. Magnusson. RDG6, carrying the *rad6::LEU2* insertion, was constructed by transforming MKP-o with a 2.3-kb *Bam*HI-*Hind*III DNA fragment having the *LEU2* gene surrounded by sequences that flank *RAD6*. RDG6 was constructed by Dr. R.D. Gietz. The yeast strains carrying the plasmid YCpMP2 (see below) are designated MKP-op, XK15-p, KAM1-p and RDG6-p. The *Escherichia coli* strain JF1754 was used to recover plasmid DNA from yeast cells. *E. coli* NR3837 and JF1754F$^+$ were used to produce single-stranded plasmid DNA. The complete genotypes of all strains used in this study are given in Table 1.

Table 1. Bacterial and yeast strains

Strain	Genotype	Source or Reference
JF1754	*Δlac, gal, metB, leuB, hisB436, hsdR*	B.A. Kunz.
JF1754^{+}	F^{+} Tn10, *Δlac, gal, metB, leuB, hisB436, hsdR, rpsL*	Pierce et al. (1987)
NR3837	*F′ pro-lac/ara, Δpro-lac, thi, trpE9777, dam*	B.W. Glickman
MKP-o	*MATα, can1-100, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901*	Pierce et al. (1987)
XK15	*MATα, can1-100, ade2-1, lys2-1, ura3-52, his3-Δ200, trp1-Δ901, rad18::LEU2*	Constructed for this study
KAM1	*MATα, can1-100, ade2-1, lys2-1, ura3-52, his3-Δ200, trp1-Δ901, rad1::LEU2*	K. Magnusson
RDG6	*MATα, can1-100, ade2-1, lys2-1, ura3-52, his3-Δ200, trp1-Δ901, rad6::LEU2*	R.D. Gietz.
BKPA18/2	*MATa, ade2-119, trp5-27, ilv1-92, rad18-2*	B.A. Kunz

2.3 Plasmid

YCpMP2 (Pierce et al. 1987) is a hybrid yeast-bacterial shuttle vector which can replicate autonomously in yeast and in *E. coli* (Figure 1). The vector contains a replication origin (*ARS1*), a centromere sequence (*CEN4*) and a selectable marker (*URA3*) from yeast and the replication origin (*REP*) and the ampicillin resistance determinant (AMP^R), the ß-lactamase gene, from the bacterial plasmid pBR322. The *CEN4* sequence allows YCpMP2 to be maintained predominantly at a copy number of one in haploid yeast cells (Newlon 1988). In addition, YCpMP2 carries *SUP4*-o, an ocher suppressor allele of a yeast tyrosine tRNA gene. The replication origin from the filamentous phage M13, which permits the generation of single-stranded plasmid DNA (Dente et al. 1983; Levinson et al. 1984; Zagursky and Berman 1984), is also part of this vector.

2.4 Media

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

per liter:	40	g	Dextrose
	6.7	g	Bacto yeast nitrogen base w/o amino acids

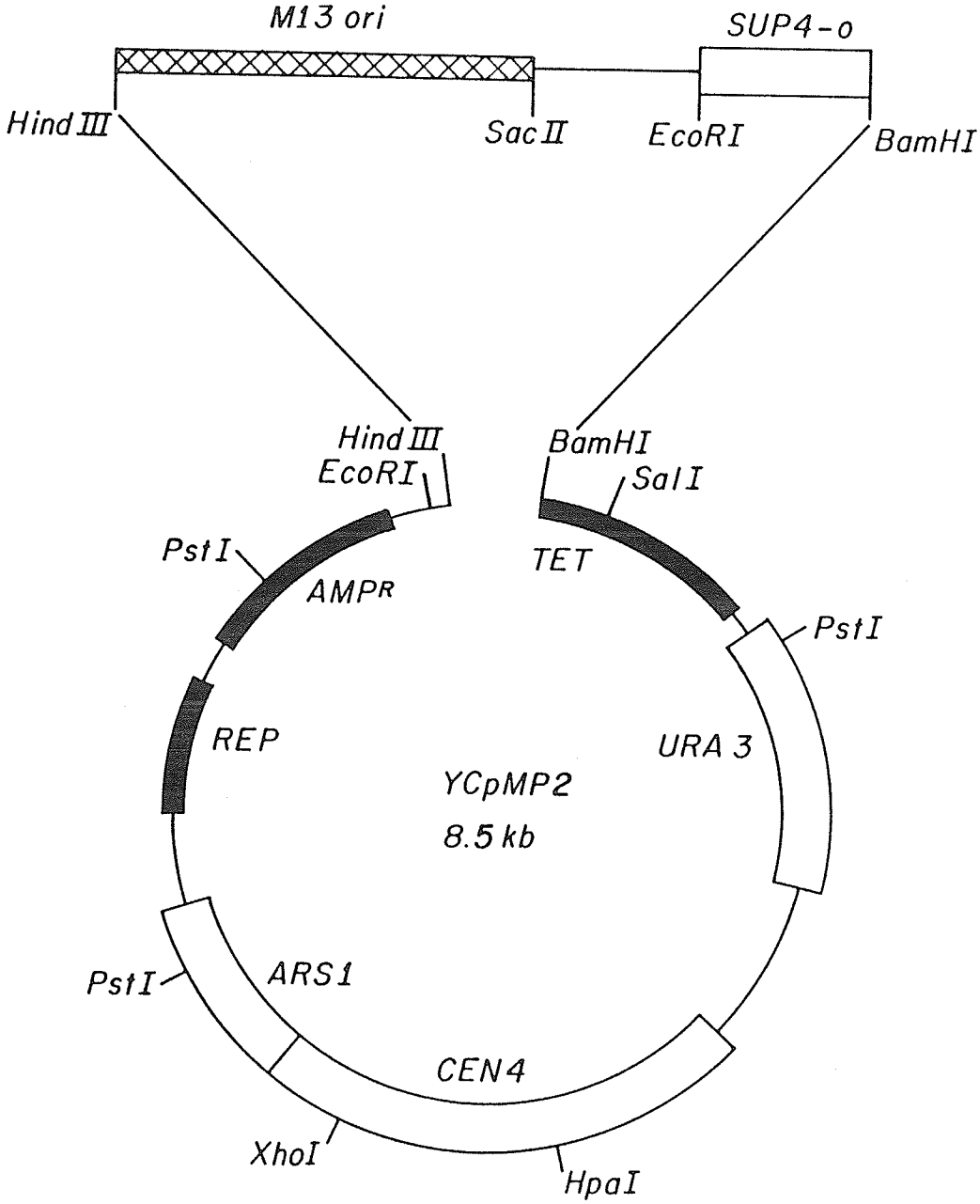
Required nutrients were added at the concentrations suggested by Sherman et al. (1983). To enhance colouring due to the *ade2-1* allele (see section **2.5**), adenine was added at half the recommended concentration (Zimmermann 1973). SD media for transformation with nicked heteroduplex plasmid DNA contained one-fourth (for MKP-o) or one-third (for XK15, KAM1 and RDG6) the recommended concentration of adenine.

B. YPD (Sherman et al. 1983):

per liter:	20	g	Dextrose
	20	g	Bacto peptone
	10	g	Bacto yeast extract

For yeast transformation, 10 mg adenine sulfate and 30 mg L-lysine HCl were added per liter of YPD and the medium was designated YPDLA.

Figure 1. Plasmid YCpMP2


M13 ori
SUP4-o
Hind III
Sac II
EcoRI
BamHI
Hind III
EcoRI
BamHI
Sal I
Pst I
AMPR
TET
Pst I
REP
URA 3
YCpMP2
8.5 kb
Pst I
ARS1
CEN4
Xho I
Hpa I

C. YT (Miller 1972):

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

When required, ampicillin (100 mg/l) 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. M9 + amp (Miller 1972):

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

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

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

2.5 Detection of *SUP4*-o Mutants

Forward mutations in the *SUP4*-o gene are detected by scoring for reduced suppression of three ocher markers. The haploid yeast strains used in this study carry ocher alleles which confer either resistance to the arginine analog canavanine (*can1-100*), cause red pigmentation (*ade2-1*) or result in lysine auxotrophy (*lys2-1*). Since these mutations are suppressed by *SUP4*-o, cells harbouring YCpMP2 are canavanine-sensitive and form white, lysine-independent colonies. Mutants lacking suppressor activity are selected as canavanine-resistant, red, or pink colonies unable to grow when replicated to lysine omission medium. Selection for reduced suppression of all three ocher 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 ocher mutations are rare (<1% of mutations analyzed) at the chromosomal *SUP4*-o locus (Kurjan and Hall 1982). Second, we have not detected sequence alterations in the plasmid copy of *SUP4*-o from mutants isolated using less stringent selection methods (Kunz et al. 1987). Third, 177 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. 1990).

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

To isolate spontaneous mutants, yeast strains were grown from low titre inocula (100 cells/3 ml) in uracil omission medium (MKP-op) or in uracil-leucine omission medium (XK15-p, KAM1-p, or RDG6-p) and grown at 30°C with shaking to stationary phase (MKP-op and KAM1-p: 1-2 x 10^7 cells/ml; XK15-p: 8 x 10^6 cells/ml; RDG6-p: 3.5 x 10^6 cells/ml) as determined by a Coulter counter. Cell suspensions were diluted and plated on uracil (MKP-op) or uracil-leucine omission medium (XK15-p, KAM1-p, RDG6-p) to measure viability and on fully supplemented medium to determine plasmid retention. To select for red, canavanine-resistant colonies, the cell suspensions were plated on uracil omission medium (MKP-op) or uracil-leucine omission medium (XK15-p, KAM1-p, RDG6-p) containing 30 mg/ml canavanine. (The canavanine-resistant derivatives of KAM1-p and RDG6-p were isolated by T. Kolodka and F. Yadao, respectively.) All plates were scored after 6 days incubation at 30°C. Red colonies that emerged on canavanine-containing medium were transferred to uracil omission medium, grown 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 or uracil-leucine 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 for Spontaneous Mutants (J.W. Drake: personal communication):

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

r = mutations in the target per DNA replication (solved algorithmically
f_m = the median mutation frequency
N_m = the median population size at the time of sampling including residual growth on the plates (3.5 generations for MKP-op and 3 generations for XK15-p, KAM1-p and RDG6-p on canavanine-containing medium).

2.7 DNA Isolation

2.7.1 Large Scale Yeast DNA Preparation

SE buffer:	900	mM	sorbitol
	100	mM	Na_2EDTA, pH 7.5

Zymolyase: Zymolyase 100,000 (Seikagaku Kogyo Comp.) was dissolved at a concentration of 6 mg/ml in SE buffer.

TE-1 buffer:
50 mM Tris, pH 7.4
20 mM Na_2EDTA, pH 7.4

TE-2 buffer:
10 mM Tris, pH 7.4
1 mM Na_2EDTA, ph 7.4

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

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), washed 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-1 buffer. Sodium dodecyl sulfate (SDS, 500 μl, 10%, w/v) was added, the contents were mixed gently by inversion and incubated for 30 min at 65°C. 1.5 ml potassium acetate (5 M) was added and the suspension was chilled for 30 min on ice. The precipitate was pelleted by centrifugation (34,800 x g, 20 min, 4°C), the supernatant 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-2 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-2 buffer and stored at -20°C.

2.7.2 Rapid Prep for Yeast DNA Isolation

SCE buffer:	77	mM	sorbitol
	10	mM	sodium citrate
	6	mM	Na_2EDTA, pH 8.0
SCEM buffer:	SCE buffer containing 10% (v/v) 2-mercapto-ethanol.		
Zymolyase:	Zymolyase 100,000 was dissolved at a concentration of 6 mg/ml in SCEM buffer.		
TE buffer:	50	mM	Tris, pH 7.5
	20	mM	Na_2EDTA, pH 7.5
RNase:	see section 2.7.1		

Total yeast DNA used for plasmid retrieval was isolated by a modification of a procedure described by Sherman et al. (1983). Yeast cultures were grown in 5 ml of uracil omission broth at 30°C with shaking to stationary phase and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 5 ml YPD broth and incubated overnight at 30°C with shaking. The cells were then collected by centrifugation (1,850 x g, 10 min, room temperature), washed in 500 μl SCE buffer, transferred to a microfuge tube and resuspended in 300 μl SCEM buffer. Zymolyase (20 μl) was added to the tube which was then vortexed to mix the contents and incubated at 37°C for 1 h. During this incubation time, the tubes were inverted at 15 min intervals to resuspend the cells. The resulting spheroplasts were pelleted by centrifugation for 20 s in a microfuge (Brinkman 5415C, room temperature), resuspended in 357 μl TE buffer and 36 μl SDS (10%, w/v) by vortexing and incubated at 65°C for 30 min. Potassium acetate (107 μl, 5 M) was added to the tube which was inverted several times to mix the contents and then chilled on ice for 15 min. The precipitate was pelleted twice by centrifugation at 4°C for

15 min, the supernatant transferred to a fresh tube and 1 ml ice-cold 95% ethanol was added. The contents of the tube were mixed by inversion, and the tube was centrifuged briefly to pellet the nucleic acids which were then washed with 1 ml ice-cold 70% ethanol, dried by aspiration and dissolved in 300 μl TE buffer. RNase (1.5 μl) was added and the tube was incubated at 37°C for 30 min. The nucleic acid solution was then extracted once with 300 μl TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and twice with 150 μl TE-saturated chloroform. After each extraction the samples were centrifuged for 2 min and the upper aqueous layer was transferred to a fresh tube. After the last extraction, isopropanol (300 μl) was added, the contents of the tube were mixed by inversion, and the tube was centrifuged briefly to pellet the DNA which was then washed twice with ice-cold 70% ethanol, dried by aspiration and dissolved in 30 μl TE buffer. DNA samples were stored at -20°C.

2.7.3 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 2.7.2

The previous yeast DNA preparation was replaced midway through this study by a glass-bead technique (modified from: Hoffman and Winston 1987) which allowed faster processing of a larger number of samples. 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 vortexed for 2 min and spun for 5 min in a microfuge (Brinkman 5415C, 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.

2.7.4 Alkaline Extraction 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.	
Sodium acetate:	3 M sodium acetate was dissolved in glacial acetic acid while being heated to 65°C to achieve a pH of 4.8.		
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 Crouse et al. (1983) 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 500 μl of GTE buffer and transferred to a microfuge tube. Cells were pelleted by centrifugation (Brinkman 5415C, room temperature), resuspended in 180 μl GTE buffer and 10 μl of lysozyme (Boehringer Mannheim) (25 mg/ml dissolved in GTE buffer) was added. The cell suspension was mixed gently and then the tube was incubated at room temperature for 5 min. Next, the tube was transferred to ice, 400 μl

NaOH/SDS was added slowly in spirals starting at the bottom of the tube and the mixture was incubated on ice for 5 min. Sodium acetate (300 μl, 3 M, pH 4.8) was added drop-wise, the contents were mixed by inverting slowly and the tube was incubated on ice for 10 min. The precipitate was pelleted by centrifugation at 4°C for 15 min and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated two more times. Then, 450 μl isopropanol (room temperature) was added, the contents were mixed by inversion, the tube was spun for 2 min, the supernatant was discarded and the pellet was washed with 70% ice-cold ethanol and dried by aspiration. The pellet was dissolved in 200 μl TE buffer and the nucleic acids were then extracted twice with 200 μl TE-saturated phenol and once with 200 μl TE-saturated chloroform. After each extraction the samples were centrifuged for 2 min and the upper aqueous layer was transferred to a fresh microfuge tube. Following the last extraction, an equal volume of ammonium acetate was added, the contents were mixed by inversion and the precipitate was pelleted by centrifugation for 15 min at 4°C. The supernatant was transferred to a fresh tube and the nucleic acids were precipitated by adding 1 ml of 95% ice-cold ethanol and pelleted by centrifugation for 1.5 min at room temperature. The pellet was washed with 1 ml 70% ice-cold ethanol, dried by aspiration and dissolved in 100 μl TE buffer. DNA samples were stored at -20°C.

2.7.5 Rapid Alkaline Procedure for Plasmid DNA Isolation

GTE buffer:	see section 2.7.4
NaOH/SDS:	see section 2.7.4
Ammonium acetate:	see section 2.7.4
TE buffer:	see section 2.7.2

During the course of this study, the alkaline extraction procedure

was replaced by a more rapid version (modified from: Morelle 1989) which uses the same solutions as described in the previous protocol. 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 500 µl of GTE buffer and transferred to a microfuge tube. Cells were spun down (Brinkman 5415C, room temperature, 30 s), resuspended in 180 µl GTE buffer and 10 µl of lysozyme (25 mg/ml dissolved in GTE buffer) was added and the cell suspension was mixed gently and then incubated at room temperature for 5 min. Next, the tube was transferred to ice, 400 µl NaOH/SDS was added drop-wise and the mixture was incubated on ice for 5 min. Ammonium acetate (300 µl) was added drop-wise and the mixture was incubated on ice for 10 min. The precipitate was pelleted by centrifugation at 4°C for 15 min and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated two more times. Then, 500 µl isopropanol (room temperature) was added, the contents were mixed by inversion and the tube was held at room temperature for 10 min. Following centrifugation for 5 min at 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.

2.7.6 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 2.7.4

NaOH/SDS: see section 2.7.4

Potassium acetate: 3 M potassium acetate was adjusted to pH 4.8 with glacial acetic acid.

Ammonium acetate:	10 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C to achieve a pH of 7.8.
PEG:	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 heteroduplex 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 M9+amp (NR3837 with plasmid) or YT+amp (JF1754 with plasmid) at 37°C with shaking. Then, 2 ml of overnight culture was transferred to 500 ml M9+amp or 500 ml YT+amp and grown overnight at 37°C with shaking. Cells were pelleted by centrifugation (2,000 x g, 15 min, 4°C), resuspended in 100 ml of ice-cold STE buffer, pelleted again, and resuspended in 18 ml GTE buffer. Lysozyme (2 ml, 10 mg/ml dissolved in GTE buffer) was added and the cell suspension was incubated at room temperature for 10 minutes and 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 15 min (12,100 x g, 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 minutes. The DNA was precipitated with 1.3% (w/v) PEG, pelleted by centrifugation (Brinkman 5415C) for 5 min at 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 for 2 min at 4°C 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 for 5 min at 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.

2.8 Preparation of Nicked Heteroduplex Plasmid DNA

2.8.1 Preparation of Single-Stranded Plasmid DNA

A. Preparation of bacterial phage M13K07

The procedure described by Vieira and Messing (1987) was used. *E. coli* JF1754F$^+$ was grown overnight in 5 ml YT containing tetracycline (5 mg/l) at 37°C with shaking. Then, 0.5 ml of the overnight culture was transferred to 50 ml YT containing 5 mg/l tetracycline and incubated at

37°C with shaking to reach $O.D._{460}$ = 0.85. Next, 200 μl of the JF1754F$^+$ cell suspension was mixed with 100 μl of phage M13K07 (10^7 - 10^8 pfu/ml), incubated at room temperature for 10 min, and 50 μl of infected JF1754F$^+$ was plated onto a YT plate containing tetracycline and kanamycin (70 mg/l) and the plate was incubated at 37°C overnight. A single colony that emerged on the YT+tetracycline+kanamycin was picked, inoculated into 30 ml of 2x YT containing tetracycline and kanamycin and the culture was grown for 24 hours at 37°C with shaking. The cells were removed by centrifuging twice (17,300 x g, 4°C, 15 min) and the phage in the supernatant were titred and used for the production of single-stranded plasmid DNA.

B. Production of single-stranded plasmid DNA

Formation of single-stranded plasmid DNA was induced by infection of NR3837 or JF1754F$^+$ cells carrying YCpMP2 or YCpMP2 derivatives with M13K07 helper phage as described previously (Messing and Vieira 1987). NR3837 or JF1754F$^+$ cells carrying plasmid were grown overnight in M9+amp (NR3837) or in YT+amp+tetracycline (JF1754F$^+$) at 37°C with shaking. Then, 1 ml of overnight culture was transferred into 100 ml M9+amp (for NR3837) or 100 ml YT+amp+tetracycline (for JF1754F$^+$) and incubated at 37°C with shaking for 1 h to reach $O.D._{460}$ = 0.15. The cells were then infected with phage (7 x 10^{10} pfu) and incubated at a lower shaking speed for 1 h. Next, 1 ml of kanamycin (7 mg/ml) was added and the culture was incubated at 37°C with shaking for 27 h. The cells were removed by centrifugation (9,150 x g, 4°C, 30 min) and the supernatant was kept at 4°C for purification of single-stranded plasmid DNA.

C. Purification of single-stranded plasmid DNA

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

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

Sodium acetate: see section 2.7.4

TE buffer: see section 2.7.4

The supernatant containing the single-stranded plasmid DNA was transferred into 30 ml volume centrifuge tubes, centrifuged (12,100 x g, 4°C, 15 min), transferred to sterile glass tubes (Corex no. 8433), centrifuged (6,780 x g, 4°C, 30 min) and transferred into a fresh Corex tube. PEG (4.2 ml) was added to the tube, the contents were mixed well and the tube was incubated at room temperature for 15 min before being centrifuged (6,780 x g, 4°C, 30 min). The supernatant was discarded, the pellet was dissolved in 300 μl TES buffer and transferred into a microfuge tube. Two lots of dissolved pellet (total = 600 μl) were transferred to each tube. The solution was extracted once with 600 μl TE-saturated phenol and once with 570 μl TE-saturated isopropanol:chloroform (1:24). After each extraction the tube was vortexed for 30 s, centrifuged for 4 min at 4°C (Brinkman 5415C), the upper aqueous layer was transferred to a fresh microfuge tube and 40 μl of sodium acetate and 1 ml of ice-cold 95% ethanol were added. The contents were mixed well and the tube was held at -20°C for 15 min. The DNA was pelleted by centrifugation (Brinkman 5415C, 4°C, 15 min), dissolved in 40 μl TE buffer and the concentration of single-stranded plasmid DNA was estimated from the relative intensities of the ethidium bromide-stained bands corresponding to the single-stranded plasmid and phage DNAs and the quantity of total DNA in the preparation

(determined by A_{260}). The DNA was stored at -20°C.

2.8.2 Linearization of Plasmid DNA

TE buffer: see section 2.7.2

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

Plasmid DNA was linearized by digestion with *Xho*I. Following digestion, the reaction mixture was extracted once with TE-saturated phenol, once with TE-saturated phenol:chloroform (1:1) and once with TE-saturated chloroform. The DNA was precipitated with 95% ice-cold ethanol, pelleted by centrifugation (Brinkman 5415C, 4°C, 15 min), washed with 70% ice-cold ethanol, dried by aspiration, and dissolved in 30 μl TE buffer. The concentration of DNA was determined by A_{260} and the DNA was stored at -20°C.

2.8.3 Construction and Purification of Nicked Heteroduplex Plasmid DNA

TE buffer: see section 2.7.2

20x SSC buffer:	3	M	sodium chloride
	0.3	M	sodium citrate

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

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

Endonuclease V dilution buffer:

20	mM	Tris-HCl, pH 7.5
10	mM	2-mercaptoethanol
0.1	mM	Na_2EDTA
500	μg	bovine serum albumin (BRL)
		Stored at -20°C.

10x Endonuclease V reaction buffer:

66.7	mM	glycine-NaOH buffer, pH 9.4
5	mM	$MgCl_2$
8.3	mM	2-mercaptoethanol
0.5	mM	ATP

Micrococcus luteus endonuclease V: 3.5 U/μl *Micrococcus luteus* endonuclease V (United States Biochemical) was diluted to a concentration of 0.35 U/μl in endonuclease V dilution buffer just prior to use.

Endonuclease V digest mix:

10	μl	10x endonuclease reaction buffer
86	μl	DNA in TE buffer (ca. 2.8 μg DNA)
4	μl	*Micrococcus luteus* endonuclease V (0.35 U/μl).
		Incubated at 37°C for 30 min.

Loenings 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.

Heteroduplex plasmid DNA was constructed from combinations of double-stranded linear and single-stranded circular DNA (Table 2) by using a modification of a thermal denaturation and renaturation procedure (Figure 2) (Kramer et al. 1989a). A 30 μg aliquot of the linearized plasmid DNA was added to single-stranded circular DNA (ca. 60 μg of plasmid DNA plus M13K07 DNA in TE) in a final volume of 956 μl SSC buffer. This hybridization mixture was incubated at 100°C for 4 minutes, transferred to a small 75°C bath, cooled slowly to 65°C, incubated at 65°C for 15 min, removed from the bath, allowed to cool slowly to 45°C, and then incubated at room temperature for 5 min. The mixture was then separated by agarose gel (0.7% w/v in Loenings buffer) electrophoresis at 2 V/cm overnight. The position of the heteroduplex-containing band on an unstained gel was estimated from the position of the same band in a lane removed from the gel and stained with ethidium bromide solution

Table 2. Combinations of plasmid DNAs used to construct heteroduplex molecules

Mismatch[a]	Site[b]	Linearized double-stranded plasmid DNA	Single-stranded plasmid DNA
A-G	32	*sup4*-o (C -> A)[c]	*SUP4*-o (WT)[d]
C-T	32	*SUP4*-o (WT)	*sup4*-o (G -> T)
T-G	32	*sup4*-o (C -> T)	*SUP4*-o (WT)
C-A	32	*SUP4*-o (WT)	*sup4*-o (G -> A)
C-A	33	*sup4*-o (T -> C)	*SUP4*-o (WT)
T-G	33	*SUP4*-o (WT)	*sup4*-o (A -> G)
G-A	56	*SUP4*-o (WT)	*sup4*-o (C -> A)
T-C	56	*sup4*-o (G -> T)	*SUP4*-o (WT)
G-T	56	*SUP4*-o (WT)	*sup4*-o (C -> T)
A-C	56	*sup4*-o (G -> A)	*SUP4*-o (WT)
C-A	69	*SUP4*-o (WT)	*sup4*-o (G -> A)
T-G	69	*sup4*-o (C -> T)	*SUP4*-o (WT)

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

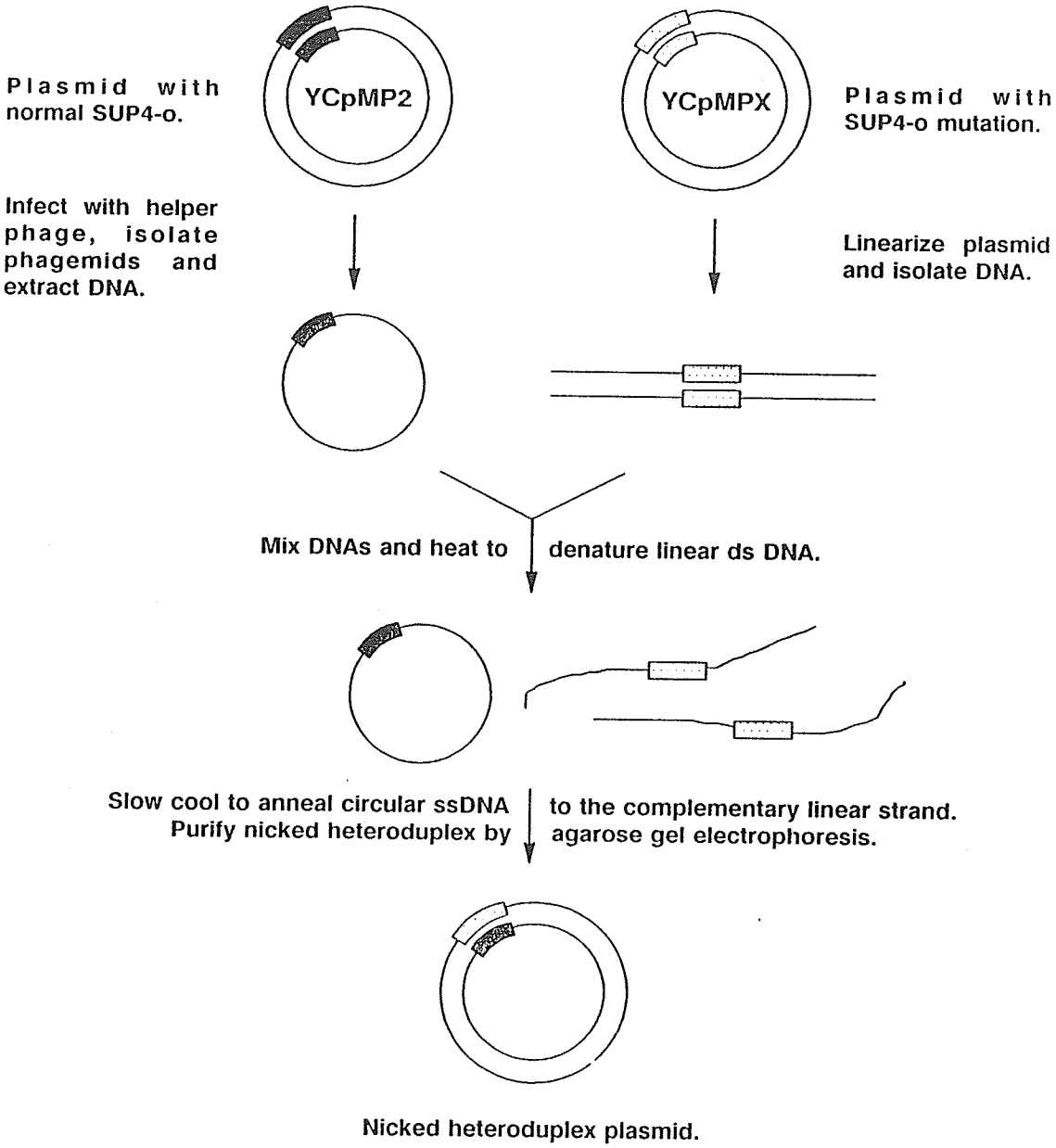
[b] Numbers correspond to sites in the *SUP4*-o gene.

[c] Substitutions at the indicated sites are given for the transcribed (double-stranded DNA) or non-transcribed (single-stranded DNA) strand.

[d] WT: wild-type.

Figure 2. Construction of heteroduplex DNA

HETERODUPLEX CONSTRUCTION



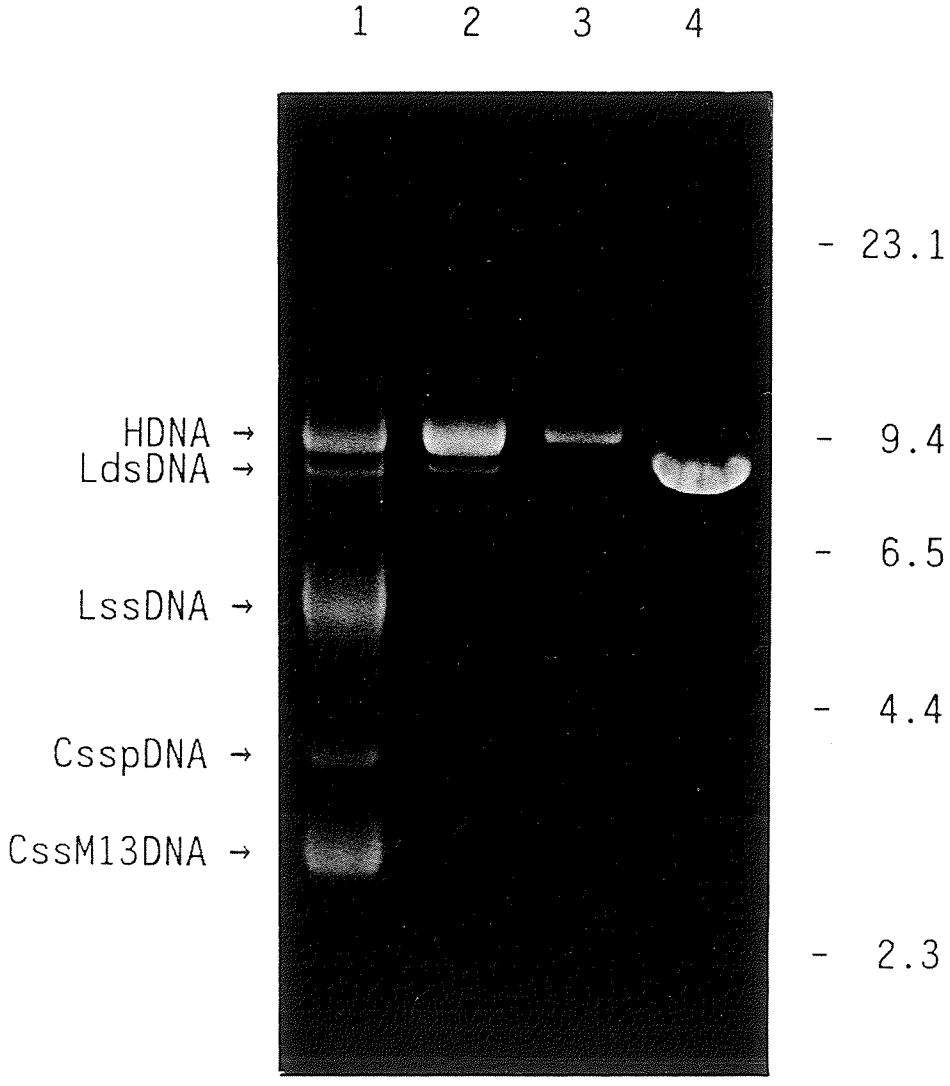
(0.5 μg/ml) and viewed on a transilluminator. The bands were cut out of the unstained gel and the heteroduplex DNA was recovered from the agarose by modification of a freeze-extraction procedure (Thuring et al. 1975). Each gel slice was frozen in a microfuge tube at -20°C overnight and the tube was centrifuged twice at maximum speed in a microfuge (Brinkman 5415C, 4°C), once for 1.5 hours and once for 30 min. After each spin, the supernatant was transferred to a sterile 13 mm diameter tube (Sarstedt). Then, 2 volumes of butanol were added to remove ca. 50% of the H_2O, the tube was vortexed briefly, and the aqueous layer was transferred to a microfuge tube. Following extraction with TE-saturated chloroform, ethanol precipitation, and two washes with 70% ethanol, the DNA was dissolved in TE buffer. Then the DNA was digested with *Micrococcus luteus* endonuclease V at 37°C for 30 min (0.25 U endonuclease V per μg DNA). Following extractions with TE-saturated phenol and TE-saturated chloroform, precipitation with 95% ice-cold ethanol and two washes with 70% ice-cold ethanol, the DNA was dissolved in 50 μl TE buffer. Agarose gel electrophoresis of samples from these preparations indicated that the heteroduplex DNA was not detectably contaminated with single-stranded circular DNA or linearized DNA (Figure 3). Yields were determined by comparing the intensities of ethidium bromide-stained heteroduplex DNA bands with the intensities of DNA bands of known quantity. The nick and the mismatch sites were at least 4 kb apart.

2.9 Transformation Procedures

2.9.1 Bacterial Transformation

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

Figure 3. Purification of heteroduplex plasmid DNA. The samples were electrophoresed in a 0.7% agarose gel at 2 V/cm overnight. The numbers on the right-hand side are the sizes of DNA markers in kb. Lane 1: annealing reaction mixture which contains nicked heteroduplex DNA (HDNA), linear double-stranded DNA (LdsDNA), linear single-stranded plasmid DNA (LssDNA), circular single-stranded plasmid DNA (CsspDNA) and circular single-stranded helper phage DNA (CssM13DNA); lane 2: heteroduplex DNA purified from agarose gel slices by the freeze-squeeze method; lane 3: heteroduplex DNA digested with endonuclease V to eliminate linear double-stranded DNA; lane 4: double-stranded plasmid DNA linearized by digestion with *Xho*I.


1
2
3
4
HDNA →
LdsDNA →
LssDNA →
CsspDNA →
CssM13DNA →
- 23.1
- 9.4
- 6.5
- 4.4
- 2.3

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

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

2.9.2 Yeast Transformation

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

PEG: 44% (w/v) polyethylene $glycol_{4000}$ (Sigma) was dissolved and filter sterilized just prior to use.

Yeast cells were transformed using the lithium acetate procedure described by Ito et al. (1983). YPDLA (100 ml) was inoculated with

stationary phase yeast cells and the culture was incubated with shaking at 30°C overnight until the cell titre reached 1 - 3 x 10^7 cells/ml. The culture was centrifuged (4,080 x g, room temperature, 5 min) to pellet the cells which were washed twice in 5 ml TE buffer and resuspended in 1 ml lithium acetate (100 mM dissolved in TE buffer). The suspension was then incubated at 30°C with shaking for 1 h and 10 μl spermidine (1 mg/ml N-[3-aminopropyl]-1,4 butane diamine, Sigma) was added. For each transformation, a 100 μl aliquot of the cell suspension was transferred to a sterile 13 mm tube (Sarstedt). The DNA to be transformed into the cells was added and the tube was incubated at 30°C for 30 min without shaking. [To disrupt *RAD18*, 7.8 μg of the isolated DNA fragment described in the Results section **3.1.1** was used. To introduce YCpMP2 into XK15, 1 μg plasmid DNA was used. To assay mismatch correction, 0.25 μg heteroduplex DNA and 200 μg sonicated salmon sperm DNA as carrier (Shiestl and Gietz 1989) were used. For cotransformation measurements, 0.125 μg of YCpMP2 and 0.125 μg of YCpMP2-32A or YCpMP2-56T DNA, or nicked versions of the same plasmids, were used.] PEG (440 μl) was added gently and the tube was incubated for an additional 1 h at 30°C without shaking and then heated at 42°C for 5 min. Cells were then pelleted by centrifugation (1,850 x g, room temperature), washed twice with, and resuspended in, 1 ml SD medium containing no supplements. 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. For transformation with heteroduplex DNA, or for cotransformation controls, the proportion of red, white and red/white sectored transformants that emerged were determined. For other transformations, selected transformants were purified by streaking onto the same type of medium used to select the

transformants and incubating the plates for 2-3 days at 30°C. The genotypes of the transformants were then characterized by testing for growth on appropriately supplemented media.

2.10 DNA Sequencing

10x Buffer 3: Reaction buffer 3 from BRL

RNase: see section **2.7.1**

*Bam*HI: 50 U/μl *Bam*HI (BRL) was diluted to a concentration of 1 U/μl in 1x buffer 3 just prior to use.

RP primer: 0.1 A_{260} U/ml M13 reverse sequencing primer (Pharmacia). This 17 bp primer has the sequence 5'-d(CAGGAAACAGCTATGAC)-3' and binds at position +167 to +183 relative to the first base-pair of the *SUP4*-o gene on YCpMP2. Stored at -20°C.

[^{32}P] dATP: 3000 Ci/mmol α-[^{32}P] dATP (DuPont). Stored at -70°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 were 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 and distilled water and urea were then added step-wise. The solution was filtered first through a double layer of Whatman filter paper #1 and then through a 0.2 μm Millipore filter. Insta-gel was stored in a brown bottle at 4°C.

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

A modification of the dideoxynucleotide chain termination sequencing procedure (Sanger et al. 1977) as described by Korneluk et al. (1985) was used. To prepare double-stranded plasmid DNA for sequencing, the DNA was first linearized by mixing 1.2 μl of 10x buffer 3, 1.0 μl RNase, 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 mixture and the tubes were spun briefly (Brinkman 5415C) to mix both solutions. These microfuge tubes were transferred to a 46°C waterbath and incubated for 20 min and 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).

2.11 Hybridization Analysis

2.11.1 Agarose Gel Electrophoresis

Enzyme digest:	1x reaction buffer 3 (10x buffer 3 supplied by (BRL) 1 μg DNA 50 units of *Eco*RI (BRL) 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 digestion mix:	1x buffer 3 (10x buffer 3 supplied by BRL) 50 μg λ DNA (Pharmacia) 200 U *Hind*III (BRL) The enzyme digest was incubated for 1.5 h at 37°C and the reaction was terminated by adding stop buffer (one-fourth of the reaction volume). The DNA was stored at 4°C and heated at 65°C for 5 min before use.
Loenings buffer:	see section 2.8.3

Total yeast DNA was digested with *Eco*RI for 1 h, stop buffer was added to terminate the reaction and the resulting DNA fragments were

separated by agarose (0.7%, w/v, dissolved in Loenings buffer) gel electrophoresis at 1 V/cm (a λ DNA standard was included). The gel was stained with ethidium bromide (0.5 μg/ml), a picture was taken for later sizing of the bands and the gel was soaked for 1 h in 0.5 M NaOH and then 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 Nick Translation

10x NT buffer:	500	mM	Tris, pH 7.2
	100	mM	$MgSO_4$
	1	mM	dithiothreitol
	500	μg/ml	bovine serum albumin (BRL)
	Stored at -20°C.		
$[^{32}P]$dATP:	see section **2.10**		
Reaction mix:	1x		NT buffer
	1	μg	DNA
	20	μM	dATP (this final concentration includes the concentration of $[^{32}P]$dATP in the reaction mix)
	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.		
DNase:	0.1 U/ml DNase I (BRL)		
	50% (v/v) glycerol		
	1 x NT buffer		
	Prepared just before use and kept on ice.		
DNA polymerase:	5 U/μl DNA polymerase I Klenow fragment from *E. coli* (FPLC pure, Pharmacia). Stored at -20°C.		
Salmon sperm DNA:	10 mg/ml salmon sperm DNA was dissolved in water 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 nick

translation (Maniatis et al. 1982). The reaction mix was added to a sterile screw cap microfuge tube and chilled to 11°C. DNase (1 μl) was added and the contents of the tube were gently mixed by tapping and then incubated at 11-13°C for 10 min. Next, 1 μl DNA polymerase was added and the tube was kept at 16°C for 50 min. Successively, 2 μl Na_2EDTA (500 mM), 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 transferred to -60°C for 1 h, the precipitate was pelleted by centrifugation (Brinkman 5415C) for 15 min at 4°C and the supernatant was removed with a Pasteur pipette and discarded. Then, 1 ml ice-cold ethanol (70%) was added to the tube which was held at -60°C for 1 h and centrifuged for 15 min at 4°C. The supernatant was removed with a pasteur pipette and the pellet dissolved in 100 μl sterile water. To this, 100 μl salmon sperm DNA was added and 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
Prepared just prior to use and heated to 65°C.

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

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

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

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

Direct DNA hybridization in agarose gels was carried out as described by Tsao et al. (1983) The dried gel was transferred into a plastic bag (Philips KB5936), prehybridization solution was added (2 ml/cm^2 of gel) and the bag was heat-sealed and incubated for 2-4 h at 65°C. The prehybridization solution was removed and replaced with hybridization solution (50 μl/cm^2 of gel), the nick-translated DNA was added and the bag was heat-sealed and incubated overnight at 65°C. The gel was then removed carefully from the bag and the following washes were performed: 1. 250 ml of solution I for 5 min at room temperature (twice). 2. 250 ml of solution II for 15 min at room temperature (twice). 3. 250 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 -70°C.

2.12 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 the Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina. For both types of test, values of $P < 0.05$ were considered significant.

3 RESULTS

3.1 Specificity of Yeast *rad* Mutators

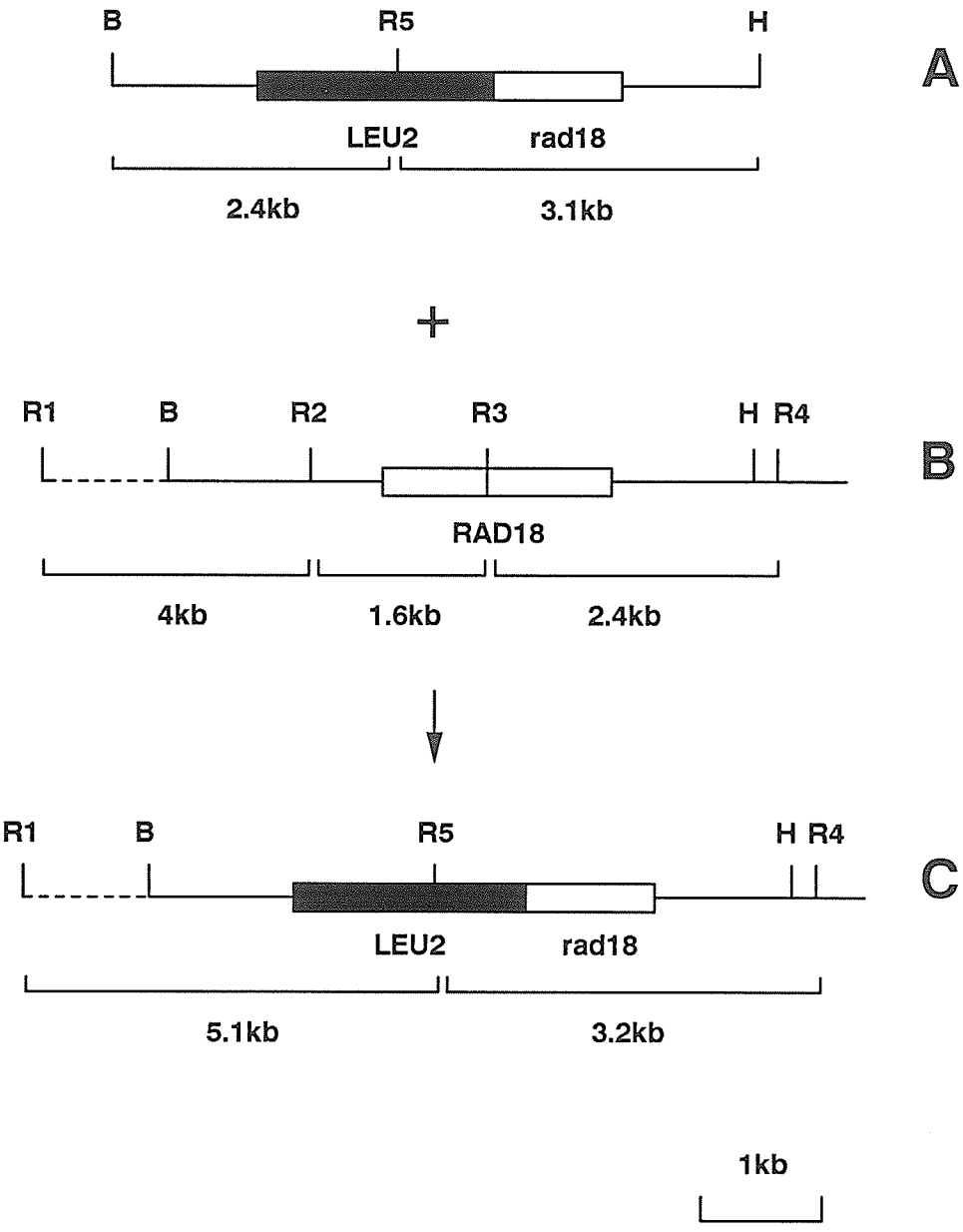
The object of this part of the study was to investigate the mutator effects conferred by defects in the *RAD1*, *RAD6* and *RAD18* genes. The approach taken was to characterize spontaneous mutations arising in the *SUP4*-o gene in *rad1*, *rad6* or *rad18* backgrounds.

3.1.1 Construction of a *rad18* Strain

In order to investigate the mutational specificities of the yeast *rad* mutators, it was first necessary to construct a *rad18* strain (the *rad1* and *rad6* strains were constructed by K.A. Magnusson and R.D. Gietz, respectively). XK15, carrying a *rad18::LEU2* insertion, was derived by transforming MKP-o with a 5.5 kb *Bam*HI-*Hpa*I DNA fragment (Figure 4) encompassing the *RAD18* gene and having a 2 kb *Hpa*I-*Hpa*I fragment containing the *LEU2* gene inserted between the *Eco*RI sites immediately 5′ to, and within, *RAD18* (Fabre et al. 1989). This replacement deletes a 1.6 kb fragment running from 660 bp upstream of the first ATG codon through the initial 950 bp (65%) of the *RAD18* coding sequence (Chanet et al. 1988; Fabre et al. 1989) (Figure 4A). The 5.5-kb *Bam*HI-*Hpa*I fragment was isolated from plasmid p*rad18Δ1* (provided by F. Fabre). Since MKP-o requires leucine due to a defective *LEU2* allele (*leu2-3,112*), the *LEU2* gene in the *Bam*HI-*Hpa*I fragment allows for positive selection of fragment integration events by growth on medium lacking leucine. Colonies that emerged on leucine omission medium were tested for sensitivity to UV light because the *RAD18* gene is involved in the repair of UV-induced DNA damage. Several UV-sensitive transformants were selected and analyzed further.

Replacement of the *RAD18* gene with the 5.5 kb *Bam*HI-*Hpa*I fragment was assessed by DNA hybridization analysis as follows. Total chromosomal DNA from MKP-o and UV-sensitive transformants was digested with *Eco*RI and

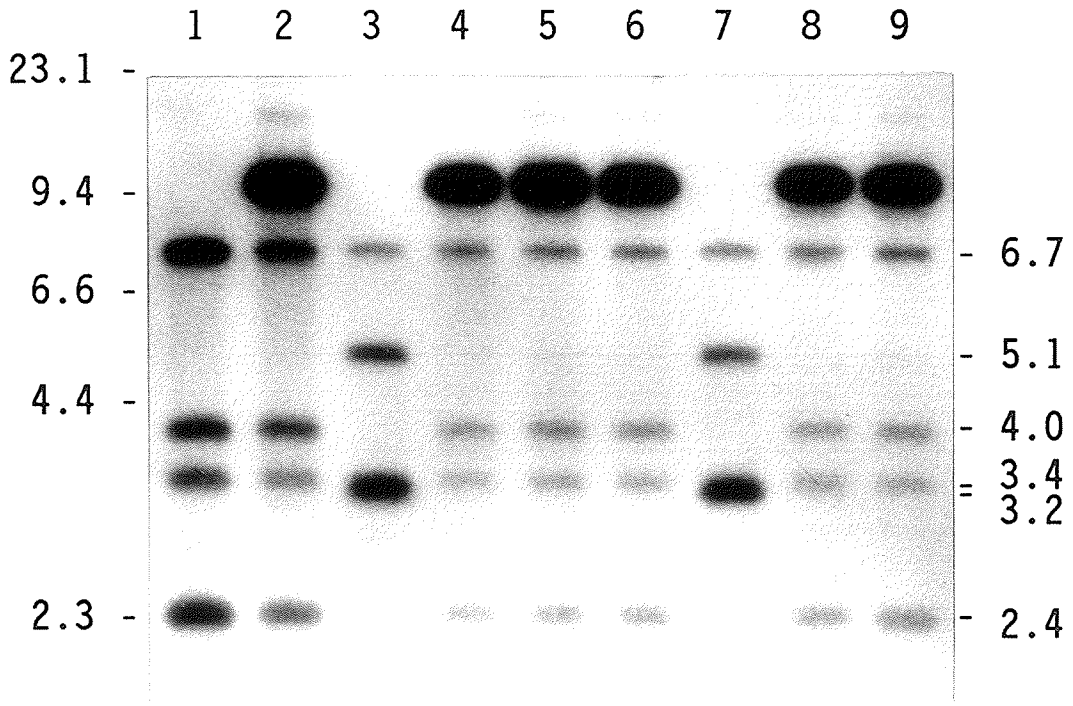
Figure 4. Inactivation of the *RAD18* gene in MKP-o. The figure shows replacement of the *RAD18* gene (B) with a partially deleted copy carried on a *Bam*HI-*Hpa*I fragment obtained from p*rad18Δ1* (A). Replacement occurs via homologous recombination and inactivates the chromosomal *RAD18* gene as shown in C. The dashed line indicates a stretch of yeast chromosomal DNA of unknown length. R1, R2, R3, R4, and R5 are *Eco*RI recognition sites; B: *Bam*HI site; H: *Hpa*I site.


B
R5
H
A
LEU2
rad18
2.4kb
3.1kb
+
R1
B
R2
R3
H
R4
B
RAD18
4kb
1.6kb
2.4kb
R1
B
R5
H
R4
C
LEU2
rad18
5.1kb
3.2kb
1kb

the resulting fragments were separated by gel electrophoresis. After appropriate treatment of the gel (as detailed in Materials and Methods), it was dried and probed with ^{32}P-labelled copies of the 5.5 kb *Bam*HI-*Hpa*I fragment. As can be determined from Figure 4, the probe should detect four fragments when hybridized to MKP-o DNA. These correspond to the R1-R2 (size unknown) and 2.4 kb R2-R4 fragments shown in Figure 4B and the 3.4 kb and 6.7 kb fragments that result from cutting at site R5 in the chromosomal copy of the *LEU2* gene (determined by hybridization of a *LEU2* probe to *Eco*RI-digested MKP-o DNA, data not shown). The 1.6 kb R2-R3 fragment (Figure 4B) would not be detected because the *LEU2* gene replaces this fragment in the 5.5 kb *Bam*HI-*Hpa*I probe (Figure 4A). Figure 5 shows that MKP-o DNA gave four hybridization bands of 2.4, 3.4, 4 and 6.7 kb. Thus, the 4 kb band corresponds to the R1-R2 fragment shown in Figure 4B. Integration of the 5.5 kb *Bam*HI-*Hpa*I fragment at the chromosomal *RAD18* locus would change this pattern. In particular, the 4 kb R1-R2 fragment should be replaced by the 5.1 kb R1-R5 fragment and the 2.4 kb R3-R4 fragment should be replaced by the 3.2 kb R5-R4 fragment (Figure 4C). Since the 3.2 kb R5-R4 fragment and the 3.4 kb *LEU2* fragment are very close in size, they might overlap to generate a single wide hybridization band. For two of the transformants, XK15 and XK27, the 2.4 kb and 4 kb bands were missing, a new band at 5.1 kb was evident and the band at 3.4 kb was slightly wider (Figure 5). This is the pattern expected for replacement of the chromosomal *RAD18* gene with the *rad18::LEU2* insertion. It appears from the autoradiogram that the 5.5 kb *Bam*HI-*Hpa*I fragment integrated at some other location in the remaining six transformants.

To confirm that the UV sensitivity of XK15 was due to inactivation of the *RAD18* gene, this isolate was crossed to an unrelated haploid strain

Figure 5. Hybridization analysis of Leu$^+$ transformants. Total DNA from each yeast strain was digested with *Eco*RI and separated by electrophoresis in a 0.7% agarose gel. The *Bam*HI-*Hpa*I fragment of plasmid p*rad18Δ1* (Fig. 4A) was labelled with ^{32}P and used as a probe. Lane 1: MKP-o, lanes 2-9: Leu$^+$ transformants, DNA from strains XK15 and XK27 is in lanes 3 and 7, respectively. The DNA size markers are in kb.


1
2
3
4
5
6
7
8
9
23.1
9.4
6.6
4.4
2.3
6.7
5.1
4.0
3.4
3.2
2.4

(BKPA18/2) which carries the *rad18-2* allele at the *RAD18* locus. The resulting diploid also proved to be very UV-sensitive. This failure to complement the UV sensitivity conferred by the *rad18-2* allele verified that the *RAD18* gene in XK15 was defective.

3.1.2 Plasmid Stability and Selection of Spontaneous Mutants

Cultures of yeast strains were grown from low titer inocula to stationary phase in medium selective for the plasmid and then plated to measure plasmid retention and to select canavanine-resistant colonies. Plasmid stability was determined by comparing the number of colonies that formed on medium selective for the plasmid with the corresponding value for nonselective medium. Of the KAM1 and XK15 cells, 87% and 91%, respectively, carried YCpMP2 (Table 3). This indicates that neither the excision repair deficiency nor the *rad18* defect influence plasmid maintenance because the values for both KAM1-p and XK15p are typical for retention of YCpMP2 in MKP-op (*RAD*) (Table 3). On the other hand, less than two-thirds of the RDG6-p cells carried the plasmid (Table 3). This difference between the plasmid retentions in MKP-op and RDG6-p, is significant ($P < 0.001$) and suggests that the *RAD6* E_2 may also contribute to assuring the stability of chromosomes or their accurate segregation at mitosis.

The fractions of the total canavanine-resistant colonies arising in the *rad1*, *rad6* and *rad18* strains that were red and Lys^-, and so were classified as *SUP4*-o mutants, were similar to the corresponding value (3%) for the wild-type strain (Table 4). The mean frequency of spontaneous mutation at the *SUP4*-o locus was 6.5-fold higher for KAM1-p, 8-fold higher for RDG6-p and 3-fold higher for XK15p compared to MKP-op. These increases were significant (in all three cases, $P < 0.001$) and reflected the

Table 3. Mitotic stability of plasmid YCpMP2

Strain	No. of colonies on selective medium	No. of colonies on nonselective medium	Percent retention
MKP-op (*RAD*)	37,299	42,231	88.3
KAM1-p (*rad1*)	9,968	11,513	86.6
RDG6-p (*rad6*)	11,387	17,866	63.7
XK15p (*rad18*)	8,488	9,355	90.7

Table 4. Characterization of canavanine-resistant colonies

Strain	Colony types[a]	Number examined[b] (% of total)		Frequency[b] (x 10^{-6})
MKP-op (*RAD*)	Total	31,923	(100)	65
	Red, Lys^-	984	(3.1)	2.0
KAM1-p (*rad1*)	Total	37,316	(100)	360
	Red, Lys^-	1,358	(3.6)	13.1
RDG6-p (*rad6*)	Total	19,703	(100)	560
	Red, Lys^-	593	(3.0)	16.8
XK15p (*rad18*)	Total	6,369	(100)	155
	Red, Lys^-	245	(3.8)	5.9

[a] The total includes canavanine-resistant mutants that are (i) white or (ii) red and Lys^+ or Lys^-. The white or red and Lys^+ colonies likely reflect mutation at the *CAN1* locus or at anti-suppressor loci, respectively.

[b] Colonies were isolated from, and frequencies are the means for 45 (MKP-op), 22 (KAM1-p), 25 (RDG6-p), or 10 (XK15p) independent cultures.

respective mutator effects. The spontaneous mutation rates were calculated to be 4.7×10^{-7} for MKP-op, 2.2×10^{-6} for KAM1-p, 2.4×10^{-6} for RDG6-p and 1.4×10^{-6} for XK15p. Since the strains tested are isogenic except for the particular *rad* loci, any differences detected should be due to the *rad* mutator effects rather than variation in genetic background.

3.1.3 Classes of Mutation Detected

To ensure the independence of the spontaneous *SUP4*-o mutations characterized by DNA sequencing, each mutant was chosen from a separate culture. Collections of 249 mutants arising in KAM1-p (*rad1*), 202 mutants arising in RDG6-p (*rad6*) and 212 mutants arising in XK15p (*rad18*) were examined (the mutants isolated in the *rad6* background were characterized by F. Yadao). The results were then compared to those for 354 mutants isolated in MKP-op [data for 32 mutants isolated as part of this study were pooled with the results for 322 mutants analyzed earlier (Kunz et al. 1990)]. Ten different mutational classes were identified among the mutants collected from the four strains: single and double (tandem and nontandem) base-pair substitutions, single and multiple base-pair deletions, insertions of single or multiple base-pairs or yeast Ty elements (Ty is a 6 kb retrotransposon), duplications and more complex changes (Table 5).

With the exception of multiple base-pair deletions, the relative proportions of those classes of mutation common to both MKP-op and KAM1-p (*rad1*) (single and non-tandem double base-pair substitution, single base-pair deletion and Ty insertion) were similar (Table 5). Increases in the frequencies of the single base-pair substitutions and deletions and Ty insertions in KAM1-p were all approximately 6-fold and together accounted for >98% of the overall *rad1* mutator effect. A tandem double substitution and a multiple base-pair insertion were detected in the *rad1* strain but

Table 5. Sequence alterations in *SUP4*-o mutants

Sequence alteration	MKP-op (*RAD*) Number (%)	MKP-op (*RAD*) Frequency (x 10^{-7})	KAM1-p (*rad1*) Number (%)	KAM1-p (*rad1*) Frequency (x 10^{-7})	RDG6-p (*rad6*) Number (%)	RDG6-p (*rad6*) Frequency (x 10^{-7})	XK15p (*rad18*) Number (%)	XK15p (*rad18*) Frequency (x 10^{-7})
Substitution								
Single	290 (81.9)	16.5	211 (84.1)	111.0	142 (70.3)	118.1	197 (92.9)	54.8
Double					-			
Tandem	-		1 (0.4)	0.5			1 (0.5)	0.3
Nontandem	2 (0.6)	0.1	1 (0.4)	0.5			-	
Deletion								
1 bp	24 (6.8)	1.4	16 (6.4)	8.5	4 (2.0)	3.3	4 (1.9)	1.1
>1 bp	7 (2.0)	0.4	2 (0.8)	1.1	1 (0.5)	0.8	2 (0.9)	0.5
Insertion							-	
1 bp	1 (0.3)	0.06	-		-			
> 1 bp			1 (0.4)	0.5	-			
Ty element	26 (7.3)	1.4	17 (6.9)	8.9	53 (26.2)	44.1	6 (2.8)	1.7
Duplication	1 (0.3)	0.06	-		1 (0.5)	0.8	1 (0.5)	0.3
Complex change	3 (0.8)	0.2	-		1 (0.5)	0.8	1 (0.5)	0.3
Total	354		249		202		212	

not in MKP-op whereas no single base-pair insertions, duplications or complex changes were recovered in KAM1-p.

As anticipated on the basis of other investigator's findings (Picologlou et al. 1990), there was a large (> 30-fold) increase in the frequency of Ty insertion into *SUP4*-o in RDG6-p (Table 5). However, even though the fraction of single base-pair substitutions was significantly less in the *rad6* strain (70% vs. 82% for MKP-op, $P < 0.02$), the frequency of these events was increased 7-fold. Furthermore, this enhancement accounted for more than two-thirds of the total increase in the *SUP4*-o mutation frequency attributed to the *rad6* mutator. Single and multiple base-pair deletions, duplications and more complex changes were recovered in each strain at similar frequencies (Table 5). Thus, these types of spontaneous mutation likely involve processes independent of *RAD6*. Neither double base-pair substitutions nor single base-pair insertions were detected in the *rad6* strain. It is possible that the preferential increase in single base-pair substitutions and Ty insertions effectively reduced the probability of detecting the rarer mutational classes. Indeed, the relative fractions of single and multiple base-pair deletions and complex changes were decreased for RDG6-p relative to MKP-op.

Single base-pair substitutions constituted a significantly greater fraction of the total mutations in XK15p than in MKP-op (93% vs. 81%, $p < 0.001$) and occurred 3-fold more frequently in the *rad18* strain (Table 5). This increase in the frequency of single base-pair substitutions was sufficient to account for the entire increase in the *SUP4*-o mutation frequency in XK15p. In addition to single base-pair substitutions, a number of mutational classes including double base-pair substitutions, single base-pair deletions, multiple base-pair deletions, insertions of

the yeast retrotransposon Ty, and more complex changes were recovered in the *rad18* strain at frequencies similar as to those for MKP-op. Thus, these classes of mutation likely represented spontaneous events that resulted from processes independent of *RAD18*.

3.1.4 Analysis of Base-Pair Substitutions

Single base-pair substitutions accounted for the majority of spontaneous mutations in all four strains. Here this mutational class is examined in greater detail.

3.1.4.1 *rad1* Strain

In the *rad1* strain, the increment in the frequency of single base-pair substitutions was responsible for 85% of the mutation frequency increase (Table 5). Both types of transition and all four possible transversions were recovered in MKP-op and KAM1-p, although there was a small excess of transversions for each strain (Table 6). The relative fractions of the different base-pair changes in the two strains were quite similar. One nontandem double substitution (A·T -> T·A at position -2 and C·G -> A·T at position 18) and one tandem double substitution (C·G -> T·A at position 26 and G·C -> T·A at position 27) were detected in the *rad1* strain. Two nontandem, double base-pair changes were detected in MKP-op. Each involved A·T -> G·C and G·C -> A·T transitions, either at positions 12 and 14 or positions 8 and 10.

The distributions, within *SUP4*-o, of the substitutions arising in MKP-op and KAM1-p, including double events, are given in Figure 6. A total of 67 sites were mutated and some similarities were noted between the two distributions. For both strains, base-pair substitutions occurred at only one position (site 51) within the tRNA intron while the remaining changes were distributed throughout the *SUP4*-o gene with 47 sites in common. In

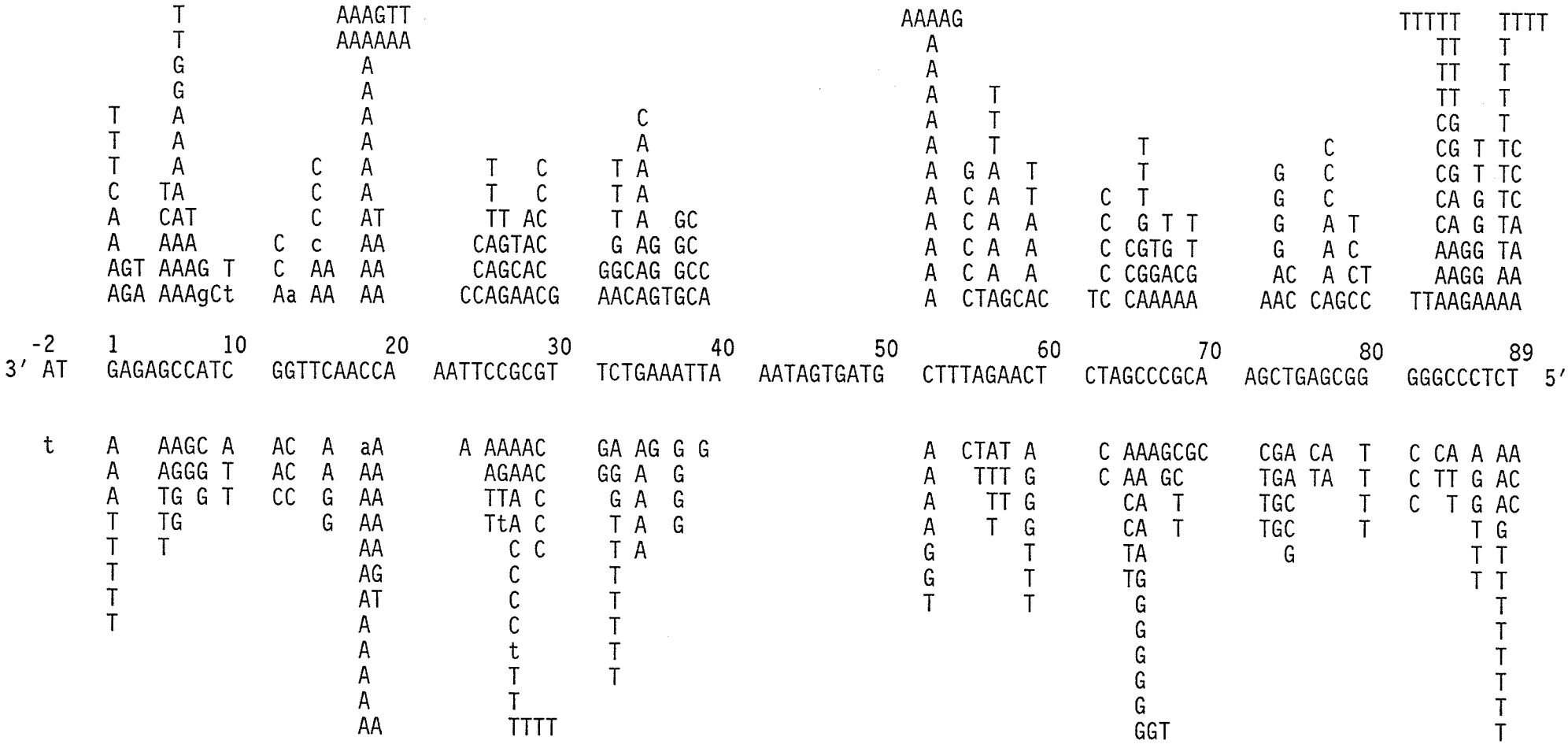
Table 6. Base-pair substitutions

Substitution	Number[a] (%)			
	MKP-op (*RAD*)	KAM1-p (*rad1*)	RDG6-p (*rad6*)	XK15p (*rad18*)
Transitions				
G·C -> A·T	76 (25.8)	53 (24.7)	39 (27.5)	22 (11.1)
A·T -> G·C	44 (15.0)	14 (6.5)	15 (10.6)	12 (6.0)
Total	120 (40.8)	67 (31.2)	54 (38.1)	34 (17.1)
Transversions				
G·C -> T·A	99 (33.7)	74 (34.4)	85 (59.8)	152 (76.4)
G·C -> C·G	59 (20.1)	56 (26.0)	3 (2.1)	7 (3.5)
A·T -> C·G	5 (1.7)	7 (3.3)	-	-
A·T -> T·A	11 (3.7)	11 (5.1)	-	6 (3.0)
Total	174 (59.2)	148 (68.8)	88 (61.9)	165 (82.9)

[a] Including events in multiple mutations.

Figure 6. Distribution of spontaneous base substitutions in the *SUP4*-o gene in the *rad1* strain. For simplicity, only the region of the transcribed strand encoding the tRNA is shown, along with the first two upstream bases. The anticodon is at 36 to 38 and the 14-base-pair intron extends from 40 through 53 (Knapp et al. 1978). Mutations isolated in MKP-op (*RAD1*) and KAM1 (*rad1*) are presented above and below the transcribed strand, respectively. Lower case letters indicate changes in double mutants.

RAD1


-2 1 10 20 30 40 50 60 70 80 89
3′ AT GAGAGCCATC GGTTCAACCA AATTCCGCGT TCTGAAATTA AATAGTGATG CTTTAGAACT CTAGCCCGCA AGCTGAGCGG GGGCCCTCT 5′

rad1

addition, two of the most frequently mutated sites (18 and 88) coincided and of the specific substitutions at the common sites, 75% of those detected in MKP-op were also found in KAM1-p. On the other hand, a number of differences were also observed. While no changes were recovered 5′ or 3′ to the *SUP4*-o coding region in MKP-op, an A·T -> T·A transversion occurring as part of a non-tandem double event was detected at position -2 in KAM1-p. This is the first substitution we have identified outside of the region encoding the tRNA. However, since this change has not yet been detected as an individual event among several thousand *SUP4*-o mutations characterized to date, it might not reduce *SUP4*-o expression or tRNA processing sufficiently to permit its recovery as a single substitution in this system. Base-pair changes were detected at fewer sites for KAM1-p than for MKP-op (50 vs. 64) and the substitution frequencies in the *rad1* strain were from 3 to 52-fold greater at 42 of the 47 common sites. Finally, 25% of the specific substitutions detected in the *rad1* background at sites common to both strains were missing from the corresponding sites in the *RAD1* distribution. By using the Monte Carlo estimate of the *P* value of the hypergeometric test (Adams and Skopek 1987) to compare the two distributions statistically, we found that the probability of random sampling error being the cause of differences in the two distributions was less than 1 in 200 (with 1,500 simulated comparisons, the upper limit on the 90% confidence interval for the estimate of *P* was 0.005).

3.1.4.2 *rad6* Strain

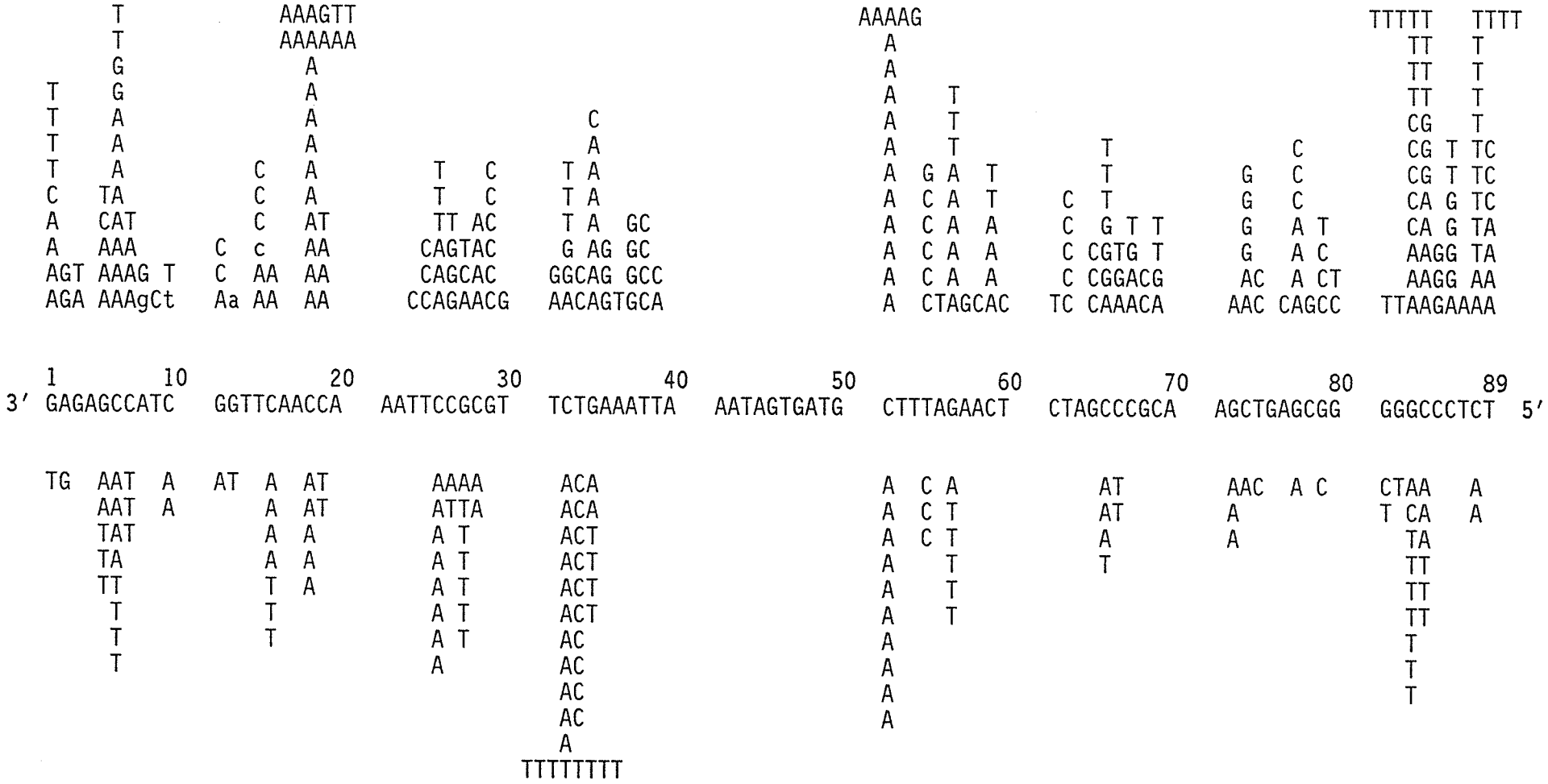
In RDG6-p, only the two transitions and transversions at G·C pairs were detected, whereas both transitions and all four possible transversions were recovered for MKP-op (Table 6). The fraction of G·C -> C·G transversions decreased and the proportion of G·C -> T·A events

increased in RDG6-p relative to MKP-op ($P < 0.001$ in both cases). Although there was a small decrease in the fraction of A·T -> G·C transitions, it was not significant ($P > 0.2$). The changes in the relative fractions of the base substitutions resulted in an 8-fold bias for changes at G·C pairs compared to a 4-fold preference in MKP-op, a significant difference ($P < 0.02$). From Table 6, it is clear that only the frequencies of the two transitions and the G·C -> T·A transversion were enhanced by the *rad6* mutator but the magnitude of the increase was greatest for the transversion.

The distributions of the base-pair substitutions arising in RDG6-p and MKP-op are presented in Figure 7. Base-pair changes occurred within the tRNA intron only at position 51 and none was recovered 5′ or 3′ to the *SUP4*-o coding region. Substitutions were detected at 65 sites but mutations were found at substantially fewer positions in RDG6-p than MKP-op (33 vs. 65, respectively). There are 12 G·C and 25 A·T pairs where single substitutions can be detected in *SUP4*-o but which were not mutated in the *rad6* strain. G·C -> T·A and/or G·C -> A·T events can be detected at all 12 G·C pairs and A·T -> G·C transitions at 19 of the 25 A·T pairs (L. Kohalmi and B.A. Kunz, personal communication). Thus, the difference in the numbers of sites mutated does not simply reflect the preference for both transitions and G·C -> T·A transversions in RDG6-p. Rather, it suggests that DNA sequence context influences the site specificity of the *rad6* mutator effect. The mutation frequencies were greater, by as much as 76-fold, at all sites mutated in the *rad6* strain. Application of a statistical test for comparing mutational spectra (Adams and Skopek 1987) indicated that the probability of random sampling error accounting for differences in the distributions was less than 1 in 500 (the upper limit

Figure 7. Distribution of base substitutions in the *SUP4*-o gene in the *rad6* strain. Mutations isolated in MKP-op (*RAD6*) and RDG6-p (*rad6*) are presented above and below the transcribed strand, respectively. Legend as for Figure 6.

RAD6


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

rad6

of the 90% confidence interval on the estimate of *P* was 0.002).

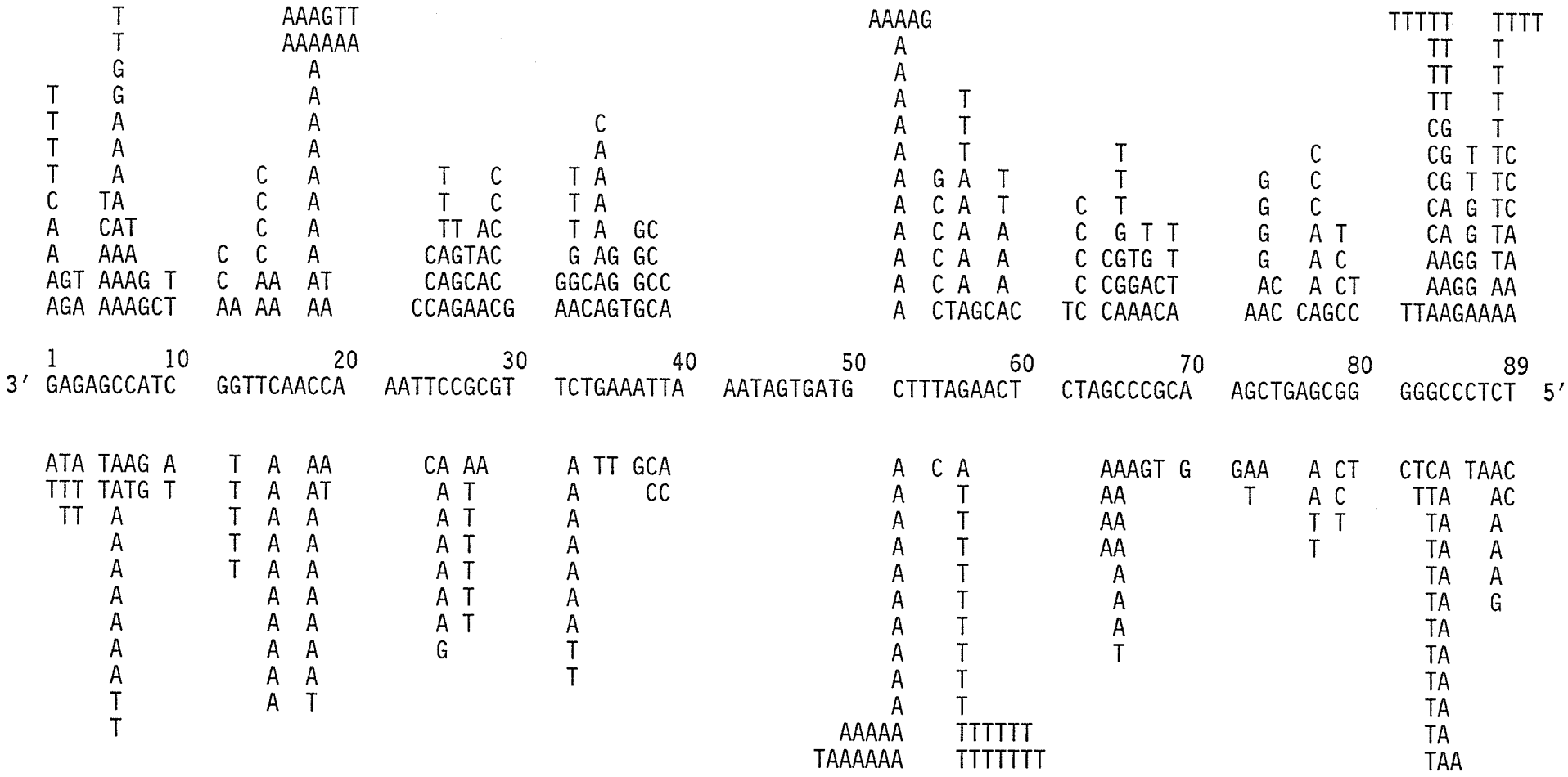
3.1.4.3 *rad18* Strain

In XK15p, only the A·T -> C·G transversion was not detected, whereas both transitions and all four possible transversions were recovered for MKP-op (Table 6). The double substitution identified in XK15p (A·T -> G·C at site 8, G·C -> A·T at site 10) was also detected in MKP-op. Five-fold more transversions than transitions occurred in XK15p and there was a 10-fold bias for substitutions at G·C pairs compared to a slight excess of transversions and a 4-fold preference for changes at G·C sites in MKP-op. These differences were significant ($P < 0.001$) and were due to an increase in the fraction of G·C -> T·A events at the expense of all other substitutions for XK15p relative to MKP-op ($P < 0.001$). From Tables 5 and 6, it can be determined that the 8-fold increase in the frequency of G·C -> T·A transversions accounted for nearly the entire increase (94%) in the overall spontaneous mutation frequency in the *rad18* strain. Although there were small increases in the frequencies of both types of transition and A·T -> T·A transversions, none of these was significant ($P > 0.1 - 0.8$) and it appears that the *rad18* mutator specifically induced G·C -> T·A transversions.

The distributions of the base-pair substitutions arising in MKP-op and XK15p are shown in Figure 8. No changes were recovered 5′ or 3′ to the *SUP4*-o coding region. In total, substitutions were detected at 67 sites, including position 51 within the tRNA intron, but mutations occurred at fewer positions in XK15p than MKP-op (45 vs. 64, respectively). This did not merely reflect the preference for G·C -> T·A transversions in the *rad18* strain since 6 of the 9 exon G·C pairs that were not mutated in XK15p are sites where G·C -> T·A transversions can be detected in *SUP4*-o

Figure 8. Distribution of base substitutions in the *SUP4*-o gene in the *rad18* strain. Mutations isolated in MKP-op (*RAD18*) and XK15p (*rad18*) are presented above and below the transcribed strand, respectively. Legend as for Figure 6.

RAD18


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

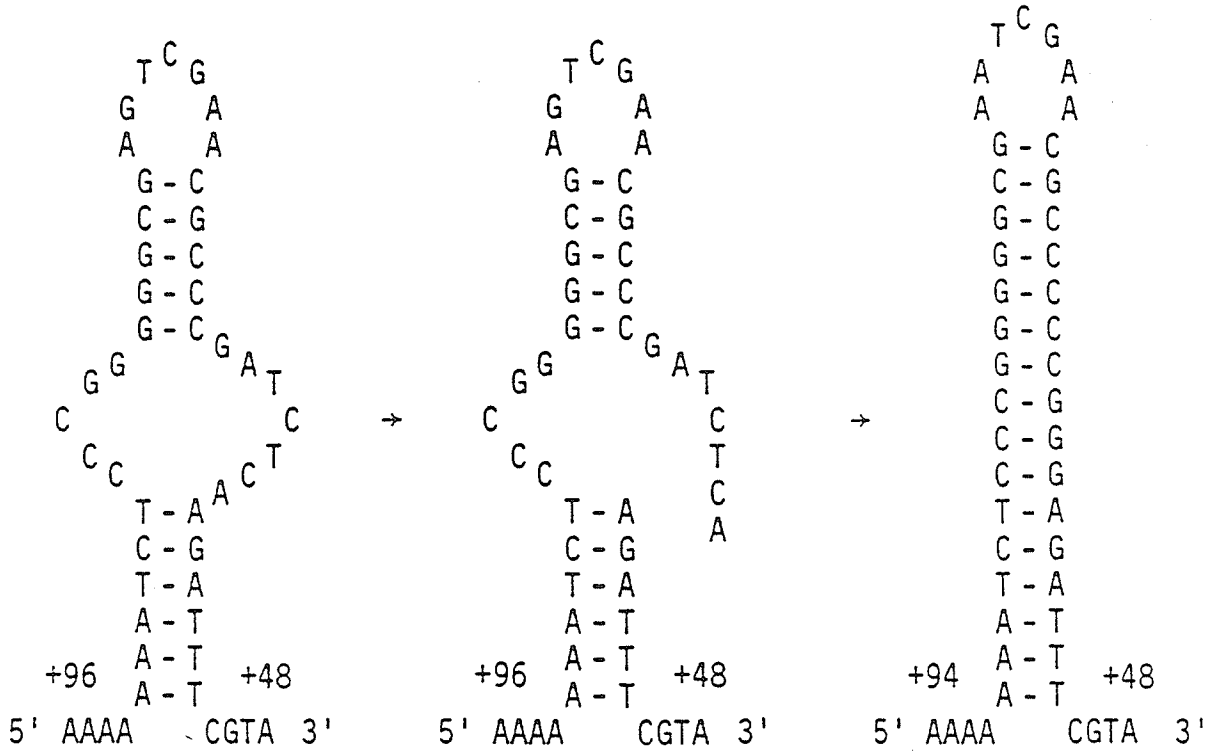
rad18

base-pairs from positions -139 through 29 (RDG6-p and XK15p) or the removal of 834 base-pairs from -655 through 79 (XK15p). The extents and positions of these deletions are only approximations because short repeated sequences (two, four, six and four base-pairs, respectively) were originally present at the deletion termini. Single copies of these sequences were retained in the mutants so that the deletions may have been generated by nonhomologous recombination between the repeats (Meuth 1989).

3.1.6 Ty Insertions

Four different Ty elements have been identified in yeast and can be distinguished, in part, by their long terminal repeats. These differ in sequence and length and are designated delta (330 bp), sigma (340 bp) and tau (370 bp). Delta is associated with Ty1 and Ty2, sigma with Ty3 and tau with Ty4 (Roeder and Fink 1983; Clark et al. 1988; Stucka et al. 1989). All Ty elements identified in this study had conserved features of delta sequences (Giroux et al. 1988) and so were presumably Ty1 or Ty2. Inactivation of *RAD1* or *RAD6*, but not *RAD18*, increased the frequency of Ty insertion into *SUP4*-o (Table 8). In MKP-op, Ty insertion occurred at three target sites, immediately 5′ (with respect to the nontranscribed strand) to positions 18, 38 or 44. The majority of events occurred 5′ to position 38 and this lab has previously reported site 38 to be a hotspot for Ty transposition in *SUP4*-o (Giroux et al. 1988). In each of the *rad* strains, the majority of Ty insertions were detected at this same site (Table 8). However, the relative fraction of Ty insertions at this target site in RDG6-p was smaller than for MKP-op ($P < 0.001$) (although this was also the case for XK15p, the small number of Ty insertions recovered in this strain makes the statistical significance uncertain). Nevertheless, the frequency of Ty insertion at position 38 was 23-fold greater in RDG6-p. In addition,

Figure 9. Inverted repeat in the *SUP4*-o gene. The inverted repeat that potentially can direct the replacement of the sequence 5′-GATCTCA-3′ with 5′-CCGGG-3′, as diagrammed. The replacement perfects the structure of the palindrome. Only the transcribed strand is shown.


+96
+48
5' AAAA CGTA 3'
+96
+48
5' AAAA CGTA 3'
+94
+48
5' AAAA CGTA 3'

and yeast excision repair patches have been roughly estimated to be 60 nucleotides long (Haynes et al. 1981). To test the ability of DNA methylation to direct mismatch correction via excision repair, the plasmid DNAs used to make the heteroduplexes were isolated from the NR3837 or JF1754 strains, so as to produce heteroduplex plasmids in which either strand, or both or neither, was/were methylated.

3.2.2 Mismatch Repair Assay

The yeast strains used in this study contain the *ade2-1* allele which confers red colouring in the absence of a functional *SUP4*-o gene. They were transformed with heteroduplex plasmids carrying defined mismatches in *SUP4*-o and plated onto medium selective for the plasmid in order to screen transformants. Under these conditions, three types of transformant colony could arise: (i) white colonies, indicating that prior to the first round of DNA replication in the transformed cells, the mismatch was corrected to the normal base-pair to give a functional *SUP4*-o allele, (ii) red colonies, indicating that the mismatch was repaired to the incorrect base-pair to yield a mutant *SUP4*-o allele, (iii) sectored red/white colonies, indicating that the mismatch was not repaired. The latter type of colony appears in the absence of mismatch correction because following the first round of DNA replication, one of the initial two daughter cells receives a plasmid with a functional *SUP4*-o allele while the other acquires a plasmid with a defective copy. A red/white sectored colony might also result from cotransformation of a cell with two heteroduplex plasmids because multiple copies of yeast centromere plasmids are mutually unstable in haploid yeast cells (Newlon 1988). By transforming strains with mixtures of nicked copies of YCpMP2 and its mutant derivatives, it was determined that less than 1% of the sectored transformants detected could

Legend to Tables 9 and 10.

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

[b] The asterisk indicates methylation. N: nicked strand; C: continuous strand.

[c] "+" represents a *SUP4*-o$^+$ allele; "-" represents a *sup4*-o$^-$ allele.

[d] Nonsectored red colonies/nonsectored white colonies.

Table 10. Mismatch correction at sites 5 bp in either direction from the GATC sequence in *SUP4*-o

Mismatch[a]	Site	Methylated strand[b]	Allele on nicked strand[c]	MKP-op (*RAD1*) No. of colonies	MKP-op (*RAD1*) Ratio, red/white[d]	MKP-op (*RAD1*) Per cent sectored colonies	KAM1-p (*rad1*) No. of colonies	KAM1-p (*rad1*) Ratio, red/white[d]	KAM1-p (*rad1*) Per cent sectored colonies
G-T	56	N/C	+	927	1.16	11	1,599	0.77	9
		N*/C	+	776	0.70	7	931	0.75	7
		N/C*	+	1,023	0.71	7	1,004	0.85	10
		N*/C*	+	665	0.87	7	1,198	0.91	11
A-C	56	N/C	-	478	1.06	11	439	0.87	11
		N*/C	-	1,406	1.05	14	1,080	1.34	11
		N/C*	-	1,049	0.93	9	1,111	0.86	10
		N*/C*	-	1,228	1.06	11	1,197	0.93	11
T-G	69	N/C	-	800	1.02	1	924	0.86	1
		N*/C	-	1,100	0.73	2	2,881	0.79	1
		N/C*	-	895	0.86	1	513	1.00	2
		N*/C*	-	1,424	0.73	0.4	1,080	0.94	1
C-A	69	N/C	+	843	0.92	1	744	1.00	3
		N*/C	+	610	0.71	2	711	0.81	2
		N/C*	+	1,321	0.84	1	1,551	0.84	2
		N*/C*	+	468	0.83	1	672	0.73	2

3.2.4 Mismatch Correction in the *rad6* Strain

With regard to base-pair substitutions, the *rad6* mutator only increased the frequencies of transitions and G·C -> T·A transversions. The increase in transitions might have been due to a reduction in the overall efficiency of mismatch correction since yeast mismatch repair appears to correct transition mismatches more efficiently than most transversion mismatches (Bishop et al. 1989; Kramer et al. 1989a). Similarly, a failure to repair G-A or C-T mismatches (the convention used for the transversion mismatches is that the correct base in the mismatch is given first) that occurred at G·C sites could have accounted for the enhanced incidence of G·C -> T·A transversions. We tested these possibilities by comparing the abilities of MKP-o and RDG6 to correct the appropriate mispairs. To do so, we constructed a series of nicked heteroduplex plasmids each carrying, in *SUP4*-o, one of the transition mismatches (G-T, C-A) at positions 32, 33 or 56 or one of the transversion mismatches (G-A, C-T) at positions 32 or 56. These sites were selected because, with the exception of the G·C -> A·T transition at site 56, the frequencies of the substitutions that could result from the mismatches were 24 to 163-fold higher in RDG6-p than MKP-op (the G·C -> AT transition frequency at site 56 was 2.5-fold greater in the *rad6* strain).

As judged by the relative proportions of sectored colonies in the two strains (Table 11), none of the mismatches was corrected any less efficiently in the *rad6* background. In addition, repair of five of the ten mismatches (A-G and T-G at 32, C-A at 33, G-A and A-C at 56) was not biased towards restoration of the incorrect base-pair in RDG6-p relative to MKP-op. Although there was such a preference for correction of the remaining mismatches, the differences were too small to account for the

increases in the substitution frequencies at the sites in question.

3.2.5 Mismatch Correction in the *rad18* Strain

Comparison of the mutational spectra for XK15p and MKP-op indicated that the *rad18* mutator effect was attributable to an increase in the incidence of G·C -> T·A transversions. Such an increase could have been due to a failure to repair G-A or C-T mismatches that occurred at G·C sites and so the ability of XK15p to correct these mispairs was assessed. To do so, nicked heteroduplex plasmids carrying G-A or C-T mismatches at each of two positions in *SUP4*-o, 32 and 56 were constructed. These sites were chosen as they had G·C to T·A transversion frequencies that were 30-fold higher in XK15p than MKP-op.

The relative proportions of sectored colonies that emerged in the two strains were quite similar (Table 12), indicating that there was no decrease in the efficiencies with which the mispairs were corrected in the *rad18* background. In addition, correction of the mismatches at site 32, and the C-T mismatch at site 56, was not biased towards restoration of the incorrect base-pair (A·T) in XK15p relative to MKP-op. Although there was a preference for correction of the G-A mismatch at position 56 to a T-A base-pair ($P < 0.02$), the difference was much too small to account for the 30-fold increase in the G·C -> T·A transversion frequency at this site in the *rad18* strain.

4 DISCUSSION

The specificity of spontaneous mutagenesis in the yeast *SUP4*-o gene in *rad1*, *rad6* and *rad18* backgrounds was examined in this study. Additionally, heteroduplex plasmids containing defined base mismatches were used to probe the functions of these *rad* genes in yeast mismatch correction. Here, the possible mechanisms by which these *rad* mutators might influence spontaneous mutation rates are considered. For the purpose of this section, the mutators are not dealt with in numerical order, *rad18* is discussed before *rad6*.

4.1 *rad1* Mutator Effect

Disruption of the *RAD1* gene clearly resulted in a mutator phenotype. Yet there has been controversy regarding the ability of *rad1* defects to enhance spontaneous mutagenesis (Sargentini and Smith 1985). Previously, detection of the mutator effect apparently depended on the assay system and the particular *rad1* allele used, suggesting that the mutator phenotype might be obscured by its own specificity and/or by leakiness of individual *rad1* alleles. Since the frequencies of all types of base-pair substitution were increased in the *rad1* strain, it is doubtful that the *rad1* mutator effect could be concealed by its specificity alone. However, base-pair changes were not detected at all sites in *SUP4*-o where substitutions occurred in the isogenic *RAD1* strain. Thus, neighbouring DNA sequences might modulate the *rad1* mutator effect at specific base-pairs. If so, this could explain why *rad1* strains were not always found to be mutators when mutation was assayed only at specific sites. Leakiness of the *rad1* alleles used might also have been a complicating factor.

Increases in the frequencies of single base-pair substitution, single base-pair deletion and Ty insertion accounted for almost the entire change in the *SUP4*-o mutation frequency in the *rad1* strain. The relative

in *REV3* (Lawrence and Christensen 1979; Lawrence et al. 1984). In this study, it was determined that the *rad1* mutator enhanced the frequencies of spontaneous single base-pair substitutions and deletions. Taken collectively, these findings suggest that the *rad1* mutator phenotype in yeast could be explained, in large part, by error-prone translesion synthesis across spontaneous damage which is a substrate for excision repair. The notion of translesion synthesis past unrepaired spontaneous damage is reminiscent of the hypothesis that spontaneous lesions are channelled through mutagenic repair pathways in strains deficient in error-free repair (Hastings et al. 1976; Quah et al. 1980). The difference is that in the case of translesion synthesis, elevated spontaneous mutagenesis would not reflect mutagenic repair of DNA damage but rather tolerance of spontaneous DNA lesions via an error-prone mechanism.

Insertion of the yeast retrotransposon Ty into *SUP4*-o was also increased in the *rad1* background. It has been found that treatment with UV or 4-NQO, mutagens that produce DNA damage which is subject to excision repair, stimulates transcription of Ty elements and activates their transposition (McEntee and Bradshaw 1988; Bradshaw and McEntee 1989). Although this activation is mutagen dose-dependent and so presumably correlates with the production of DNA damage, the precise mechanism(s) at work has not yet been elucidated. Nevertheless, these results plus the findings reported here argue that activation of Ty transposition by spontaneous DNA damage, which would usually be repaired in a *RAD1* strain, contributes to the *rad1* mutator phenotype.

It should also be considered that the *rad1* mutator effect could be unrelated to spontaneous damage (Lawrence 1982; Sargentini and Smith 1985). One possible explanation would be that defects in the *RAD1* gene

might somehow reduce the accuracy of DNA synthesis. Since Ty retrotransposition is increased in the *rad1* strain, it seems improbable that altered replicational fidelity alone could be responsible for the mutator phenotype. Still, it could be that a decrease in the fidelity of replicative DNA synthesis is responsible for the enhanced frequencies of single base-pair substitution and deletion in the *rad1* strain. However, this would be difficult to reconcile with the ability of *rev3* alleles to eliminate the *rad3-12* mutator phenotype, since the predicted *REV3* polymerase is not required for replicative DNA synthesis (Morrison et al. 1989).

Alternatively, it has been suggested that excision repair functions might recognize mismatches generated either as DNA replication errors, or produced via heteroduplex formation during intrachromosomal recombination (Morrison et al. 1989; Bailis and Rothstein 1990). Accordingly, the increase in single base-pair deletions and substitutions in the *rad1* strain might reflect a deficiency in mismatch correction. In yeast, a base-pair deletion mismatch (single nucleotide loop opposite a missing nucleotide) can be repaired to restore the missing information or fix the deletion (Bishop et al. 1989; Kramer et al. 1989a). Furthermore, in one study (Kramer et al. 1989a), reductions in mismatch repair were found to enhance the incidence of deletion. So a defect in the repair of frameshift mismatches could account for the increased deletion frequency in the *rad1* background. On the other hand, it seems less likely that disruption of the *RAD1* gene enhanced substitution mutagenesis by perturbing mismatch correction. There was no significant increase in the fraction of a particular type(s) of base-pair change in the *rad1* strain arguing that the specificity of mismatch correction was not altered. If instead the *RAD1*

allele (*his1-7*) and forward mutation to ocher suppression, but not locus reversion of an ocher allele (*lys1-1*), were increased in *rad18* strains (von Borstel et al. 1971; Quah et al. 1980). The analysis of the *rad18* mutator effect in this study explains these earlier results. Most likely reversion or intragenic suppression of *his1-7* can result from G·C -> T·A transversion. At least 8 tyrosine, 4 serine and 6 leucine-inserting tRNA genes can mutate to produce ocher suppressor tRNAs in yeast (Guthrie and Abelson 1982; Sherman et al. 1982). Only the tyrosine and serine tRNA genes give rise to ocher suppressors having moderate or high efficiency (Sherman et al. 1982) and the anticodons for the 8 tyrosine tRNA genes and 3 of the 4 serine tRNA genes are GTA and TGA, respectively (Guthrie and Abelson 1982). Since G·C -> T·A transversion could convert both of these to the ocher anticodon TTA, the specificity of the *rad18* mutator would enable it to induce ocher suppressors but not revert the *lys1-1* ocher mutation. The reversion rate of the putative frameshift allele *ura4-11* was also increased in a *rad18* background (von Borstel et al. 1971). However, the nature of the DNA sequence alteration in *ura4-11* is uncertain since it was designated a frameshift allele (Magni 1969) on the basis of inadequate evidence (enhanced meiotic reversion associated with outside marker recombination). My findings imply that the change in *ura4-11* is a base-pair substitution.

The specificity of the *rad18* mutator effect suggested that *RAD18* might play a role in correcting G-A or C-T mismatches to G·C pairs. This potential explanation was very interesting because the *mutY* mutator in *E. coli* specifically enhances G·C -> T·A transversions (Nghiem et al. 1988) and it has been determined that *mutY*$^{+}$ encodes an adenine glycosylase active on G-A mispairs (Au et al. 1989). Yet, we found that G-A and C-T

G·C -> T·A transversions (Loeb et al. 1986) and this was not observed in the *rad18* strain. Still, a number of yeast AP endonucleases have been described (Wallace 1988) and the available data do not allow one to unequivocally exclude the possibility that *RAD18* encodes or regulates a minor AP endonuclease which only recognizes apurinic lesions occurring at G·C sites. However, there is no precedent for an AP endonuclease with this specificity. Similarly, a defect in base excision repair subsequent to the endonuclease step, but only for repair of apurinic lesions at G·C sites, does not seem reasonable. For these reasons, it is unlikely that *RAD18* is involved in the repair of AP sites.

An intriguing alternative is that the *RAD18* gene product functions in minimizing specific DNA replication errors. In *E. coli*, the *mutT* mutator enhances only A·T -> C·G transversions (Yanofsky et al. 1966) and the *mutT*$^+$ protein functions during *in vitro* DNA replication to prevent misincorporation of dGTP opposite template adenine (Akiyama et al. 1989). The influence of *rev3* mutations on the *rad18* mutator effect might indicate a similar role for the *RAD18* gene in DNA synthesis. In particular, the *RAD18* gene product might participate, directly or in a regulatory context, in a process which limits the formation of G-A or C-T mismatches at template guanines or cytosines, respectively, during DNA synthesis. At present, it is not clear whether such a role for *RAD18* in DNA replication would be restricted to translesion synthesis. *REV3* defects confer an antimutator phenotype but spontaneous mutation in *rev3 rad18* double mutants is reduced to the wild-type rather than *rev3* antimutator levels (Quah et al. 1980). This implies that a fraction of the mutations produced by the *rad18* mutator is independent of *REV3*. Interestingly, formation of G-A mispairs was found to be the most frequent error made by purified

yeast DNA polymerase I during *in vitro* DNA synthesis (Kunkel et al 1989). Furthermore, it appeared that neighbouring DNA sequences modulated the production of this mispair since the error frequency varied by as much as 14-fold at different template guanines (Kunkel et al. 1989). It was suggested that proteins required for achieving high fidelity replication *in vivo* were not present in the *in vitro* reactions (Kunkel et al. 1989). Possibly the *RAD18* gene product, or a protein whose expression might be regulated by *RAD18*, is one of these missing fidelity components.

4.3 *rad6* Mutator Effect

Characterization of the mutator effect conferred by deletion of *RAD6*, a gene encoding a ubiquitin-conjugating enzyme, demonstrated that it increases the frequency of base-pair transitions and G·C -> T·A transversions, confirmed the recent finding (Picologlou et al. 1990) that it also enhances Ty transposition in yeast, and showed that it does not influence other mutational classes. Together, these features distinguish the *rad6* mutator phenotype from those of other yeast mutators characterized in this study and elsewhere. In particular, the others increase the frequencies of either all six possible base-pair substitutions (*rad1*), only the three changes at G·C pairs (*rad52*) or just the G·C -> T·A transversion (*rad18*) and increase (*rad1*), decrease (*rad52*) or do not influence (*rad18*) the frequency of Ty transposition (Kunz et al. 1989; this study).

As noted above, deletion of *RAD1*, which functions in excision repair, also enhances Ty transposition and this might reflect Ty activation by unexcised spontaneous DNA damage. The effect of the *RAD6* deletion on Ty transposition cannot be explained in the same way. There is evidence that excision repair is active in a *rad6* background (Prakash

1977) and the magnitude of the *rad6* mutator effect on the transposition frequency was approximately 6-fold greater than the corresponding value for the *rad1* mutator. Consequently, it seems reasonable to accept the suggestion (Picologlou et al. 1990) that a failure to ubiquitinate Ty proteins, or altered chromatin structure resulting in increased access of transposition complexes to DNA, might account for enhanced Ty transposition in a *rad6* background. Not only did deletion of *RAD6* increase the frequency of Ty insertion into *SUP4*-o, but it also led to insertion of Ty at a larger number of sites (nine for RDG6-p vs. three for MKP-op). This did not simply reflect the greater number of Ty insertions detected in the *rad6* strain since the fraction of Ty elements found in this strain at the hotspot position (5′ to site 38) was significantly smaller than in the wild-type parent. Although target site specificity might be affected by the conditions used to select mutants due to Ty insertion (Wilke et al. 1989), the same selective conditions applied to isolation of *SUP4*-o mutants from all strains used in this study. Thus, our results suggest that the *RAD6* gene product functions to limit the target site specificity, as well as the frequency, of Ty transposition.

An increase in the frequencies of both transitions and the G·C -> T·A transversion was the major effect attributable to the *rad6* mutator. This specificity might partially explain the reported failure of *rad6-1* and *rad6-3* defects to enhance reversion of several different nutritional markers (Kern and Zimmermann 1978; Monteleone et al. 1981). However, one of the alleles whose reversion was not increased, *trp5-48*, is an ocher allele as is *lys1-1* which was stimulated to revert (locus reversion was not distinguished from suppression) by the *rad6-1* mutator (Hastings et al. 1976). These seemingly contradictory results might

reflect, in part, the fact that the *rad6-1* and *rad6-3* alleles can be easily suppressed (Lawrence and Christensen 1979) and the influence of such suppression on the *rad6* mutator phenotype has yet to be investigated in detail. In any event, the *rad6* mutator could enhance spontaneous base-pair substitution in at least three ways: by preventing pre-replicative repair of spontaneous DNA damage, by decreasing the efficiency or specificity of post-replicative mismatch correction or by reducing the fidelity of DNA replication.

Pre-replicative repair of spontaneous DNA damage could be diminished in a *rad6* strain if the relevant repair enzymes were denied access to DNA lesions or if enzyme activity required *RAD6*-mediated ubiquitination. Yet, repair mechanisms that can deal with spontaneous damage may function in *rad6* strains. A *rad6* defect did not prevent intracellular excision repair of UV-induced DNA damage (Prakash 1977) and extracts from wild-type yeast cells or a strain carrying a *rad6-1* allele had the same levels of apurinic/apyrimidinic endonuclease activity (Chlebowicz and Jachymczyk 1977). Additionally, a single type of DNA damage that gives rise to base-pair substitutions with the same specificity as the *rad6* mutator has yet to be identified. Thus, it seems unlikely that failure to repair a particular form of DNA damage accounts for the enhancement of the three classes of substitution that are increased spontaneously in the *rad6* background. Similarly, it is doubtful that a general inability to repair spontaneous DNA lesions would be the cause of such a distinctive mutator phenotype.

The base-pair substitution specificity of the *rad6* mutator suggested that the overall efficiency of mismatch correction and/or the ability to repair G-A or C-T mismatches might depend on *RAD6*-mediated ubiquitination.

However, correction of mismatches that could give rise either to the transitions or the G·C -> T·A transversion was at least as efficient in the *rad6* strain as in its wild-type parent. Furthermore, the magnitudes of any biases for restoring the mismatches to the incorrect base-pairs were too small to account for the increased substitution frequencies due to the *rad6* mutator. These results demonstrate that the mismatch correction enzymes were operating effectively in the *rad6* strain and argue that neither their intrinsic activity nor their specificity requires that they be ubiquitinated by the *RAD6* gene product. Still, the possibility that a defect in *RAD6* does influence the efficiency and/or specificity of repairing mismatches in chromatin cannot be discounted. The mismatch correction experiments involved the transformation of naked heteroduplex DNA into yeast cells and whether the heteroduplex DNA was present as chromatin before being repaired remains to be determined. Conceivably, one function of the *RAD6* ubiquitin conjugating enzyme is to provide mismatch correction enzymes with access to mispairs in chromatin. If so, mismatch correction might appear to occur normally in a *rad6* strain when the heteroduplexes are not in the form of chromatin.

It was suggested above that the *RAD18* gene product might contribute to the fidelity of DNA replication by involvement in a process which limits the formation of G-A or C-T mismatches at template guanines or cytosines, respectively. This hypothesis is based, in part, on the finding that the yeast *rad18* mutator specifically increases G·C -> T·A transversions. Defects in *RAD6* confer the same phenotypic properties as mutations in *RAD18*. These include sensitivity to the same DNA damaging agents (*RAD6* and *RAD18* belong to the same epistasis group for the repair of UV-induced DNA damage) and the antifolate trimethoprim (Game et al.

1975; Haynes and Kunz 1981), a deficiency in post-replication repair (Prakash 1981), enhanced spontaneous and induced mitotic recombination (Kunz and Haynes 1981) and defective induction of targeted mutagenesis by UV (Cassier-Chauvat and Fabre 1991) [*RAD6* also affects other processes not influenced by *RAD18* including cell growth and sporulation (Haynes and Kunz 1981; Kupiec and Simchen 1984; Fabre et al. 1989)]. In addition, transcription of the *RAD6* and *RAD18* genes is increased by DNA damage and meiosis but remains constant during the mitotic cell cycle (Madura et al. 1990; Jones and Prakash 1991). Furthermore, the two genes share a potential regulatory sequence not found in other *RAD* genes whose expression is also enhanced by DNA damage (Jones and Prakash 1991). These striking similarities point to a close relationship between *RAD6* and *RAD18* leading to the speculation that the activities of their gene products might be linked. If so, and since the *rad6* and *rad18* mutators both increase the frequency of G·C -> T·A transversions, the *RAD6* ubiquitin conjugase might play a role in maintaining the fidelity of DNA replication by interacting with the *RAD18* protein. Two forms of interaction between *RAD6* and *RAD18* have been suggested. First, *RAD6*-mediated ubiquitination might be required for activation of the *RAD18* gene product (Sung et al. 1990). Second, since the *RAD18* protein might be capable of DNA binding (Chanet et al. 1988; Jones et al. 1988) whereas the *RAD6* protein is not (Koken et al. 1991), interaction of the *RAD6* ubiquitin conjugating enzyme with the *RAD18* gene product might serve to guide the *RAD6* protein to its sites of action (Koken et al. 1991). More frequent formation of G-A and/or C-T mismatches at G·C pairs coupled with a reduced efficiency of correcting mismatches in chromatin could explain why G·C -> T·A transversions predominated over transitions in the *rad6* strain.

4.4 Concluding Remarks and Future Work

In conclusion, all three *rad* strains showed mutator phenotypes. The *rad1* mutator increased the frequencies of single base-pair substitution and deletion and Ty transposition, *rad6* enhanced base-pair transitions, the G·C -> T·A transversion and Ty transposition and the *rad18* mutator specifically enhanced G·C -> T·A transversions. Possible mechanisms by which the three *RAD* genes might contribute to limiting the rate of spontaneous mutagenesis in yeast are as follows. The *RAD1* protein might reduce the frequency of transposition and the availability of spontaneous damage for error-prone translesion synthesis. The *RAD18* gene product, or a protein whose expression might be regulated by *RAD18*, might increase the fidelity of DNA replication. The *RAD6* ubiquitin-conjugating enzyme might limit the frequency of transposition and enhance the fidelity of DNA replication by facilitating mismatch correction and interacting with the *RAD18* protein.

Two areas of particular interest that are amenable to future study are the involvement of translesion synthesis in the mutator phenotypes and the role of *RAD6* in mismatch correction. To investigate the first problem, the predicted *REV3* translesion polymerase could be eliminated in the mutator strains by deleting *REV3*. Subsequently, the effect of this deletion on the specificity of the mutators could be assessed to identify classes of mutation that might be attributed to translesion synthesis. Second, one approach to determine whether *RAD6* gives mismatch correction enzymes access to chromatin would be to delete the *PMS1* gene in a *rad6* strain. If there was no change in the magnitude and/or specificity of the *rad6* mutator, it would indicate that the *RAD6* gene product does not function in mismatch correction.

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