Specificity of Yeast Mutators Conferred by Alterations in Genes Required for DNA Replication Fidelity

By

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- Medicine and Surgery 0564
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- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology .. 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
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- Radiation 0754

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- Acoustics 0986
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- Atmospheric Science 0608
- Atomic 0748
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- Molecular 0609
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Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics ... 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
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SPECIFICITY OF YEAST MUTATORS CONFERRED BY ALTERATIONS IN GENES REQUIRED FOR DNA REPLICATION FIDELITY

BY

KARTHIKEYAN RAMACHANDRAN

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

of

DOCTOR OF PHILOSOPHY

Dedicated to my beloved wife and parents

Abstract

The specificity of the mutator phenotype conferred by mutations in a proofreading domain of *pol3* (DNA polymerase δ) as well as deletion of the *PMS1* gene involved in mismatch correction in the yeast *Saccharomyces cerevisiae* were examined. To determine the specificity of the *pol3* and *pms1*Δ mutators, spontaneous mutations arising in the *SUP4*-o gene (ochre supressor allele of a yeast tyrosine t-RNA gene) maintained in the genetic background of these mutator strains were characterized by DNA sequence analysis. A *pol2* (DNA polymerase ε) mutator could not be characterized due to its incompatibility with the genetic screen used to select *SUP4*-o mutations. In order to determine the effect of strand identity on these two mutators (to determine if there was a strand bias in the formation of a premutational leasion), mutations were characterized with the *SUP4*-o gene in two orientations such that, the transcribed strand was the leading strand template in one orientation and lagging strand template in the other. The effect of strand identity on mismatch correction was further examined by determining the efficiency of correction of heteroduplex DNA containing defined mismatches on the *SUP4*-o gene and comparing it with the data obtained from similar experiments using heteroduplex DNA carrying the same mismatch but, with the orientation of the *SUP4*-o gene reversed. Such heteroduplex DNAs were also used to determine the effect of proofreading defects on mismatch correction.

Comparison of the data suggest that proofreading may have a broad specificity, although predominantly involved in correcting replication errors that lead to single base pair substitutions. Mismatch correction on the other hand seems to display a preference

for correcting replication errors that lead to single base pair deletion and insertion mutations with a higher efficiency for correcting those that lead to single base pair insertion events. This is contrary to the situation in *E. coli* where proofreading corrects transversions errors with high efficiently while mismatch correction mainly corrects transitions errors. Among the heteroduplexes tested, T/T mismatches were the least efficiently corrected events while C/T and G/A were the most efficiently corrected ones. My data also suggest that C/C mismatches are rarely formed during replication while T/T are formed more frequently, but proofread very efficiently. Strand identity seem to modulate the *pol3* mutator while no such effect was seen for the *pms1*Δ mutator. However, sequence context or neighboring nucleotides seem to modulate the extent of both the *pol3* and *pms1*Δ mutators. Interestingly, the mismatch correction efficiency was enhanced significantly in the *pol3* background. This is in contrast to the situation in *E. coli* where a proofreading defect leads to a deficiency in the mismatch correction efficiency due to the saturation of the mismatch correction pathway with excessive replication errors left behind by the deficiency in proofreading. My results suggest that, in yeast, mismatch correction is induced significantly in the event of generation of excessive replication errors.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

+1bp	single base-pair insertion
-1bp	single base-pair deletion
5-meC	5-methylcytocine
A	adenine
amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base-pairs
C	cytosine
dAMP	2'-deoxyadenosine-5'- monophosphate
dATP	2'-deoxyadenosine-5'- triphosphate
dCMP	2'-deoxycytidine-5'- monophosphate
dCTP	2'-deoxycytidine-5'- triphosphate
ddATP	2',3'-dideoxyadenosine-5'- triphosphate
ddCTP	2',3'-dideoxycytidine-5'- triphosphate
ddGTP	2',3'-dideoxyguanosine-5'- triphosphate
ddTTP	2',3'-dideoxythymidine-5'- triphosphate
dGMP	2'-deoxyguanosine-5'- monophosphate
dGTP	2'-deoxyguanosine-5'- triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds DNA	double stranded DNA

dTMP	2'-deoxythymidine-5'- monophosphate
dTTP	2'-deoxythymidine-5'- triphosphate
FdUMP	5-fluoro-2'-deoxy-uridine-5'-monophosphate
FOA	5-fluoro-orotic acid
FUMP	5-fluoro-uridine-5'-monophosphate
G	guanine
g, mg, ng, μg	gram, milligram, nanogram, microgram
h(rs)	hour(s)
HNPCC	hereditary non-polyposis colon cancer
kb	kilobase pairs
kDa	kilodalton
l, ml, μl	litre, milliliter, micrometer
M, mM, μM	molar, millimolar, micromolar
MCB	*Mlu* I cell cycle box
min	minute
Na_2EDTA	disodium ethylenediaminetetraacetic acid
no.	number
NTBPS	non-tandem base-pair substitution
nts	nucleotides
PAS	primosome assembly site
ORF	open reading frame
PCNA	proliferating cell nuclear antigen

PEG	polyethylene glycol
PMS	post-meiotic segregation
pol	polymerase
pol α	DNA polymerase alpha
pol β	DNA polymerase beta
pol δ	DNA polymerase delta
pol ε	DNA polymerase epsilon
pol γ	DNA polymerase gamma
pol I	DNA polymerase I
pol II	DNA polymerase II
pol III	DNA polymerase III
pur	purine
pyr	pyrimidine
RF-C	replication factor C
RP-A	replication protein A
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s	second
SBPS	single base-pair substitution
SDS	sodium dodecyl sulphate
SSB	single stranded DNA binding protein

ss DNA	single stranded DNA
T	thymidine
TEMED	N,N,N',N'-tetramethylethylenediamine
Tr	transition
tRNA	transfer RNA
Tv	transversion
UV	ultraviolet
v/v	volume/volume
VSP	very short patch
w/v	weight/volume
w/w	weight/weight
WC (base-pair)	Watson and Crick

1. Introduction

1.1 DNA Polymerases

There are two major classes of DNA polymerase based on the type of template used for DNA synthesis. One type is the DNA-dependent DNA polymerase (designated DNA polymerase) which synthesizes DNA using DNA as the template. The second type is the RNA-dependent DNA polymerase (designated reverse transcriptase) which synthesizes DNA using RNA as the template. A third, minor type, is the template-independent polymerase (designated terminal transferase) that synthesizes DNA from the 3'-OH-terminus of an existing DNA fragment independently of a template. This literature review focuses on: 1. DNA polymerases, with emphasis on their structure, properties, and functional role in replication; and 2. two processes that influence replication fidelity, proofreading and mismatch correction.

1.1.1 Prokaryotic DNA Polymerases

Escherichia coli has been used as a model system for the study of DNA polymerases for several decades, and we now have a considerable body of knowledge on the process of DNA replication in this organism. Three DNA polymerases that are immunologically and genetically distinct from one another have been identified in *E. coli*. They are DNA polymerases III, I and II (Kornberg and Baker 1992).

1.1.1.1 DNA Polymerase III

Soon after its discovery in the early 70s (Kornberg and Gefter 1971), DNA polymerase III was identified as the major replicative polymerase in *E. coli* (Gefter *et al.* 1971). It also plays a potential role in the repair of DNA damage caused by some mutagenic agents (Hagensee *et al.* 1987a; Hagensee *et al.* 1987b). Initial purification of this enzyme was difficult due to its very low abundance (10 to 20 copies per cell), rendering the purification process very tedious (McHenry and Kornberg 1977). DNA polymerase III is believed to be a multi-subunit protein of about 900 kDa comprising at least 10 subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ and β) which, together have been designated the "DNA polymerase III holoenzyme" (Wickner and Kornberg 1974). This enzyme has been purified in three different forms from cell extracts. One form is comprised of three subunits (α, ε and θ) and is designated the catalytic core (hereafter the core) (Maki and Kornberg 1987). A second form is comprised of two cores and a dimer of the τ subunit and has been designated pol III' (McHenry 1982; Studwell and O'Donnell 1991). The third form designated pol III*, is comprised of nine subunits excluding the β subunit (Maki *et al.* 1988). Although these different forms of the enzyme may exist in the cell, it is likely that they are subassemblies of the holoenyme which is most probably the functional form (McHenry 1991).

Arthur Kornberg, among others, has contributed significantly to the understanding of the structure of this enzyme and its function in *E. coli* DNA replication. The structure of the holoenzyme as described by Kornberg (Maki *et al.* 1988; Kornberg and Baker 1992) has provided an excellent frame work for the study of the function of this enzyme.

Table 1. Subunits of the *E. coli* DNA Polymerase III Holoenzyme.

Subunit	Size (Mr)	Gene	Reference
α	129,900	*dnaE*	Welch and McHenry (1982)
ε	27,500	*dnaQ*	Scheuermann *et al.* (1983)
θ	10,000	*holE*	Patricia and O'Donnell (1993)
τ	71,100	*dnaX*	Kodaira *et al.* (1983); Mullin *et al.* (1983)
γ	47,500	*dnaX*	Kodaira *et al.* 1983; Mullin *et al.* (1983)
β	40,600	*dnaN*	Burgers *et al.* (1981)
δ	34,000	*holA*	Ziming *et al.* (1993)
δ'	32,000	*holB*	Carter *et al.* (1993); Ziming *et al.* (1993)
χ	12,000	*holC*	Xiao *et al.* (1993)
ψ	14,000	*holD*	Xiao *et al.* (1993)

Genes encoding all the subunits of pol III have now been identified (Table 1). The holoenzyme is a very processive DNA replication machine capable of synthesizing several thousand nucleotides at a stretch, without dissociation from the template (Mok and Marians 1987), at a rate of about 750 nucleotides (nts)/s (Studwell and O'Donnell 1990). The core on the other hand is one of the weakest polymerases with a synthesis rate of about 20 nt/ s and a processivity of about 11 nts (Fay *et al.* 1981). The processivity and speed of DNA synthesis of the different forms of the DNA polymerase III increases with the complexity of the form, emphasizing the importance of its component subunits (Fay *et al.* 1982).

The catalytic core exists free in the cell at about 40 copies, and about half of the population is assembled into the holoenzyme (Maki and Kornberg 1985). The α subunit by itself is a very weak polymerase (Maki and Kornberg 1985), and the ε subunit by itself is a weak 3' → 5' exonuclease (Scheuermann and Echols 1984). The α and ε subunits form a tightly associated complex and this enhances the rate of polymerization and proofreading (Studwell and O'Donnell 1990; Maki and Kornberg 1987). The θ subunit binds to ε but not to α indicating a linear relationship in the core complex formation, i.e. α–ε–θ in that order (Studwell and O'Donnell 1993). The θ subunit stimulates the ε exonuclease activity, but the function of this subunit has yet to be determined (Studwell and O'Donnell 1993).

A β subunit-dimer is believed to act as a clamp that can glide along a DNA molecule, and is loaded on the DNA by the γδ complex in an ATP-dependent, core-independent manner (O'Donnell 1987; Maki and Kornberg 1988). Assembly of the β–

clamp occurs at the primed DNA terminus to form a preinitiation complex (O'Donnell 1987; Maki and Kornberg 1988). Subsequently, the catalytic core assembles with the β–clamp to form the initiation complex (O'Donnell 1987). X-ray structure analysis has shown that the β dimer is a ring-shaped, head-to-tail, dimer with an internal diameter capable of accommodating duplex DNA (Kong *et al.* 1992). When circular duplex DNAs bound by β–clamps are linearized with restriction enzymes, the clamp dissociates off the DNA molecule, providing evidence that the clamp has mobility on the DNA and can glide off the ends (Stukenberg *et al.* 1991). So the β–clamp must be associating with the DNA by encircling the DNA, and not through chemical binding (Stukenberg *et al.* 1991). A recent study identified a UV-inducible form of the β* subunit (Skaliter *et al.* 1996; Paz-Elizur *et al.* 1996). β* is synthesized from an UV-inducible internal gene (designated *dnaN**) of the *dnaN* gene encoding the β subunit (Paz-Elizur *et al.* 1996). It stimulated the polIII mediated polymerization activity by about 10 fold by increasing its processivity (Paz-Elizur *et al.* 1996). Hence, β* is suggested to assemble an alternative form of the β–clamp in a process associated with DNA synthesis in UV-irradiated cells (Skaliter *et al.* 1996).

Assembly of the γ complex from purified proteins suggests a composition of $\gamma_2,\delta_1,\delta'_1,\chi_1,\psi_1$ (Onrust *et al.* 1995a). The β–clamp binds the γ complex via the δ subunit in an ATP-dependent manner (Naktinis *et al.* 1995). Interestingly, the binding of the β and δ subunits does not require ATP hydrolysis. Based on this and structural analysis of the γ subunit, it has been hypothesized that the β binding surface of the δ subunit is partially buried in the γ complex and an ATP-dependent conformational change exposes the

surface for interaction (Naktinis *et al.* 1995). Both the β dimers and the γ complex (Stukenberg *et al.* 1991) exist in the cell in significant abundance compared to the holoenzyme or the core. After loading the β–clamp to a primed DNA terminus, the γ complex easily dissociates from the β-DNA complex but still retains its activity and is able to load multiple β–clamps to primed DNA (Stukenberg *et al.* 1991). The actual mechanism by which the β dimer clamp is loaded on the DNA is unknown. A monomer-dimer equilibrium study suggests that β dimers are inherently unstable and can easily dissociate at both surfaces during assembly (Griep and McHenry 1988; Griep and McHenry 1990). However, there is a more recent contradictory report that the β dimer is inherently stable suggesting that further information is necessary to elucidate the actual mechanism by which the β–clamp is loaded on the DNA (Kelman and O'Donnell 1995).

The τ (71 kDa) and γ (47 kDa) subunits are both encoded by the *dnaX* gene, and γ is formed by an efficient translational frameshift event (Flower and McHenry 1990). The γ subunit is composed of the N-terminal 430 residues of τ with an unique C-terminal glutamic acid residue (Hawker and McHenry 1987). Based on binding assays, it has been proposed that a τ dimer is involved in binding the γ subunit on one side and two core complexes on the other side, i.e. one core is attached to each τ subunit (Xiao *et al.* 1995). Mutational studies revealed that the C-terminal sequence of τ that is absent in γ is involved in binding the core, and that the dimerization of the core is an essential function for viability (Blinkova *et al.* 1993).

Overall, the holoenzyme may be represented by a horseshoe shaped τ dimer with a γ complex attached to its base and a catalytic core to each arm. This holoenzyme is

thought to be loaded on to the preinitiation complex through γ mediated binding of the α subunit in each core with the β–clamp on the leading or lagging strand (Onrust *et al.* 1995b; Kelman and O'Donnell 1995). This possibility is consistent with the outcome of a compositional study of pol III* which suggested a α_2, ε_2, θ_2, τ_2, γ_2, δ_1, δ'_1, χ_1, ψ_1 subunit composition (Onrust and O'Donnell 1993). The binding of α to the β–clamp is thought to be γ-driven because the α and γ bind to the same region on the C-terminal side of the β ring (Kelman and O'Donnell 1995). This is a useful feature as the γ subunit loads as well as unloads the β–clamp from the DNA (Stukenberg *et al.* 1994). Such an arrangement would prevent premature unloading of the clamp while the core was polymerizing, as well as provide a possible mechanism for the multiple initiations of replication required in the lagging strand (Stukenberg *et al.* 1994; Kelman and O'Donnell 1995).

1.1.1.2 DNA Polymerase I (pol I)

Pol I, also known as the Kornberg enzyme (Kornberg 1974), appears to be involved DNA replication and post-replicative repair (Lehman 1981). This enzyme consists of a single subunit of 130 kDa which is readily cleaved into a large 68 kDa fragment (Klenow fragment), containing the polymerizing and 3' → 5' proofreading activity, and a smaller 35 kDa N-terminal fragment with a 5' → 3' exonuclease activity (Klenow and Henningsen 1970). It is possible that pol I has a dual role in the cell: 1. maturation of Okazaki fragments via its 5' → 3' exonuclease activity and accurately fill in the gap between successive Okazaki fragments via it polymerizing and proofreading function; and 2. accurate DNA repair synthesis via the polymerase and proofreading

function while degrading the DNA ahead of the growing point via the 5' → 3' activity. Thus, it is an ideal candidate for a polymerizing as well as repair enzyme (Kornberg and Baker 1992). Indeed, pol I has been shown to play an important role in the repair of UV-damaged DNA, emphasizing its role as a repair enzyme (Sharma and Smith 1987; Kornberg and Baker 1992). However, since *polA,* the gene encoding pol I is not an essential gene (Joyce and Grindley 1984), pol I is either not required for replication or other polymerases can compensate for pol I *in vivo* (Bonner *et al.* 1992). Post-replicative repair analysis in a strain lacking pol I activity suggests a potential role for this enzyme in the repair of daughter-strand gaps and double-strand DNA breaks (Sharma and Smith 1987). The crystal structure of this protein is known. The structure shows a major groove that may function like the threads on a nut, making the enzyme spiral around the template during DNA synthesis (Joyce and Steitz 1987). Electrostatic potential pattern analysis and additional crystal structure analysis have confirmed the presence of a double-stranded (ds) DNA binding cleft capable of accommodating 8 bp of duplex DNA (Beese *et al.* 1993; Yadav *et al.* 1994).

It has been shown that pol I in some cases may substitute for pol III (Niwa *et al.* 1981). Although temperature-sensitive pol III strains generally arrest in the S-phase boundary indicative of a defect in DNA synthesis, one such mutant has been shown to be viable (Niwa *et al.* 1981). This specific mutation, designated *pcbA1*, is an alteration in the catalytic α–subunit creating a temperature-sensitive deficiency in pol III. Cells harbouring this mutation are viable at restrictive temperatures if pol I is functional, suggesting that pol I, under certain circumstances, can compensate for pol III in DNA

synthesis (Bryan *et al.* 1990). It has also been suggested that a strain which has the potential to utilize pol I in the place of pol III will show a preference towards pol III unless it is forced to use pol I due to a deficiency in pol III. If so, pol I might be a facilitative enzyme for replication and might substitute for pol III *in vivo* under certain conditions (Bryan *et al.* 1990). *Streptococcus pneumoniae* pol I is required for the replication of exogenous pBR322 and pLS1-derivative plasmids providing a replicative role for the enzyme (Diaz *et al.* 1994).

1.1.1.3 DNA Polymerase II (pol II)

A third and comparatively less studied polymerase designated DNA polymerase II has been identified in *E. coli* and represents a very small amount of *in vivo* DNA polymerase activity (Moses and Richardson 1970). The gene encoding pol II has been cloned and designated *polB* (Chen *et al.* 1989). Pol II is comprised of a single 89.9 kDa subunit (Iwasaki *et al.* 1991) which contains both the polymerizing and proofreading activities (Kornberg and Baker 1992). It is inhibited by aphidicolin, a DNA replication inhibitor, suggesting a role in DNA synthesis (Chen 1990). P*olB* is a non-essential gene and deletion mutants do not confer any mutator phenotype (Campbell *et al.* 1972; Hirota *et al.* 1972) suggesting that it is either not involved in DNA replication or its absence can be compensated *in vivo* by other enzymes (Chen *et al.* 1989). However, it has been shown that *polB* is a part of the SOS regulon suggesting a possible role in repair (Bonner *et al.* 1990). Additionally, the inherent processivity of pol II, which is low, can be enhanced several-fold by the pol III accessory proteins demonstrating a possible functional

relationship between the two enzymes (Bonner *et al.* 1992). A study on proofreading-defective pol II suggested that pol II in conjunction with pol III may be involved in synthesizing episomal DNA in non-dividing cells (Foster *et al.* 1995). A comparative study of the pol II wild type and proofreading-deficient enzymes showed that pol II was inherently accurate and the proofreading deficient mutant displayed a 250 fold increase in error rate, suggesting a possible role for pol II in the fidelity of DNA synthesis (Cai *et al.* 1995).

1.1.2 Eukaryotic DNA Polymerases

To date, five distinct DNA polymerases have been identified in yeast and mammalian cells. According to the revised nomenclature, they are designated DNA polymerases α, β, δ, ε and γ (Burgers *et al.* 1990) (hereafter referred to as pol α, pol β, pol δ, pol ε and pol γ, respectively). This section of the review is mainly focused on the yeast polymerases and mammalian polymerases. The letters m and y will be used to specify human and yeast proteins. Some information from other eukaryotic organisms is also provided in the relevant sections.

1.1.2.1 DNA Polymerase α

Pol α was first isolated in 1960 from calf thymus tissue (Bollum 1960). In general it is a multi-subunit protein that copurifies with a tightly associated primase activity (hence, often designated pol α/primase) (Plevani *et al.* 1984; Plevani *et al.* 1985; Wang 1991). It has been accepted that this enzyme functions in DNA replication based

on the sensitivity of yeast and mammalian pol α to DNA replication inhibitors, such as aphidicolin, and their cell-cycle regulation with transcript levels reaching a peak at the G1/S boundary (Wang 1991). A potential role for this enzyme in DNA repair synthesis has also been speculated based on the finding that ypol α transcript levels increase significantly upon inducing DNA damage with UV radiation (Johnston *et al.* 1987). However, pol α is not required for repair of DNA damaged by X-rays, suggesting that it may perform a repair synthesis specific for certain DNA damaging agents (Budd *et al.* 1989b). Mutational studies on ypol α suggest a role for the enzyme in the regular maintenance of genomic tandem repeats (Ruskin and Fink 1993). The replicative role of this enzyme has been confirmed by several findings. Ypol α is an indispensable protein (Johnson *et al.* 1985) and temperature-sensitive ypol α mutants display a terminal phenotype with an S-phase arrest at the restrictive temperature (Budd and Campbell 1987). Neutralizing monoclonal antibodies against mpol α inhibit DNA replication in permeabilized cells (Miller *et al.* 1985). Additionally, mpol α is required for *in vitro* replication of DNA by the SV40 DNA replication system, supporting a potential role for pol α in DNA replication *in vivo* (Murakami *et al.* 1986). Pol α, although lacking a 3' → 5' exonuclease activity, is also the only known eukaryotic polymerase with an associated primase activity and hence, is an ideal candidate for synthesising the primer during replication (Plevani *et al.* 1984, Plevani *et al.* 1985; Wang 1991).

It has been recently demonstrated that the ypol α transcript is present throughout the cell cycle with only a small peak at the G1/S boundary and that yeast cells can undergo several rounds of replication when the level of pol α is abolished to below

physiological levels (Falconi *et al.* 1993). This suggests that physiological levels of functional pol α can be inherited by the daughter cells and that *de novo* synthesis of this enzyme is not required to enter the S-phase (Falconi *et al.* 1993). However, two copies of the 5'-*Mlu* I cell cycle box (MCB) present in several genes regulated during the cell cycle have been observed in Y*POL1* (Pizzagalli *et al.* 1992). Additionally, the mpol α catalytic subunit which is phosphorylated throughout the cell cycle is hyper-phosphorylated at the G_2/M boundary while its 70 kDa accessory subunit is phosphorylated only in the G_2/M boundary (Nasheuer *et al.* 1991). Phosphorylation may play a role in the cell cycle dependant regulation of the functional form of the enzyme since the hyper-phosphorylated form has a reduced affinity for ss DNA(Nasheuer *et al.* 1991). In general, pol α together with its tightly associated primase is thought to be involved in the initiation of DNA replication at the origin, in lagging strand replication and in DNA repair (Tsurimoto *et al.* 1990; Wang 1991).

1.1.2.1.1 Structure of DNA Polymerase α

Pol α is a multi-subunit complex with tightly associated polymerizing and primase activities (Plevani *et al.* 1984; Plevani *et al.* 1985; Wang 1991). In the 1980s, pol α was isolated to homogeneity from several eukaryotic sources including calf thymus (Wahl *et al.* 1984), human KB cells (Wong *et al.* 1986), yeast (Plevani *et al.* 1985), rat liver (Philippee *et al.* 1986), *Drosophila melanogaster* (Kaguni *et al.* 1983), rabbit bone marrow (Goscin and Byrnes 1982) and monkey CV-1 cells (Yamaguchi *et al.* 1985). The pol α holoenzyme (pol α /primase) is, in general, a four subunit complex comprised of: a

160 to 180 kDa catalytic subunit (pol α); a 70 kDa subunit of unknown function; and two subunits 60 and 50 kDa in size that are associated with a primase activity (Wong *et al.* 1986; Biswas 1993). The three smaller subunits are immunologically distinct from the catalytic subunit and from each other proving that they are not proteolysis products of the catalytic subunit (Wong *et al.* 1986). The catalytic subunit has been isolated in smaller but functionally active forms, immunologically indistinguishable from the 180 kDa subunit, and these are believed to be proteolysis products of the 180 kDa subunit (Wong *et al.* 1986). Although mpol α and ypol α have similar subunit composition (Burgers 1989), and share a significant degree of homology (32% AA sequence homology in the catalytic polypeptide) between each other, there is a very high degree of *in vivo* species specificity (Francesconi *et al.* 1993a). Although a 4 subunit structure for ypol α and mpol α holoenzymes is widely accepted (Kaguni and Lehman 1988; Lehman and Kaguni 1989), a ypol α complex has been recently purified as a very high molecular weight (~650 kDa) complex comprising at least five additional proteins that are immunologically distinct from the four principal subunits (Biswas *et al.* 1993). This complex has polymerase, primase, 5' → 3' exonuclease and ss DNA dependent ATPase activities (Biswas *et al.* 1993).

1.1.2.1.2 The catalytic subunit

The catalytic subunit of ypol α, like that of mpol α is a 180 kDa protein (Plevani *et al.* 1985). *POL1* (previously *CDC17*), the gene encoding the ypol α catalytic subunit, was isolated from a lambda gt11 library using polyclonal antibodies to ypol α, and was

mapped to the left arm of chromosome XIV (Lucchini *et al.* 1988). The sequence of *POL1* predicts a protein containing regions of homology to conserved catalytic motifs of several known polymerases (Pizzagalli *et al.* 1988). The serine and threonine residues on the catalytic subunit of mpol α are phosphorylated during the cell cycle dependant phosphorylation process at the G_2/M boundary, potentially by the p34cdc2 kinase (Nasheuer *et al.* 1991).

1.1.2.1.3 The 86 kDa Subunit (Subunit B)

The yeast gene (*POL12*) encoding the 86 kDa subunit (also known as pol α subunit B) has been cloned and shown to be present as a single copy in the genome (Brooke *et al.* 1991). Although this subunit is thought to stabilize the polymerase activity at 30°C (Brooke *et al.* 1991), monoclonal antibodies against the human as well as mouse homologues of the yeast 86 kDa subunit inhibit the primase but not the polymerase activity (Yagura *et al.* 1987). Based on binding assays, the mpol α B subunit has been suggested to play a role in replication by aiding in the physical association of the polymerase and primase subunits (Collins *et al.* 1993). It may also have a regulatory function based on the finding that the processivity and rate of DNA replication *in vitro* are reduced in the absence of this subunit (Plevani *et al.* 1985). This subunit, like the catalytic subunit, is a phosphoprotein and the phosphorylated AA are exclusively serine and threonine (Wong *et al.* 1986). This feature is consistent with a regulatory role for the subunit, although dephosphorylation of either of these two subunits does not affect polymerization or primase activity (Wong *et al.* 1986). However, phosphorylation of the

mpol α B subunit is cell cycle-dependent, adding emphasis to a potential regulatory role for the subunit (Nasheuer *et al.* 1991). A recent mutational analysis on the ypol α B subunit suggests a role for this subunit in the initiation stages of chromosomal DNA replication (Foiani *et al.* 1994).

1.1.2.1.4 The Primase Subunits

All known α polymerases have a tightly associated primase activity capable of synthesizing short RNA primers that can be extended by the polymerase (Lehman and Kaguni 1989). This primase activity is capable of initiating the synthesis of Okazaki fragments (Hay and Russell 1989) and is most likely involved in the initiation of DNA synthesis (Stillman 1989). The yeast primase activity is associated with the 58 kDa subunit encoded by the *PRI2* gene and the 49 kDa subunit encoded by the *PRI1* gene (Foiani *et al.* 1989a; Plevani et. al. 1987). The primase activity lies on the 49 kDa subunit and the 58 kDa subunit seems to stabilize the primase activity and might have a stabilizing and a regulatory role in replication (Santocanale *et al.* 1993). The 49 kDa subunit has an ATP binding site, consistent with the presence of the primase active site on this subunit (Foiani *et al.* 1989a; Foiani *et al.* 1989b). Furthermore, polyclonal antibodies against the 58 kDa subunit reduce the primase activity markedly while antibodies against the 49 kDa subunit completely inhibit primase activity. The 49 kDa subunit seems to exist in a free form in cell extracts, and is capable of synthesizing primers although its activity is highly unstable (Santocanale *et al.* 1993). A study on temperature-sensitive yeast *pri1, pri2* and double mutants suggest that while the *PRI2*

product is involved in the actual primer synthesis, the *PRI1* gene product interacts with pol α and either mediates or stabilizes the association of the *PRI2* gene product in the pol α /primase complex (Longhese *et al.* 1993). Molecular mass studies on the pol α /primase complex from several organisms suggest that the primase may be present as an oligomer (Podust *et al.* 1992).

1.1.2.1.5 Role of pol α in DNA Replication

Knowledge about eukaryotic DNA replication has been derived mainly from *in vitro* studies with specific emphasis on the SV40 DNA replication system (Kelly 1988). Mpol α was the first eukaryotic polymerase to be designated as a replicase, identified using the SV40 DNA replication system (Li and Kelly 1984). Pol α is the only known eukaryotic polymerase with an associated primase activity, and capable of synthesizing DNA in the absence of a primer and hence must be involved at least in the priming stages of DNA replication (Fry and Loeb 1986; Hay and Russell 1989). It has been suggested that the initiation of replication by ypol α may be via a licensing protein, such as the origin recognition complex in yeast, that provides the signal for initiation of DNA replication (Stillman 1994). Pol α is an essential enzyme *in vivo*, and pol α together with pol δ can replicate SV40 DNA *in vitro* further emphasizing the involvement of pol α in DNA replication (Tsurimoto *et al.* 1990; Melendy and Stillman 1993). SV40 DNA replication studies show that in the absence of pol α, there was no DNA replication (Weinberg *et al.* 1990). However, when pol α was the sole polymerase in the system, only short fragments of DNA were synthesized, suggesting that pol α can synthesize the

primers necessary to initiate synthesis of both strands and may also function as the lagging strand replicase (Weinberg *et al.* 1990). However, a study on the fidelity of DNA synthesis by pol α suggested that it may not function as a lagging strand replicase (Kunkel *et al.* 1991). The average error rate for DNA synthesis by pol α alone was about 1/9900 bp and 1/12000 bp for single base-pair substitutions and minus-one frameshift errors, respectively, consistant with the values obtained from other non-proofreading or proofreading-deficient polymerases. This suggests a general absence of proofreading activity for pol α (Kunkel *et al.* 1989) and implies that pol α is probably only involved in priming the two strands unless, it associates with an external 3' → 5' exonuclease activity during replication (Kunkel *et al.* 1991).

1.1.2.2 DNA polymerase δ

Pol δ was first identified in the mid 70s as a distinct mammalian polymerase with a 3' → 5' exonucleolytic activity capable of proofreading during replication, which could account for the high fidelity of eukaryotic DNA replication (Byrnes *et al.* 1976). Pol δ was similar to pol α in several aspects and it was initially speculated to be pol α contaminated with a nonspecific 3' → 5' exonuclease activity (Burgers and Bauer 1988; So and Downey 1988; Bambara and Jessee 1991). For example, both polymerases were high molecular weight acidic proteins with similar sensitivity to inhibitors of replication, such as, high sensitivity to inhibition by arabinosyl nucleotides and amphidicolin and resistance to dideoxynucleotides (So and Downey 1988).

1.1.2.2.1 Structure and properties of pol δ

Biochemical and immunlogical studies have proven pol δ to be a distinct polymerase (Bauer *et al.* 1988; Burgers and Bauer 1988; Wong *et al.* 1989; Syvaoja *et al.* 1990). Pol δ has been isolated from several organisms including calf thymus (Lee *et al.* 1984), HeLa cells (Syvaoja *et al.* 1990), *Drosophila melanogaster* (Chiang *et al.* 1993; Aoyagi *et al.* 1994), *Physarum polycephalum* (Achhammer *et al.* 1995) and yeast (Bauer *et al.* 1988) as a holoenzyme with a large catalytic subunit of about 125 kDa containing both the polymerizing and proofreading activities and an associated smaller subunit of about 50 kDa whose function is not yet known. The gene encoding pol δ, designated *POL3,* has been cloned in yeast (Boulet *et al.* 1989). The cDNA encoding the *Drosophila melanogaser* pol δ has been recently isolated and sequenced (Chiang and Lehman 1995). It is interesting to note that certain *POL3* containing plasmids are unstable in *E. coli* (Simon *et al.* 1991) possibly due to toxicity caused by the expression of *POL3* in *E. coli* (Brown *et al.* 1993). Expression of *POL3* in a new T7 derivative vector was however, not toxic in *E. coli* suggesting that the toxicity seen in some cases is vector specific (Brown *et al.* 1993). The yeast *POL3* (previously *CDC2*) is essential for viability and temperature-sensitive *pol3* mutants exhibit a terminal morphology with an S-phase arrest indicative of a defect in DNA replication (Sitney *et al.* 1989). The protein predicted from the sequence of Y*POL3* shares several regions of homology to other known eukaryotic pol δ (Boulet *et al.* 1989). The gene encoding the 50 kDa subunit is yet to be cloned. Mpol δ as well as

ypol δ are regulated in a cell cycle dependent manner with a peak in transcript levels at the G1/S boundary consistant with a role in DNA replication (Zeng *et al.* 1994a). A feature unique to pol δ is that it is not inherently very processive and requires the presence of proliferating cell nuclear antigen (PCNA) to become highly processive (Tan *et al.* 1986). Interestingly, the PCNA-dependent stimulation of ypol δ processivity was not species specific probably because the PCNA binding domain has been conserved during evolution (Bauer and Burgers 1988; Burgers 1988). In addition to PCNA, there are at least two other accessory proteins involved in enhancing or stabilizing the pol δ activity. They are: 1. replication protein A designated RP-A (also known as the replication factor A [RF-A] and single strand DNA binding protein [SSB]) (Lee *et al.* 1988); and 2. replication factor C (RF-C) (Tsurimoto and Stillman 1991).

An analysis of the terminally arrested *pol3* mutant cells in temperature shift experiments reveal that about 70% of the genome is replicated before the arrest. This suggests a defect in the assembly of DNA replication complexes preventing the cells from entering new rounds of replication or a defect in a later stage of replication (Conrad and Newlon 1983a). A similar study in *Schizosaccharomyces pombe* indicates that pol δ may be involved in the replication dependent control of mitosis. Essentially, at restrictive temperatures, cells exhibit a cell division cycle phenotype (Francesconi *et al.* 1995b) and there is a defect in the replication dependent feedback control of mitosis and the cells enter a lethal mitosis resulting in a terminal phenotype (Francesconi *et al.* 1995).

There is ample evidence for the involvement of pol δ in DNA repair (Sugino 1995). Mpol δ is involved in the cellular response (Zeng *et al.* 1994b) as well as repair synthesis (Nishida *et al.* 1988; Budd and Campbell 1995) of DNA damage caused by UV-radiation. Mpol δ is thought to be responsible for the bulk of the repair synthesis in stationary phase cells (Dresler and Frattini 1986; Dresler and Kimbro 1987) as well as actively growing cells (Hunting *et al.* 1991) damaged by UV-radiation. Additionally, involvement of ypol δ in the repair of DNA damage caused by alkylating agents has been documented (Suszek *et al.* 1993; Blank *et al.* 1994).

1.1.2.2.2 Structure and properties of the pol δ accessory proteins

A. PCNA

Pol δ requires at least three accessory proteins. They are PCNA, RF-C and RP-A (Tan *et al.* 1986; Lee *et al.* 1988; Tsurimoto and Stillman 1991). PCNA is the eukaryotic equivalent of the prokaryotic β–clamp associated with the pol III holoenzyme (Kuriyan and O'Donnell 1993). Yeast PCNA is a trimer of 29 kDa subunits (Bauer and Burgers 1990), encoded by an essential gene designated *POL30* (Bauer and Burgers 1990). The crystal structure of the yPCNA was solved recently (Krishna *et al.* 1994). Briefly, each subunit is a wide v-shaped structure and three subunits are placed in an end to end fashion to form a hexagonal ring or torus shaped trimer, with a circular groove at the center that is wide enough to accomodate duplex DNA (Krishna *et al.* 1994). The yeast as well as human PCNA have also been shown to be involved in DNA repair (Ayyagari *et al.* 1995).

It has been suggested that upon induction of DNA damage in proliferating cells, the human kinase inhibitor protein p21 binds to PCNA and stalls replication but not repair, thus allowing DNA repair to proceed preferentially over replication (Ayyagari *et al.* 1995).

B. RF-C

The RF-C from human and yeast cells have been characterized. The mRF-C comprises a large 130 kDa subunit and 4 small subunits of 36 to 41 kDa (Tsurimoto and Stillman 1989; Lee *et al.* 1991) while the yRF-C comprises a large 110 kDa subunit and only three additional small subunits of 37 to 41 kDa (Yoder and Burgers 1991; Fien and Stillman 1992; Li *et al.* 1992). YRF-C has been shown to be required for DNA replication and repair (Mcalear *et al.* 1996; Sancar 1995). Both yeast and mammalian RF-C proteins have an ATPase activity that is stimulated by template-primer DNA and PCNA (Tsurimoto and Stillman 1990; Yoder and Burgers 1991; Lee *et al.* 1991). The RF-C is the eukaryotic functional equivalent to the *E. coli* γ complex that loads the β–clamp on the DNA (Sugino 1995). In the presence of linear ds DNA, yPCNA is loaded passively, probably by the diffusion of the DNA into the central groove in the PCNA trimer (Burgers and Yoder 1993). In the absence of DNA ends (i.e. when only circular DNA was used), RF-C as well as ATP hydrolysis was required for loading of yPCNA (Burgers and Yoder 1993). A recent study suggests that RF-C initially binds

nonspecifically to DNA. It then loads the PCNA on to the DNA in an ATP-dependent manner, probably by transiently opening one of the joints of the PCNA trimer. This complex then slides along the duplex DNA until it comes in contact with a 3'-OH primer-template junction and undergoes conformational change to become a competent clamp which is likely to be involved in replication (Podust *et al.* 1995).

C. RP-A

The RP-A is a ss DNA binding protein complex and has been isolated from human (Wold and Kelly 1988) as well as yeast (Brill and Stillman 1989) cells as a three subunit complex of 70, 32 and 14 kDa. It has been recently shown that mutations in the 32 kDa subunit causes defective S-phase progression (Santocanale *et al.* 1995). mRP-A preferentially bind ss DNA covering about 30 nts (Kim and Wold 1995). The study also showed that these two proteins preferentially bind to the pyrimidine rich strand of a homologous origin of replication, inducing strand separation, suggesting their involvement in the initiation of replication (Kim and Wold 1995). Recently, mRP-A was shown to be involved in nucleotide excision repair (Aboussekhra *et al.* 1995).

1.1.2.2.3 Role of pol δ in replication:

The replicative role of pol δ is well documented. Mpol δ, as well as mPCNA, are required for the *in vitro* SV40 DNA replication system (Prelich *et al.* 1987; Weinberg and

Kelly 1989). In the SV40 replication system, in the presence of inhibitors against both pol α and pol δ, there was complete inhibition of DNA synthesis (Decker 1987). However, when only pol δ was inhibited using monoclonal antibodies, short fragments were synthesised showing the involvement of pol δ in the leading strand to synthezise long DNA fragments (Weinberg *et al.* 1990). Moreover, at the restrictive temperature, y*pol3* temperature-sensitive mutants arrest in S-phase with about 30% of the genome unreplicated (Conrad and Newlon 1983a). Pol δ has been proposed to be the leading strand polymerase in *in vitro* systems based on its functional properties such as its requirement for the processive synthesis of long strands in the SV40 system (Prelich and Stillman 1988). Recent studies have shown that preferential lagging strand synthesis occurs *in vitro* in the absence of mpol δ and its accessory proteins, further emphasizing that pol δ could function as a leading strand polymerase (Tsurimoto *et al.* 1990; Weinberg *et al.* 1990). There is also a possibility that pol δ is the leading strand polymerase by virtue of its high PCNA induced processivity (Burgers 1991). The requirement of PCNA, RF-C and PR-A for processive synthesis by pol (Tan *et al.* 1986; Lee *et al.* 1988; Tsurimoto and Stillman 1991) and the fact that pol δ can be switched from non-processive to highly processive mode by PCNA makes it an ideal candidate for the lagging strand replication which requires efficient recycling of the polymerase (Morrison *et al.* 1990). Further analysis is required for identifying the precise role and strand specificity of the eukaryotic polymerases (Sugino 1995). The current models for replication are discussed in section 1.2.2.

1.1.2.3 DNA Polymerase Epsilon

Yeast pol ε was originally described in 1970 as a polymerase activity distinct from pol α (Wintersberger and Wintersberger 1970). Since the processivity of this polymerase seemed to be unaffected by proliferating cell nuclear antigen it was suggested to be a form of pol δ and was called PCNA-independent pol δ (Syvaoja and Linn 1989; Focher *et al.* 1989). Immunological and genetic evidence have shown that pol ε is a distinct enzyme, and monoclonal antibodies against either pol α or pol δ do not inhibit its activity (Burgers and Bauer 1988). Both pol ε and pol δ are sensitive to amphidicolin (Byrnes 1984). However, in contrast to pol δ, pol ε is relatively resistant to inhibition by carbonyldiphosphonate, a triphosphate analog (Syvaoja *et al.* 1990). Initially, it was shown that the transcript levels of the ypol ε was higher in stationary phase cells than in exponentially growing cells, suggesting that pol ε was involved in repair and not in replication (Chang 1977). Interest in pol ε was brought back after the cloning of the y*POL2* gene encoding the catalytic subunit of pol δ and demonstration that it was an essential gene (Morrison *et al.* 1990). It was also shown that disruption of the gene resulted in dumb-bell shaped terminal morphology indicative of a defect in DNA replication (Morrison *et al.* 1990). Although pol ε was initially distinguished from pol δ on the basis that pol ε was not stimulated by PCNA (Syvaoja and Linn 1989; Focher *et al.* 1989), evidence is now available to suggest a possible stimulation of ypol ε and mpol ε by PCNA under certain conditions (Lee *et al.* 1991; Maga and Hubscher 1995). Recent studies on calf thymus pol ε, indicate that PCNA interacts with pol ε in the primer recognition and elongation steps of DNA replication *in vitro* (Maga and Hubscher 1995).

Lee *et al.* (1991) showed that ypol ε catalyzed *in vitro* DNA synthesis is inhibited in physiological levels of salt and that activity can be restored by the addition of yRP-A and yPCNA. Maga and Hubscher (1995) showed that pol ε binds primed ss DNA randomly, resulting in unproductive binding and PCNA can reduce this effect by enhancing the primer binding of pol ε followed by stimulating the activity of the enzyme.

A protein complex known to stimulate the activity of pol ε has been isolated from yeast cells and designated stimulatory factor I (SF-I) (Budd *et al.* 1989a). It is a three subunit complex of 113.5 66 and 37 kDa proteins with ss DNA binding activity (Brown *et al.* 1990). Although it is very similar to the yRP-A, these two complexes are immunologically distinct (Brown *et al.* 1990). Further analysis has shown that the 66 and 37 kDa subunits are identical to the *HSP60* encoded mitochondrial heat-shock proteins. A second factor designated SF-II has been identified but not characterized (Budd *et al.* 1989a).

1.1.2.3.1 Structure of pol ε

Pol ε has been isolated as a single polypeptide from HeLa cells (Syvoaja and Linn 1989), human placenta (Lee and Toomey 1987; Lee *et al.* 1991) and rabbit bone marrow (So and Downey 1992). This enzyme has been isolated as a single polypeptide form of about 150 kDa as well as a multi-subunit form comprising four to five subunits from calf thymus (Crute *et al.* 1986) and yeast (Budd *et al.* 1989a; Hamatake *et al.* 1990). Immunological and activity gel analysis showed that the single polypeptide form as well as the largest subunit of the multi-subunit form are catalytically and immunologically

similar (Morrison *et al.* 1990). A single polypeptide form (145 kDa) as well as a multi-subunit complex form comprising five subunits of 200, 80, 34, 30 and 29 kDa (designated subunits A, B, C, C' and D, respectively) (Hamatake *et al.* 1990) have been identified in yeast. The 145 kDa subunit as well as the 200 kDa subunit of the multi-subunit complex were both catalytically active (Hamatake *et al.* 1990) and were immunologically indistinguishable, suggesting that the multi-subunit complex is the holoenzyme form of the ypol ε (Morrison *et al.* 1991). The *POL2* encodes 256 kDa predicted protein and the catalytic polypeptide contains both the polymerizing as well as the proofreading functions (Morrison *et al.* 1991).

The 80 kDa B-subunit copurifies with the catalytic subunit (Hamatake *et al.* 1990). *DPB2* the gene encoding this subunit, is essential and its DNA sequence predicts a polypeptide of 79.5 kDa (Araki *et al.* 1991a). Disruption of *DPB2* was lethal and resulted in a dumb-bell shaped terminal morphology indicating a role in replication. Additionally, an analysis of temperature-sensitive mutants showed that mutant cells were partially defective in replication. Moreover, intact holoenzyme could not be isolated from such cells (Araki *et al.* 1991a). The gene sequence of *DPB2* contained the *Mlu*I cell cycle box found in genes regulated in a cell cycle dependent manner. A study of the transcript level showed that *DPB2* was in fact cell cycle regulated with a peak in transcript level at the G_1/S boundary (Araki *et al.* 1991a).

The 34 and 30 kDa subunits of the ypol ε holoenzyme, designated C and C', respectively, are encoded by the same gene, *DPB3,* located on the right arm of chromosome II (Araki *et al.* 1991b). *DPB3* is dispensable for cell growth and sporulation,

and its inactivation confers a mild mutator effect increasing the spontaneous mutation rate by two to three-fold. The activity of ypol ε in *dpb3* deletion strains was reduced by about 50%, and the pol ε holoenzyme in such strains eluted as a two subunit complex consisting of subunits pol ε and B. This observation suggests that the binding of subunit D is dependent on the presence of subunits C and C', which may, therefore, play a stabilizing role in holoenzyme formation (Araki *et al.* 1991). The predicted size of the *DPB3* gene product is only 23 kDa, and so subunits C and C' are probably post-transcriptionally modified forms of the protein (Morrison and Sugino 1993). The *DPB3* gene contains a putative *Mlu*I cell cycle box and is regulated in a cell cycle dependent fashion with a peak in transcript levels at the G_1/S boundary (Araki *et al.* 1991).

Subunit D has been shown to be 29 kDa in size and immunologically distinct from the other subunits (Hamatake *et al.* 1990). Not much work has been done on this protein and its gene is yet to be cloned. As mentioned above, deletion of *DPB3* results in a two subunit pol ε suggesting that subunit D interacts with the holoenzyme via subunits C and C' and is probably a dispensable protein unless it is involved in some other vital function (Araki *et al.* 1991b).

1.1.2.3.2 Role of pol ε in DNA Replication

The precise role of pol ε in replication is unclear at this time. Mammalian pol ε may be involved in DNA repair as it is required for DNA repair synthesis in permeabilized UV-irradiated human diploid fibroblasts (Nishida *et al.* 1988). It has been

suggested that base excision repair in yeast is catalyzed by pol ε and modulated by pol α and pol δ (Wang *et al.* 1993). A recent study shows that ypol ε alone is sufficient to perform repair synthesis and the presence of PCNA, RF-C and RP-A additionally modulated its efficiency (Shivaji *et al.* 1995). It has also been demonstrated that ypol ε or ypol δ can perform the repair synthesis in UV-damaged cells in a mutually complementary manner i.e., one pol can substitute for the other in such repair synthesis (Budd and Campbell 1995). Although ypol ε is an essential protein *in vivo*, and temperature-sensitive mutants arrest in S-phase indicating a role in replication (Morrison *et al.* 1991), it is not required for the SV40 *in vitro* DNA replication system (Weinberg and Kelly 1989). From these observations, and the fact that all known proteins functioning only in repair are dispensable for cell viability, pol ε must function either in replication or in an unknown form of DNA repair occurring during replication, absence of which is lethal to the cell (Stillman 1994). A recent study has shown that pol ε may function as a DNA-damage sensor, that sends out signals for the expression of DNA repair genes (Navas *et al.* 1995). However, based on its relatively small mutator effect compared to pol δ, pol ε has been suggested to be the lagging strand polymerase (Burgers 1991), while based on its high inherent processivity, it has been suggested to be the leading strand polymerase (Morrison *et al.* 1990) or only in repair synthesis based on *in vitro* experiments (Stillman 994).

1.1.2.4 DNA polymerase beta:

Pol β, the smallest known eukaryotic polymerase, was first identified in vertebrates as a 39 kDa enzyme, and was postulated to be involved in DNA repair (Chang 1973). Pol β is an ideal candidate for DNA repair as it is capable of efficient filling of short gaps in oligo-hybridized single stranded DNA molecules (Singhal and Wilson 1993). It has been shown that the mpol β can substitute for the *E. coli* pol I *in vivo* (Sweasy and Loeb 1992). Additionally, mpol β (Singhal *et al.* 1995; Sobol *et al.* 1996) and ypol β (Clairmont and Sweasy 1996) have a role in the gap filling step of base excision repair. The yeast equivalent of the mpol β was first reported as the DNA sequence of an open reading frame (ORF) on chromosome III that could encode a potential DNA polymerase (Oliver *et al.* 1992). The predicted protein sequence had partial homology to the mammalian pol β and this ORF was designated YCR14C (Bork *et al.* 1992). Later, YCR14C was cloned and over expressed in *E. coli* (Prasad *et al.* 1993). The expressed protein was purified to homogeneity and was found to be 67 kDa in size corresponding to the size predicted from the DNA sequence. The protein had several mpol β like characters such as distributive DNA synthesis, gap-filling synthesis and sensitivity to ddNTP. This polymerase has been tentatively designated pol IV, a β-like polymerase in yeast (Prasad *et al.* 1993). The ORF is now provisionally called *POLX* (Leem *et al.* 1993). It is not an essential gene since deletion of *POLX* doess not affect growth, sporulation or spore viability (Prasad *et al.* 1993). The pol IV transcript is

induced several fold during meiosis although *polx*Δ mutants did not show any meiotic phenotype except for hyper-recombination and hence, it is postulated that pol IV may be involved in double strand break DNA repair (Prasad *et al.* 1993).

1.1.2.5 DNA polymerase γ (pol γ)

Pol γ is the mitochondrial polymerase and is associated with a proofreading function. The gene encoding the ypol γ is a nuclear gene and has been cloned and designated *MIP1* (Foury 1989). It contains the conserved 3' → 5' exonuclease domains (Foury and Vanderstraeten 1992) that has been reported in other proofreading-proficient polymerases (Bernard *et al.* 1989). The proofreading and the polymerase activity of ypol γ reside in the same subunit of the ypol γ and is essential for the accurate replication of the mitochondrial genome (Foury and Vanderstraeten 1992).

1.1.2.6 The Rev3 *protein*

The Rev3 protein was initially identified using mutants that showed a reduced frequency of UV mutagenesis (Lemontt 1971). The *REV3* gene sequence predicted a 172 kDa potential polymerase, with sequence homology to Epstein-Barr virus polymerase and eukaryotic pol α, based on a computer model (Morrison *et al.* 1989). Haploid yeast *rev3* deletion strains are viable and do not express any growth defects (Morrison *et al.* 1989). The REV3 protein is not under cell cycle control (Singhal *et al.* 1992) and *rev3* deletion does not result in a dumb-bell phenotype. These results show that REV3 protein is neither

an essential one nor is involved in replication (Morrison *et al.* 1989). It has been suggested that the REV3 protein might participate in the production of spontaneous base-substitution, deletions and other related events because, mutations in *REV3* reduced the spontaneous mutation rates (Cassier *et al.* 1980; Quah *et al.* 1980) as well as UV induced mutation rates (Lemontt 1972; Lawrence and Christensen 1979; Lawrence *et al.* 1984) of several reporter genes. Based on this and similar findings it is possible that the REV3 protein is only involved in translesion synthesis (Morrison *et al.* 1989). The antimutator phenotype caused by the inactivation of *REV3* might be a result of translesion synthesis playing a major role in spontaneous mutagenesis in yeast (Quah *et al.* 1980).

1.1.2.7 The YEL055C ORF

In a recent report, Sugino (Sugino 1995) has mentioned that the *S. cerevisiae* genome project has identified on ORF designated YEL055C that could encode a potent polymerase of 130 kDa. In the report, he has also mentioned that his group has identified a 135 kDa protein immunologically distinct from the other identified ypols with a polymerizing activity that it could be the product of this new ORF (Sugino 1995).

1.2 Role of polymerases at the replication fork

1.2.1 Prokaryotic DNA replication

A considerable body of knowledge has been accumulated on the replication process of the *E. coli* genome. In simple terms, replication in *E. coli* consists of synthesis

of short RNA primers by the primosome and elongation of both the strands by pol III holoenzyme followed by the removal of the primers and gap-filling by pol I and ligation by DNA ligase (as reviewed by Kornberg and Baker 1992).

The priming step of *E. coli* replication is characterized by three distinct sequential steps; assembly of the preprimosome, addition of the primase to form the primosome and finally synthesis of the primer (Marians 1984). Lack of stable association of the primase to the preprimosome and the availability of several primosomal forms (Marians 1992) has lead to the redefining of the term primosome which now defines "the multi-subunit complex" (previously designated preprimosome) with the knowledge that the primase will act on this complex to synthesise a primer (Kornberg and Baker 1992). The primosome comprises at least five proteins. These are the dnaB protein (Reha-Krantz and Hurwitz 1978), along with the PriA, PriB, PriC and dnaT proteins (Allen and Kornberg 1993) (previously designated n', n, n'' and i proteins by Kornberg's group or replication factors X, Y and Z [a mixture of two proteins] by Hurwitz's group). The dnaC protein although not part of the primosome, functions in the assembly process (Kobori and Kornberg 1982) Before a primer can be synthesized, a primosome had to be formed on the SSB coated DNA template. The formation of a primosome on the DNA is an ATP dependent step and involves all the proteins required for primer synthesis with the exception of the primase (Sholmai and Kornberg 1980). Primosome assembly requires a specific DNA binding site, designated PAS (**p**rimosome **a**ssembly **s**ite) within the single stranded SSB-coated DNA template (Marians 1984). The PAS is usually a short 50 to 70

nucleotide region capable of forming a stem loop secondary structure (Shlomai and Kornberg 1980).

Primosome assembly is initiated by the binding of PriA to PAS within the SSB-coated DNA template (Lee and Marians 1990). Once a PriA binds to a PAS, the site is closed to binding by additional PriA (Allen and Kornberg 1993). PriB then binds to the PriA-DNA complex. dnaB and dnaC proteins form an ATP-dependent complex in solution made up of one dnaB hexamer and six dnaC monomers (Kobari and Kornberg 1982). dnaT transfers the dnaB hexamer from the complex onto the PriA-PriB-DNA complex forming the primosome (Arai *et al.* 1981). The complete set of proteins that remain bound to the DNA-primosome complex is yet to be distinguished as is the functional role of the PriC protein(Allen and Kornberg 1993). The primosome is capable of bi-directional movement by virtue of its helicase activity. In the 3' $\rightarrow$ 5' direction, it draws its energy through PriA mediated (d)ATP hydrolysis and in the 5' $\rightarrow$ 3' direction, it derives energy through the dnaB mediated rNTP hydrolysis (Lee and Marians 1989). In response to signals not yet understood, the dnaG protein which is the primase (Rowen and Kornberg 1978), associates with the primosome-DNA complex and synthesizes a primer. It is thought that while the primosome can travel along the DNA template, the primase dissociates off the complex after synthesis of a primer. So a primase must associate with the preprimosome for each new Okazaki fragment to be synthesized (Allen and Kornberg 1993).

The bulk of DNA synthesis is performed by the pol III holoenzyme (Gefter *et al.* 1971). There is much evidence available to support a bi-directional, coupled, leading and lagging strand synthesis by the heterodimeric pol III holoenzyme, as proposed by the Kornberg (Maki *et al.* 1988; Kornberg and Baker 1992). *In vitro* experiments using G4 DNA circles show that the holoenzyme is highly stable on the DNA and one holoenzyme could replicate the entire genome in a highly processive manner (Burgers and Kornberg 1983). Additionally, the holoenzyme displays a very long intramolecular recycling time which is about 60 fold higher than its intermolecular recycling time. In other words, the holoenzyme could replicate multiple primed DNA substrates as well as single primed DNA substrates very efficiently but at the same time was very inefficient in recycling between DNA molecules (Burgers and Kornberg 1983). For the coupled synthesis to be accomplished by a heterodimeric holoenzyme, the lagging strand has to be looped around to co-ordinate the lagging strand synthesis to the leading strand synthesis (Kornberg 1988; Stillman 1994). When the holoenzyme was bound *in vitro* to DNA primed at two nearby locations, one on each strand, the presence of loops were confirmed using electron microscopy. Loops were however absent when only the catalytic core was used adding evidence to the heterodimeric holoenzyme theory (Marians 1984). Additionally the holoenzyme is present in a very low abundance in the cell (ca 20 /cell) (McHenry and Kornberg 1977). A heterodimeric polymerase mediated, coordinated synthesis hypothesis is attractive because, it provides for a possibility of a very few number of holoenzyme molecules replicating the genome and a mechanism of pausing replication when synthesis on one strand stops (reviewed in McHenry 1991 and Kelman and O'Donnell 1995). If the

replication was conducted by an independent polymerase on each strand and one of them stalls at a site of damage, the other polymerase will continue synthesis, spooling out a length of ss DNA on the stalled strand (Kelman and O'Donnell 1995). There are about 800 SSB complexes in the cell capable of covering 50 kb ss DNA exposing additional regions to nuclease attack. Contrary to this, a heterodimer will come to a halt when one of its polymerases stalls (Kelman and O'Donnell 1995). The heterodimer however, poses a potent problem in the recycling of the lagging strand core between Okasaki fragments. In other words, the lagging strand core is at a kinetic disadvantage for rapid disassociation and reassociation required in the lagging strand (Kelman and O'Donnell 1995).

A novel idea for the recycling of the core at the lagging strand has been provided by the O'Donnell group (Stukenberg *et al.* 1994). According to their model, the recycling of the core replicating the lagging strand is enabled through the γ complex. While the lagging strand core, attached to a β–clamp, is replicating an Okasaki fragment, the γ complex assembles a new β–clamp at the next primed terminus. Once the lagging strand core has completed an Okasaki fragment to a nick, it readily dissociates off the DNA-β–clamp complex and can now recycle to the β–clamp on the next primed terminus (Stukenberg *et al.* 1994). There are several additional evidence to support this hypothesis. Assembly studies have shown that β–clamps can be assembled at the rate of one per half a second at physiological salt concentrations providing evidence that β–clamp assembly is rapid enough to account for each Okasaki fragment (Stukenberg *et al.* 1994). At physiological salt concentrations, the pol III* dissociates spontaneously from a β–clamp after completing synthesis to a nick (Stukenberg *et al.* 1994). This as well as the relative

abundance of β dimers compared to the holoenzyme support the hypothesis of the O'Donnell group (Stukenberg *et al.* 1994). The process of replication is completed upon removal of the RNA primer and filling in the gap, potentially by pol I, and ligation of the nick by ligase (Kornberg and Baker 1992).

1.2.2 Eukaryotic DNA Replication:

The precise functioning of eukaryotic DNA replication is not well understood. Work done using the SV40 replication system and yeast have contributed significantly to the current database on this topic. As opposed to the one major replicative polymerase situation in *E. coli* (Kornberg and Baker 1992), at least three polymerases i.e., pol α, pol ε and pol δ seem to be involved in eukaryotic DNA replication based on evidence such as the requirement of these proteins for viability and the dumb-bell shaped terminal morphology of temperature-sensitive yeast mutants (Conrad and Newlon 1983a; Conrad and Newlon 1983b; Morrison *et al.* 1990; Tsurimoto *et al.* 1990; Morrison *et al.* 1991; Campbell *et al.* 1993). It is widely accepted that pol α is the priming polymerase as it is the only known polymerase with an associated primase activity (Fry and Loeb 1986; Hay and Russell 1989). Several models have been proposed for eukaryotic DNA replication using these polymerases. In an overall picture, there are three major scenarios: 1. The pol δ (leading) and pol α– pol δ (lagging strand) mediated replication process based on the SV40 replication system, proposed by the Stillman group; 2. the pol ε (leading strand) and pol α- pol δ (lagging strand) mediated replication process, proposed by the Sugino

group; and 3. the pol δ (leading strand) and pol α- pol ε (lagging strand) mediated replication process, proposed by the Burgers groups. These three hypotheses are discussed below.

The SV40 replication system seems to suggest a two polymerase replication system. Only pol α and pol δ are required for this system to function (Tsurimoto *et al.* 1990). When pol α was removed, DNA replication was completely blocked. However, short strands were synthesized when pol α was the only polymerase in the system, suggesting that pol α primes both the strands and synthesizes the lagging strand while pol δ synthesizes the leading strand by virtue of its high PCNA induced processivity (Weinberg *et al.* 1990). Although this seems to suggest a role for pol α in DNA synthesis in addition to priming the strands, it is likely that it is not the case. This conclusion is based on fidelity assays on the accuracy of the polymerase *in vitro*. Pol α has a relatively high error rate of about 1/10000 to 1/12000 nts polymerized. This high error rate which is in part due to the absence of an associated proofreading activity seems to suggest that pol α is probably involved only in priming both the strands (Kunkel *et al.* 1991). There is, however, a possibility that pol α could efficiently serve as a lagging strand polymerase with pol δ functioning as the leading strand polymerase if they function as an asymmetric dimer with pol δ proofreading both the strands (Perrino and Loeb 1990). This SV40 system derived two polymerase system is probably not true as pol ε in addition to pol α and pol δ is required for replication *in vivo* (Morrison *et al.* 1991). The Stillman group (Waga *et al.* 1994; Waga and Stillman 1994) proposed that initially, pol α binds the SSB

bound DNA and synthesizes a primer via its primase and then initiates replication on that strand. Soon after a short strand is synthesized, RF-C and PCNA bind the 3'-OH terminus of the growing strand, stalling and subsequently displacing pol α. PCNA now recruits pol δ to commence continuous synthesis (leading strand synthesis). Simultaneously, pol α dissociates off and binds the next priming site on the opposite strand (lagging strand) and performs initial synthesis. Pol δ subsequently takes over from pol α in a similar manner as explained for the leading strand. It is also suggested that the two polymerases interact with each other and perform a coordinated leading-lagging strand synthesis. In this scenario, pol δ which is the major replicase synthesises DNA using a DNA primer synthesised by a polymerase (pol α) as opposed to an RNA primer synthesised by a primase as in the case of *E. coli* (Waga and Stillman 1994). To account for the essentiality of pol ε *in vivo*, they suggest that pol ε is probably involved in an unidentified, vital, replication related repair process (Tsurimoto *et al.* 1990) or that the essential function performed by pol ε *in vivo* might be duplicated in the SV40 system (Waga and Stillman 1994). It is also been suggested that while pol ε may not be required to replicate the relatively short SV40 replicon, while it might be indispensable for replication of the relatively large replicon seen in eukaryotic chromosomes (Waga and Stillman 1994). It is however, cautioned that it might not be appropriate to speculate on *in vivo* replication using information gained from the SV40 system since there are several differences beetween the two processes, such as size of the replicon and proteins and sequences required at the origin (Campbell 1993). Development of *in vitro* systems based on yeast proteins in which synthesis is *ARS* (**a**utonomous **r**eplication **s**equence required

for initiation of replication) dependent may be a quick process to understand the functions of the proteins involved in the replication fork (Campbell 1993).

Pol ε was initially suggested to function only in DNA repair, based on the observation that mpol ε was required for DNA repair synthesis in permeabilized UV-irradiated human diploid fibroblasts (Nishida *et al.* 1988). It was not thought to be important in replication because it was not required by the SV40 DNA replication system (Tsurimoto *et al.* 1990). It was however pointed out later that ypol ε was an essential protein and temperature-sensitive defects in pol ε resulted in exactly the same dumb-bell morphology indicative of a defect in replication, exhibited by pol a and pol δ mutants. This coupled with the fact that defects in no known protein that functions only in DNA repair results in such a phenotype, pol ε has been suggested to be involved in replication *in vivo* (Morrison *et al.* 1990; Morrison *et al.* 1991). Since pol ε is inherently processive, the Morrison-Sugino group has suggested a replication model with pol ε as the leading strand polymerase. In their model, pol α is thought to initiate synthesis by priming both the strands. Pol δ then takes over the lagging strand synthesis. The recycling of pol δ from one Okasaki fragment to the next is probably carried out with the aid of PCNA as pol δ can be efficiently moved in and out of a processive mode based on its dependency on PCNA for processivity. Pol ε takes over in the leading strand by virtue of its high processivity in physiological levels of salt in the presence of PCNA (Morrison *et al.* 1990). Since pol α is relatively inaccurate, defective primers synthesized by pol α may result in non-synthesis or pol δ may function in proofreading for pol α during primer

synthesis. The latter possibility is more probable as it has been shown that pol δ can proofread for pol α in vitro (Perrino and Loeb 1990).

Proofreading mutants of pol δ confer a 10 fold higher spontaneous mutation rate than similar pol ε mutants (Morrison and Sugino 1993). It is not possible to conclude if this reflects the relative contribution of the two polymerases to DNA synthesis as the ratios of their polymerase and proofreading activities in the cell is not known (Morrison and Sugino 1993). However, assuming a relatively higher contribution by pol δ, Burgers suggested that it may possible that pol δ conducts the leading strand replication while pol ε functions in lengthening the primers synthesized by pol α in the lagging strand (Burgers 1991).

1.3 DNA replication fidelity

A very high DNA replication fidelity is required for the accurate transmission of genetic information from one generation to the next. The *in vivo* spontaneous mutation rate is less than $1x10^{-9}$ (Drake 1991). This incredible accuracy of DNA replication is a result of at least four cellular processes. They are: 1. base selectivity by DNA polymerases i.e., ability of the replicating polymerase to choose a correct base over an incorrect base; 2. proofreading by the polymerase i.e., ability of the polymerase to recognize an incorrectly inserted base as soon as it is inserted, remove it using a 3' → 5' exonuclease activity and continue synthesis and; 3. mismatch correction, a post-replicative correction mechanism that tracks down mispairs left behind by the replication

machinery, excises a portion of the newly synthesized strand including the mismatch, followed by gap-filling by a polymerase and ligation by ligase (Echols and Goodman 1991); and 4. the processes involved in maintaining a balance in the levels of the nucleotides as well as those involved in clensing the nucleotide pool (such as the degradation of of 8-oxo-7,8-dihydrodeoxyguanosine-5'-triphosphate).

1.3.1 Selectivity of base insertion by DNA polymerases:

The catalytic role of a DNA polymerase is the template directed polymerization of deoxynucleotides onto a primer in accordance to the Watson-Crick (here after WC) rule (A-T or G-C pairing). Base selectivity is the ability of the polymerase to selectively discriminate against non-WC base-pairs (Echols and Goodman 1991).

Several possible factors or control points are involved in the nucleotide insertion selectivity mechanism and in an over all picture, it is a result of the kinetic balance of the steps involved in the insertion process (Kunkel 1992a; Kunkel 1992b). Initial discrimination probably occurs at the substrate binding stage when a dNTP binds the polymerase. The enzme-substrate complex then undergoes a conformational change that positions the dNTP for bond formation and provides the second stage of discrimination. A kinetically slow reaction follows the incorporation of a non-WC base, providing the proofreading activity an opportunity to act. It is also possible that the tertiary complex undergoes a conformational change favoring proofreading when a wrong base is bound. Finally, the elongation step after insertion of a non-WC is slower, providing a kinetic

advantage for the proofreading activity (Echols and Goodman 1991; Kunkel 1992a; Kunkel 1992b; Von Hippel *et al.* 1994). This base selectivity pathway has been verified by several pre-steady state and steady state kinetic methods (Goodman *et al.* 1993; Von Hippel *et al.* 1994). Another possible factor in base insertion selectivity is the geometry effect i.e., bond distances and bond angles. The polymerase may have a requirement for equivalent geometry as displayed by WC base-pairs for efficient polymerization (Sloane *et al.* 1988). Based on geometry, a correct base may be preferentially incorporated due to the requirement of a rate limiting conformational change of the enzyme through an "induced-fit process" before the chemical step of phosphodiester bond formation (Kuchta *et al.* 1987).

1.3.2 Proofreading by DNA polymerases:

The 3' → 5' exonucleolytic editing or proofreading was first identified in the early 70s when it was found that the 3' → 5' exonuclease activity of *E. coli* pol I removed incorrectly paired terminal nucleotides much more rapidly than correctly paired ones (Brutlag and Kornberg 1972). Among the proofreading proficient polymerases identified so far, the polymerizing and exonuclease activity of a polymerase usually co-reside on the catalytic subunit with an exception in the case of the *E. coli* pol III where they reside in two different subunits i.e., the polymerizing activity on the α subunit and the exonuclease on the ε subunit (Scheuermann *et al.* 1983).

Among the eukaryotic polymerases, pol δ, pol ε and pol γ have an associated proofreading activity (Morrison and Sugino 1994; Foury and Vanderstraeten 1992). It is

widely believed that pol α lacks proofreading capability although there are reports that have indicated otherwise: it has been shown that, *Drosophila* pol α may have a cryptic 3' → 5' exonuclease activity (Cotterill *et al.* 1987), the human lymphocyte pol α copurifies with a proofreading activity (Bialek *et al.* 1989), the calf thymus pol α copurifies with a 3' → 5' exonuclease activity (Bialek and Grosse 1993). There has been a report that ypol α excised dCMP from the 3'-OH terminus of a single-stranded substrate (Brooke *et al.* 1991). The enzyme, however, did not excise 3'-OH-terminally mispaired nucleotides from oligonucleotide substrates, even after very long incubation periods with a large excess of the enzyme (Kunkel *et al.* 1991). This was been confirmed by Brooke *et al.* (1991) who demonstrated that ypol a did not degrade the radiolabelled oligomer (dC)15 mispaired portion of an oligomer under conditions in which several proofreading proficient polymerases degraded this substrate. Moreover, fidelity assays have shown that the *in vitro* replication error rate of ypol α is in accordance with proofreading deficient polymerases (Kunkel *et al.* 1991), and so it is reasonable to assume that in general pol α lacks a proofreading activity.

Polymerization and proofreading are competing processes and the accuracy of a polymerase depends on the precise balance of these two activities (Goodman *et al.* 1993; Von Hippel *et al.* 1994) as indicated by the mutator/antimutator phenotypes observed when this balance is offset in mutant *E. coli* strains (Langhammer and Piechocki 1984; Oller and Schaaper 1994). These two activities require a precise balance in the kinetics of their catalytic rates (Goodman *et al.* 1993) since the proofreading activity in addition to removing incorrect bases, also removes about 5 to 15% of the correctly inserted bases

(Fersht *et al.* 1982). Kinetically, the fidelity of a polymerase is therefore dependent on the rates of three critical steps (Von Hippel *et al.* 1994): 1. $k_{FORWARD}$, the rate at which the polymerase can proceed to insert the next base; 2. $k_{REVERSE}$ the rate at which the exonuclease can take over and excise the inserted base before the polymerase can insert the next base; and 3. $k_{RELEASE}$ the rate at which the polymerase can dissociate off the template. These rates are expected to vary with the nucleotide under consideration, for correct vs. incorrect base-pairs as well as for different mispairs and template primer positions (Petruska and Goodman 1985; Kunkel 1992a; Kunkel 1992b). Biochemical studies using isotope quenching (Kuchta *et al.* 1987) and nuclear magnetic resonance (Ferrin and Mildvan 1986) have added evidence to support these factors. These studies show that once a dNTP is bound to the enzyme, a first order isomerisation of the enzyme-polynucleotide-dNTP complex occurs and this positions the substrate dNTP for bond formation. This is a rate limiting conformational change and is substantially slower for an incorrect base than for a correct base. This rate limiting process can slowdown polymerization and allow proofreading to take over (El-Deiry *et al.* 1984; Maki and Kornberg 1987; El-Deiry *et al.* 1988).

1.3.2.1 Specificity of proofreading in prokaryotes

All the three polymerases identified, to date, in *E. coli* have an associated 3' → 5' exonuclease activity capable of proofreading function (Kornberg and Baker 1992). Although it has been shown that the proofreading activities of pol II may be involved in synthesizing episomal DNA in non-dividing cells (Foster *et al.* 1995) and that it may

contribute to the overall fidelity of DNA synthesis, its specificity has not been assessed (Cai *et al.* 1995). The exonuclease active sites of pol I has been well characterized, genetically, biochemicaly and by crystallography (Joyce and Steitz 1987; Derbyshire *et al.* 1988 Derbyshire *et al.* 1991). Very little has been done to characterize its specificity since the extent of DNA synthesized by it is very small (Kunkel 1992b). However, the specificity of proofreading by pol III, the major *E. coli* replicase (Kornberg and Baker 1992) has been characterized to a significant extent (Schaaper 1993).

The ε subunit, encoded by the *dnaQ* gene, of the pol III catalytic core is responsible for the proofreading activity of the polymerase (Scheuermann and Echols 1984). Mutations in the *dnaQ* gene results in a marked reduction of the proofreading activity (DiFrancesco *et al.* 1984) and an increase in the spontaneous mutation frequency relative to a wild type strain (Fowler *et al.* 1986). The *dnaQ* mutant strains showed an increase in the rate of all the types of sequence alterations detected while the single base-pair substitutions (SBPS) displayed the largest increase (Wu *et al.* 1990). The literature available on the effect of proofreading on spontaneous mutagenesis, studied mostly using mutant *dnaQ* strains, points out a remarkable specificity of this mutator (Piechocki *et al.* 1986; Wu *et al.* 1990; Fowler *et al.* 1992; Schaaper 1993). In general, proofreading was able to correct premutational events (PMEs) that would lead to transversions more efficiently than those that would lead to transitions. There was not much of a difference in the efficiency of correction between the subclasses of transition PMEs. There was, however, a significant difference in the efficiency of correction between the subclasses of transversion PMEs with those that lead to A·T → T·A events being the most efficiently

corrected one (Piechocki *et al.* 1986; Wu *et al.* 1990; Fowler *et al.* 1992; Schaaper 1993). In a recent study analyzing the SBPS arising in a reporter gene in *E. coli* strains deficient in proofreading or mismatch correction or both, it was noted that while transversion PMEs were corrected most efficiently by proofreading, transition PMEs were corrected more efficiently by mismatch correction (Schaaper 1993). It is interesting to note that for some unknown reasons, the specificity of proofreading-deficient *E. coli* strains are dependent on the medium of growth with transitions predominating when grown in rich medium and transversions predominating in minimal medium (Schaaper 1988). It has also been mentioned that there is a mismatch deficiency in proofreading deficient strain probably due the saturation of the mismatch correction pathway by the numerous replication errors left behind due to defective proofreading (Schaaper and Radman 1989; Damagnez *et al.* 1989).

1.3.2.2 Specificity of proofreading in eukaryotes

The eukaryotic pol δ and pol ε are proofreading proficient enzymes and are believed to participate in error correction during replication (Morrison and Sugino 1994; Sugino 1995). Amino acid sequence homology has identified three conserved segments, designated Exo I through III on several prokaryotic and eukaryotic polymerases corresponding to 3' → 5' exonuclease active sites (Bernard *et al.* 1989). The validity (Reha-Krantz 1992) as well as the pattern of arrangement of these domains were subsequently challenged (Wang 1991; Morrison *et al.* 1991; Reha-Krantz *et al.* 1991). A modified version of the original domain arrangement has been published and these

domains seem to be present in ypol δ and ypol ε (Morrison and Sugino 1993). It has been shown that alteration of some of these conserved residues in ypol δ (Simon *et al.* 1991) as well as ypol ε (Morrison *et al.* 1991) result in elevated spontaneous mutation rates and are associated with considerable reductions in exonuclease activity while maintaining wild-type levels of polymerase activity. This shows a functional relationship between the conserved domains and proofreading activity (Simon *et al.* 1991; Morrison *et al.* 1991). It has, however been pointed out that it may not be appropriate to speculate that there is a high degree of conservation of DNA polymerase structure from short sequence homology (Reha-Krantz 1992). Very little has been done in terms of mapping the specificity of errors caused by defects in proofreading due to problems such as the lethality of *pol2-pol3* (Morrison and Sugino 1994) and *pol3-pms1*(mismatch correction) double mutants (Morrison *et al.* 1993). Limited data is available and shows that mutations associated with such a defect are predominantly SBPS with no apparent specificity (Morrison *et al.* 1993; Morrison and Sugino 1994). The small sample size of these experiments precluded any definite conclusions or meaningful statistical analysis (Morrison *et al.* 1993; Morrison and Sugino 1994). *In vitro* studies done with crude extracts from cells, mostly from Kunkel's group suggest that there might be differential proofreading by the leading and lagging strand replication complexes both in *E. coli* (Veaute and Fuchs 1993) as well as mammalian cells (Roberts *et al.* 1991; Minnick *et al.* 1994: Izuta *et al.* 1995). In either case, the leading strand replication machinary seem to be more accurate than the one on the lagging strand. However, since assignment of the leading and lagging polymerases in

eukaryotes is not yet known, the cause for this differential proofreading cannot be accurately determined (Izuta *et al.* 1995).

1.3.3 Mismatch Correction:

The third step in DNA replication fidelity is the post-replicative mismatch correction. A major source of mismatches are DNA replication errors although it can also be caused by spontaneous chemical base modifications such as deamination of 5-methyl cytosine to form thymine. Mismatch correction plays a vital role in maintaining a high degree of replication accuracy and a low spontaneous mutation rate in prokaryotes as well eukaryotes (Modrich 1987; Grilley at al. 1990).

1.3.3.1 Mismatch correction in E. coli

The process and proteins involved in mismatch correction in *E. coli* are well characterized and it seems to possess three major pathways. They are: 1. the MutHLS-dependent, methyl-directed mismatch correction system; 2. the G/T specific very short patch (VSP) mismatch repair system that specifically corrects G/T mismatches; and 3. the *mutY*-dependent mismatch correction system that specifically corrects G/A or 8-oxoG/A mispairs (Grilley *et al.* 1990; Au *et al.* 1992).

1.3.3.1.1 The methyl-directed mismatch correction system

This is a well characterized pathway with a very broad specificity correcting all possible base mispairs as well as short addition and deletion errors with varying

efficiencies (Kramer *et al.* 1984; Su *et al.* 1988; Lahue *et al.* 1989). It involves several proteins comprising the following proteins: MutH, MutL, MutS, MutU, SSB, DNA Ligase, exonuclease I or exonuclease VII, helicase II and pol III and ATP and dNTPs (Modrich 1987; Grilley *et al.* 1990 Grilley *et al.* 1993). This repair pathway is highly strand specific in that it preferentially corrects the newly synthesized strand over the template strand and this strand discrimination is attributed to the methylated state of adenine in the template strand at d(GATC) sites (Wagner and Meselson 1976; Pukkila *et al.* 1983; Kramer *et al.* 1984; Wagner *et al.* 1984). Adenine methylation at the d(GATC) site is performed by the *dam* gene product (Marinus 1976, Marinus 1984). This dam specific methylation follows DNA replication so that the newly synthesized daughter strands are transiently undermethylated (only one strand methylated) DNA during replication (Glickman *et al.* 1978; Lyons and Schendel 1984). The hemimethylated state of the DNA provides for strand bias as fully methylated heteroduplex DNA is immune to repair while there is no strand bias in unmethylated heteroduplex DNA (Pukkila *et al.* 1983; Wagner *et al.* 1984; Dohet *et al.* 1986).

MutS is a 97 kDa protein and is the mismatch recognition protein of the correction pathway. It binds to all the eight possible mispairs with varying efficiencies (Su and Modrich 1986; Su *et al.* 1988). The physical basis of mismatch recognition is yet unknown. However it is known that MutS has a weak ATPase activity and mediates formation of a DNA loop and that the loop formation is ATP dependent and requires the presence of a mismatch (Grilley *et al.* 1990). The MutHLS pathway corrects transition

mismatches (G/T and A/C) much more efficiently than transversion mismatches (C/T, G/A, G/G, A/A, T/T and C/C) (Kramer *et al.* 1984; Wagner *et al.* 1984; Dohet *et al.* 1986). This seems to correlate to some extent to the binding efficiency of MutS which binds G/T mispairs with the highest efficiency and T/C mispairs with the least efficiency. It is possible that the binding efficiency along with other factors contributes to the mismatch specificity (Grilley *et al.* 1990).

MutH is a 25 kDa d(GATC) specific endonuclease and has been isolated in its homogeneous form (Welsh *et al.* 1987). It nicks the unmethylated strand at a hemimethylated d(GATC) site and provides the strand discrimination for correction (Modrich 1987; Welsh *et al.* 1987). The MutH is a latent endonuclease and requires complex formation with MutS and MutL in the presence of ATP and Mg^{2+} for its activity (Au *et al.* 1992). The endonuclease nicks the unmethylated strand immediately 5' to the methylated d(GATC) site leaving a 5' phosphate and a 3' hydroxy terminus (Modrich 1989). This process has no obligate directionality relative to the mismatch site as it recognizes the d(GATC) site 3' as well as 5' to the mismatch with equal efficiency (Au *et al.* 1992). However circular heteroduplexes are better substrates than linear ones showing that the efficiency of the MutH endonuclease is influenced by DNA topology (Au *et al.* 1992).

MutL is a 70 kDa protein with a high affinity to bind to a MutS-heteroduplex DNA complex and is required for mismatch correction (Grilley *et al.* 1989). The biochemical function of this protein in the correction process is not yet known. The binding efficiency of MutL is independent of the pattern of methylation or the presence of

d(GATC) site (Bende and Grafstorm 1991). It, however, has a higher affinity for unmethylated single-stranded DNA compared to methylated single stranded DNA and methylated or unmethylated double stranded DNA (Bende and Grafstorm 1991). Purified MutL protein interacts with MutS-heteroduplex DNA complex in an ATP dependent manner. It is possible that MutL acts as a bridge interfacing the mismatch recognition by MutS with the binding and activation of the latent d(GATC) endonuclease of MutH (Grilley *et al.* 1990).

The MutU is the *uvrD* gene product and is a DNA helicase II/DNA-RNA helicase, which can unwind duplex DNA in an ATP-dependent manner (Langle-Rouault *et al.* 1987; Runyon and Lohman 1989). It is possible that once the MutH has nicked the daughter-strand containing the mismatch, the MutU initiates unwinding of the heteroduplex DNA either at the nick or at the mismatch site (Langle-Rouault *et al.* 1987; Runyon and Lohman 1989) and aids in the removal of the section of DNA containing the wrong nucleotide (Maples and Kushner 1982; Kumura and Sekiguchi 1984; Langle-Rouault *et al.* 1987; Runyon and Lohman 1989).

In addition to the MutHLS and MutU proteins, the mismatch correction process requires several additional proteins such as the SSB, ligase, exonuclease I or exonuclease VII, and pol III in the presence of ATP and dNTPs (Modrich 1987; Grilley *et al.* 1990 Grilley *et al.* 1993). The SSB protein possibly provides protection to the single stranded regions of the correction region against nuclease attack while enhancing the sensitivity of the nicked single stranded region to hydrolysis by exonuclease I, which is another required protein probably functioning in degradation of the mismatch containing daughter

strand (Molineux and Gefter 1975). SSB bound DNA is also a preferred template for pol III which possibly functions in DNA synthesis after the mismatch containing daughter strand region is degraded (Chase and Williams 1986).

The possible mechanism of methyl-directed mismatch correction requires the sequential action of several proteins and has been discussed in detail (Au *et al.* 1992; Grilley *et al.* 1990; Modrich 1989; Modrich 1994). The correction process begins with the recognition of the mismatch in the heteroduplex region by MutS to which it binds, followed by the addition of MutL and MutH (Grilley *et al.* 1989). This complexing activates the latent endonuclease activity of MutH which nicks the unmethylated daughter-strand at the hemimethylated d(GATC) site in an ATP-dependent manner (Au *et al.* 1992). It is possible that once the MutH has nicked the daughter-strand containing the mismatch, a helicase such as MutU initiates unwinding the heteroduplex DNA either at the nick or at the mismatch site (Langle-Rouault *et al.* 1987; Runyon and Lohman 1989). The daughter-strand containing the mismatch is then degraded by exonuclease I in the 3'→ 5' direction if the nick is present 3' to the mismatch or by exonuclease VII or RecJ exonuclease in the 5' → 3' direction if the nick is present 5' to the mismatch (Cooper *et al.* 1993; Grilley *et al.* 1993). This excision removes a section of the unmethylated DNA strand, usually a few kb long (Au *et al.* 1992), starting from the nick and spanning past the mismatch (Au *et al.* 1992). The resulting gap is filled by pol III (Kornberg 1980) followed by an NAD^+ dependent ligation reaction by DNA ligase (Lahue *et al.* 1989).

1.3.3.1.2 Very short patch (VSP) mismatch correction

VSP can be distinguished from methyl-directed mismatch correction by its very small, usually 10 nucleotide (Jones *et al.* 1987), excision tract as opposed to the removal of a few kb in the case of methyl-directed mismatch correction (Au *et al.* 1992). It is dependent on the *vsr* gene product and pol I and is strongly stimulated by MutL and MutS (Jones *et al* 1987; Lieb 1987; Zell and Fritz 1987; Sohail *et al.* 1990). It is highly specific to the correction of G/T mispairs and is mainly responsible for repairing deaminated 5-methyl cytocine (5-meC) (Raposa and Fox 1987; Lieb 1991). In *E. coli* K-12, 5-meC is formed by the DNA methyltransferase (dcm) at the second position within the sequence d(CCATGG) which can be spontaneously deaminated into a thymine leading to a G/T mismatch (Marinus 1984). The *vsr* gene product is a 18 kDa protein with a mismatch specific endonuclease activity (Hennecke *et al.* 1991). It is a G/T mismatch dependent and strand specific endonuclease that nicks double stranded DNA within the sequence d(CTATGN) or d(NTATGG) 5' to the first T position (Hennecke *et al.* 1991). After the nick is made, the dTMP is removed along with a few neighboring bases (usually less than 10 nucleotides) through a 5' $\rightarrow$ 3' exonuclease activity and the gap is filled in a template dependent manner, potentially by pol I (Bambara *et al.* 1978).

1.3.3.1.3 The MutY dependent mismatch correction

The MutY is a 39 kDa protein with a significant homology to endonuclease III (Michaels *et al.* 1990). This system requires MutY, a polymerase (potentially pol I) and DNA ligase and is highly specific for the correction of G/A mismatches when the A is in

the newly synthesized daughter strand and is independent of DNA methylation (Au *et al.* 1988; Nghiem *et al.* 1988). The repair tract is usually longer than 10 but shorter than 30 nucleotides and always extends 3' from the mispaired adenine (Radicella *et al.* 1993). *In vivo* MutY primarily functions in the degradation of 8-oxo-7,8-dihydrodeoxyguanosine-5'-triphosphate, which is an oxidatively damaged form of dGTP that can pair with adenine during replication leading to a G/A mismatch (Michaels *et al.* 1992a; Michaels *et al.* 1992b).

1.3.3.1.4 Other potential mismatch repair pathways

In addition to the above discussed mismatch correction pathways, there may be several other correction systems. Some potential candidates identified are: 1. The *mutA* and *mutC* genes which are transversion-specific mutator loci i.e., inactivation of these genes leads to a high degree of transversion mutations (Michaels *et al.* 1990); 2. The *mutM* gene product which possibly involves in avoidance of G·C → A·T transversions (Cabrera *et al.* 1988) by the removal of 8-oxo-7,8-dihydrodeoxyguanine which can pair with adenine during DNA synthesis (Tchou *et al.* 1991; Michaels *et al.* 1992a; Michaels *et al.* 1992b); and 3. the MutT protein that probably provides a complementary function to the MutY protein by removing the 8-oxo-7,8-dihydrodeoxyguanine when it is inserted in the daughter-strand opposite an adenine (Cox 1976; Akiyama *et al.* 1989; Schaaper and Dunn 1987).

1.3.3.2 Mismatch correction in yeast

Evidence for mismatch correction in mitotic as well as meiotic yeast cells was established in the late 80s (Hastings 1984; Bishop and Kolodner 1986; Bishop *et al.* 1987; Kramer *et al.* 1989a). *In vivo* mismatch correction studies were done by studying the outcome of transforming covalently closed heteroduplex DNA into intact cells. Such studies show that all single base-pair substitution errors are corrected, although with varying efficiencies (Bishop and Kolodner 1986; Bishop *et al.* 1987; Bishop *et al.* 1989; Kramer *et al.* 1989a). While C/C and T/T mispairs were poor substrates for correction single nucleotide loops which would eventually lead to single base addition or deletion were corrected most efficiently (Bishop *et al.* 1989; Kramer *et al.* 1989a). *In vitro* band shift assays to monitor the binding activity of cell extracts have shown the existence of mismatch binding activity with a high preference for single nucleotide loops followed by all single base mismatches with the exception of C/C mismatches adding support to the *in vivo* results (Miret *et al.* 1993). Yeast DNA is not detectabily methylated and hence, strand discrimination is not likely to be methyl-directed as in the case of *E. coli* (Proffitt *et al.* 1984). Although several speculations such as generation of single strand breaks or small gaps in the daughter-strand for strand discrimination have been suggested (Aprelikova *et al.* 1989; Tomilin and Aprelikova 1989). There is much discrepancy in the issue and for now the mechanism of strand discrimination in yeast and higher eukaryotes in unknown (Impellizzeri *et al.* 1991; Verri *et al.* 1992).

Yeast genes encoding the *E. coli MutL* and *MutS* homologues have been identified, demonstrating that components of the mismatch correction system have been

conserved during evolution (Kramer *et al.* 1989b; Reenan and Kolodner 1992; New *et al.* 1993; Prolla *et al.* 1994a).

1.3.3.2.1 The PMS (post meiotic segregation) genes:

The *PMS* genes were among the first identified eukaryotic genes thought to be involved in mismatch correction. Four PMS genes, *PMS1, PMS2, PMS3* and *PMS6* are thought to be required for mismatch correction in yeast (Kramer *et al.* 1989a; Jeyaprakash *et al.* 1994). The *pms* mutants in general showed an increase in PMS frequency and spontaneous mutation rate as well as reduced spore viability (Williamson *et al.* 1985; Kramer *et al.* 1989a). A comparison of the correction efficiency of heteroduplex transformed into intact cells showed that *pms* mutants were deficient in mismatch correction of all mismatches including single nucleotide loops (Bishop and Kolodner 1986; Bishop *et al.* 1987; Bishop *et al.* 1989; Kramer *et al.* 1989a). The *pms1* mutants also showed a significant level of instability of palindromic repeat sequences (Henderson and Petes 1993). The *PMS1* gene has been sequenced and predicted to encode a 103 kDa protein with a high degree of homology to the MutL protein (Kramer *et al.* 1989b). Although the nature and functional roles of PMS proteins in mismatch repair are still unknown (Jeyaprakash *et al.* 1994), it is believed that the *PMS1* and *MSH2* (see following section) act in the same repair pathway as the single as well as double mutant phenotypes of these two genes are mutually indistinguishable (Alani *et al.* 1994).

1.3.3.2.2 The yeast MSH genes (MutS homologue)

Presently, three MSH genes, *MSH1*, *MSH2*, and *MSH3* have been isolated and predicted to encode proteins homologous to the *E. coli* MutS protein. The *MSH1* gene encodes a 109.3 kDa protein with 28.6% amino acid homology to MutS while the *MSH2* gene encodes a 109.1 kDa protein with, a relatively lower, 25.2% amino acid homology to MutS. (Reenan and Kolodner 1992a). Although mismatch correction has not been demonstrated in the mitochondria, the *MSH1* gene product is thought to be involved in the mismatch correction of mitochondrial DNA as disruption of this gene causes an increase in the rate of mutation and rearrangement of mitochondrial DNA (Reenan and Kolodner 1992a).

The MSH2 gene product is thought to be involved in nuclear mismatch correction based on the observation that *msh2* mutants show an elevated rate of spontaneous mutation, and PMS events (Reenan and Kolodner 1992a). It is possible that the *MSH2* protein is involved in the multiple base-pair insertion/deletion repair as *msh2* mutants significantly decreased the gene conversion and increased the PMS of two *his4* alleles containing two 4-base insertion mutations (Reenan and Kolodner 1992a). Interestingly, the mismatch binding activity is completely absent in *msh2* mutants suggesting a mismatch recognition role for its protein (Miret *et al.* 1993). Additionally, it has been shown that the Msh2 protein specifically binds to DNA containing mismatches and deletion loops, further emphasizing the significance of a recognition role for this protein in mismatch correction (Alani *et al.* 1995).

A third MutS homologue, designated *MSH3,* has been recently identified (New *et al.* 1993). *MSH3* mutants do not display any significant spontaneous mutator phenotypes (Alani *et al.* 1994) or a reduction in binding activity (Miret *et al.* 1993). The effect of *MSH3* disruption on the reversion rate of several markers is significant although much less than the effect of other repair genes (New *et al.* 1993). Sequence comparisons show that *MSH3* may be more closely related to the mouse *Rep-3* gene and similar eukaryotic *MutS* homologs than to the yeast *MSH2* and similar genes thought to be involved in mismatch correction (New *et al.* 1993). It is possible that the *MSH3* may be analogous to the one of the accessory functions of MutS such as protection against recombination of non-homologous sequences (New *et al.* 1993).

1.3.3.2.3 The yeast MLH *genes (*MutL *homologue)*

The yeast *MLH1* gene was identified by PCR techniques, using conserved sequences from *PMS1* gene as a probe (Prolla *et al.* 1994a). The *MLH1* gene encodes a 769 amino acid predicted protein with sequence homology to the *E. coli* MutL and yeast *PMS1* proteins. It is interesting to note that yeast seems to have two classes of MutL homologs i.e., PMS and MLH proteins. The *mlh1* mutants show a mutator phenotype, increased PMS and reduced spore viability. Similar to the *pms1-msh2* relationship (Alani *et al.* 1994), the *mlh1, pms1* and *mlh1-pms1* mutants are phenotypically indistinguishable suggesting that *PMS1, MSH1* and *MLH1* are components of the same repair pathway (Prolla *et al.* 1994b).

1.3.3.2.4 Mechanism of mismatch correction in yeast:

Yeast possibly shares at least some similarity to the *E. coli* mismatch system as it has the MutL and MutS conserved in its genome. However it is fair to assume that it is most probably not methyl-directed for strand discrimination as the yeast genome is not detectabily methylated (Proffitt *et al.* 1984). Based on the properties of the Msh2, Mlh1 and Pms1 proteins, a model for yeast mismatch correction has been proposed (Marx 1994; Prolla *et al.*1994b) as follows: Mlh1 and Pms1 can interact with each other to form a heterodimer. Mismatch correction is initiated once a mismatch is recognized and bound by the Msh2. Msh2 then undergoes conformational change that increases its DNA binding activity as well as its binding potential with the Mlh1-Pms1 heterodimer. The Mlh1-Pms1 heterodimer then binds the Msh2-DNA complex. This complex then recruits other proteins, possibly an endonuclease, in a strand discriminating fashion, and a helicase followed by a polymerase and ligase to perform the actual correction process (Marx 1994; Prolla *et* a*l.*1994b).

1.3.3.3 Mismatch correction in mammalian cells

The existence of a mismatch correction system has been demonstrated in several higher eukaryotes including human cell lines, *Drosophila melanogaster*, *Xenopus* eggs and African green monkey cell lines (Hare and Taylor 1985a; Hare and Taylor 1985b; Brown and Jiricny 1988; Brown *et al.* 1989; Holmes *et al.* 1990; Varlet *et al.* 1990; Thomas *et al.* 1991; Yeh *et al.* 1991). Strand discrimination may be methyl directed in

the African green monkey cell lines (Hare and Taylor 1985a; Hare and Taylor 1985b) and directed by strand break in *Drosophila melanogaster* (Holmes *et al.* 1990).

Several human mismatch repair genes have been cloned using bacterial and yeast genes as probes. The cloned human genes are hMSH2 (Fishel *et al.* 1993), hPMS1 (Nicolaides *et al.* 1994), hPMS2 (Bronner *et al.* 1994) and hMLH1 (Bronner *et al.* 1994; Papadopoulos *et al.* 1994). It is interesting to note that HNPCC which affects 1 in 200 individuals in the western world (Lynch *et al.* 1993) is attributed to a deficiency in mismatch correction (Fishel *et al.* 1993; Leach *et al* 1993). The hMSH2 was the first attributed cause of HNPCC when it was found that such tumor cells lines exhibited microsatellite instability (Parsons *et al.* 1993) and had a mutated *hmsh2* gene (Fishel *et al.* 1993; Leach *et al* 1993). Later it was shown that HNPCC, in some patients, was associated with homozygous mutations in the *pms1* or *pms2* genes (Bronner *et al.* 1994; Nicolaides *et al.* 1994; Papadopoulos *et al.* 1994). It has also been established that *hmlh1* mutations may be involved in as many as 30% of HNPCC cases (Fishel *et al.* 1994; Service 1994). Additionally, a germline mutation has been identified in the *msh2* gene of patients with ulcerative colitis, a disease which increases the risk of HNPCC (Brentnall *et al.* 1995). A *msh2* mutation in mice renders carriers susceptible to lymphoid tumours (Fishel *et al.* 1994). Yeast as well as human cells have at least three MutL homologs ie., *MLH1, PMS1* and *PMS2* suggesting unique functions for each of these genes (Prolla *et al.* 1994a) and it is highly possible that they act as a complex (Prolla *et al.* 1994b). This is strongly favoured by the fact that inactivation of any one of the human MutL homologs or *MSH2* can result in HNPCC and it is likely that deficiencies in the mismatch correction

is an early event in the carcinogenesis process (Leach *et al* 1993). Defects in mismatch correction probably destabilizes the genome leading to other genetic aberrations such as activation of oncogenes and inactivation of tumour suppression genes, eventually leading to cancer such as HNPCC (Marx 1994; Modrich 1994).

1.4 Purpose of this study

The Hypotheses to be tested

1. For a given gene, the functional form of the polymerase duplicating the leading strand is different from that of the polymerase duplicating the lagging strand.

2. Proofreading by polymerases and mismatch correction contribute significantly to the fidelity of DNA replication.

3. The fidelity of replication of the transcribed strand is higher than that of the non-transcribed strand.

4. There is a reduction in the level of mismatch correction in a proofreading deficient strain of yeast caused by that saturation of the mismatch correction pathway by the excessive replication errors left behind by the proofreading deficient replication process as seen in *E. coli.*

5. Sequence context modulates the proofreading and mismatch correction processes.

In this study, I have sought to characterize the specificity of the mutator phenotypes conferred by reduction in the 3' $\rightarrow$ 5' exonuclease activities of yeast pol δ (*POL3*) and pol ε (POL2) , required for proofreading (Morrison *et al.* 1993; Morrison and

Sugino 1994) as well as deletion of the *pms1* gene, required for post-replicative mismatch correction (Bishop and Kolodner 1986; Bishop *et al.* 1987; Bishop *et al.* 1989; Kramer *et al.* 1989a; Kramer *et al.* 1989b). It has been shown that proofreading and mismatch correction defects enhance the spontaneous mutation rates (Simon *et al.* 1991; Morrison *et al.* 1993) and defects in mismatch correction have been observed in several forms of cancer (Marx 1994; Modrich 1994). Although a significant volume of information is available on such mutators in prokaryotes, little is known about these activities in eukaryotes. Presently, investigations of the DNA replication fidelity processes in eukaryotes have relied on the use of *in vitro* systems which may not accurately reflect *in vivo* processes. There is limited amount of information available on the *in vivo* specificity of proofreading in yeast, but, none for any other eukaryotic organisms. The data available are much too sparce to make any meaningful statistical comparisons (Morrison *et al.* 1993; Morrison and Sugino 1994). In this study, I have begun to characterize the specificity of proofreading and mismatch correction in yeast by DNA sequencing the spontaneous mutations occurring in a reporter gene (yeast *SUP4*-o gene) in the genetic background of the proofreading (*pol3* and *pol2*) and mismatch correction (*pms1*Δ) mutators. The *pol2* mutator could not be characterized extensively due to the incompatibility of the mutator with the system used to assay its specificity. In *E. coli*, defects in proofreading have been shown to cause a deficiency in the mismatch correction due to its saturation with the excessive amounts of errors caused by the proofreading defects (Damagnez *et al.* 1989). In order to determine if that was the case in yeast, I have assessed the efficiency of mismatch correction in a proofreading deficient genetic

background. I have also determined the effect of strand identity and sequence context on the proofreading and mismatch correction mutators in an attempt to gain some information on the identity of the yeast replication machinary(s).

In order to characterize the mutations arising in the *SUP4*-o gene, I have used a yeast system developed in our laboratory. Although the development of the system has been published (Pierce *et al.* 1987), a brief description is provided here for the benefit of the reader. The system essentially comprises a haploid yeast genome with suppressible markers on the chromosome and harboring a *SUP4*-o gene on an centromeric plasmid. The *SUP4*-o gene is a mutant tyrosine tRNA gene. The gene has been altered such that the anticodon on the tRNA has been converted from the wild type $^{5'}GUA^{3'} \rightarrow {}^{5'}UUA^{3'}$ This essentially converts the tRNA to an ochre suppresser as it would now recognize an ochre stop codon $^{5'}UAA^{3'}$ instead of its usual tyrosine codon $^{5'}UAC^{3'}$ or $^{5'}UAU^{3'}$ by wobble. The wild type genetic background (MKP-o) used in experiments contains three suppressible markers, *can1-100*, *ade2-1* and *lys2-1*. These three markers carry mutations that result in the insertion of an ochre codon in the mRNA. The *CAN1* gene encodes the enzyme arginine permease which functions in the uptake of arginine (2-amino-5-guanidinoraleric acid [$HN{=}CNH_2\text{-}NH\text{-}\mathbf{CH_2}\text{-}CH_2\text{-}CH_2\text{-}CHNH_2\text{-}COOH$]) from the medium. This enzyme also facilitates the uptake of canavanine (2-amino-4-guanidinooxy-butyric acid [$HN{=}CNH_2\text{-}NH\text{-}\mathbf{O}\text{-}CH_2\text{-}CH_2\text{-}CHNH_2\text{-}COOH$]), which is an arginine analog. Canavanine is toxic to the cell and its uptake results in cell death, usually after two to three cell doublings. *CAN1* cells are therefore sensitive to canavanine. The *ADE2* gene encodes the enzyme P-ribosyl amino imidazole carboxylase which is involved in the

multi enzyme adenine biosynthesis pathway. This enzyme is involved in the carboxylation of P-ribosylamino imidazole to P-ribosylamino imidazole carboxylate. Hence, *ade2* mutants have a block in the adenine biosynthesis pathway and as a result accumulate large amounts of P-ribosylamino imidazole which undergoes oxidation readily to form a red pigment. Therefore, *ade2* mutants are adenine auxotrophs and red in color. The *LYS2* gene encodes the enzyme α–amino adipate reductase which is one of the several enzymes involved in the biosynthesis of lysine. Therefore, *lys2* mutants are lysine auxotrophs.

In the absence of suppression, MKP-o cells are CAN^R, ade^- and red, and lys^-. In the presence of the *SUP4*-o gene, they are CAN^S, ade+ and white, and lys+. Spontaneous *SUP4*-o mutants are selected by plating cells on appropriate medium containing canavanine and picking off red colonies. These are then tested for lysine auxotrophy and the CAN^R , red and lys^- colonies are designated *SUP4*-o mutants. To determine the sequence alteration in the *SUP4*-o gene, the plasmid is rescued by isolating total yeast DNA, transforming in to *E. coli* and selecting for cells that harbor the plasmid. The plasmid in amplified in *E. coli*, isolated and the *SUP4*-o gene is sequenced on the plasmid.

2 Materials and methods

2.1 Chemicals and Media Components

All chemicals were analytical reagent grade and were obtained commercially from Fisher Scientific, Mallinckrodt, Sigma, or Bethesda Research Laboratories (BRL). Media components such as yeast extract, tryptone, peptone, yeast nitrogen base without (w/o) amino acids, and agar were purchased from CanLab.

2.2 Bacterial and Yeast Strains

The complete genotypes of all strains used in this study are given in Table 2. The *Escherichia coli* strains JF1754 and KC8 were used to recover plasmid DNA from yeast cells. *E. coli* JF1754^{+} and NR3837 were used to produce single-stranded plasmid DNA. MKP-o, a haploid wild type strain of the yeast *Saccharomyces cerevisiae* has been described (Pierce *et al.* 1987). Isogenic derivatives of MKP-o having mutations in the exonucleolytic domain of *POL3* (SMpol3, kindly provided by M. Simon) or *POL2* (KRpol2), or the *PMS1* gene deleted (KRpms1), were also used. The construction of the mutant strains is described in the relavent results sections. To study the effect of gene orientation on spontaneous mutation in the *SUP4*-o gene, MKP-o, SMpol3, KRpol2 and KRpms1were transformed with plasmids, YCpKR-Y or YCpMS-I to make WT-Y and WT-I, pol3-Y and pol3-I, pol2-Y and pol2-I and pms1-Y and pms1-I, respectively.

Table 2. Strains Used in the Study

Strain	Genotype	Reference
MKP-o	*MATα, can1-100, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-Δ200, trp1Δ901*	Pierce *et al.* (1987)
KRCMKP-o	as for MKP-o but *CAN1*	This Study
SMpol3	as for MKP-o but *pol3-4DA* exonuclease-deficient (on YCp50-4DA)	M. Simon
KRpol3	as for MKP-o but *pol3-4DA* exonuclease-deficient (on YCpKR-4DA)	This Study
KRpol2	as for MKP-o but *pol2* exonuclease-deficient	This Study
KRCpol2	as for KRpol2 but *CAN1*	This Study
KRpms1	as for MKP-o but *pms1Δ*	This Study
WT-Y	MKP-o transformed with YCpKR-Y*	This Study
WT-I	MKP-o transformed with YCpMS-I*	This Study
pol3-Y	SMpol3 transformed with YCpKR-Y*	This Study
pol3-I	SMpol3 transformed with YCpMS-I*	This Study
pol2-Y	KR1pol2 transformed with YCpKR-Y*	This Study
pol2-I	KR1pol2 transformed with YCpMS-I*	This Study
pms1-Y	KRpms1 transformed with YCpKR-Y*	This Study
pms1-I	KRpms1 transformed with YCpMS-I*	This Study
JF1754	*Δlac, gal, metB, leuB, hisB436, hsdR*	Pierce *et al.* (1987)
NR3837	*F' pro-lac/ara, Δpro-lac, thi, trpE9777, dam*	B.W. Glickman
JF1754+	as for JF1754 but F'	Pierce *et al.* (1987)
KC8	*hsdR17, hisB436, leuB6, pyrF::Tn5 Km^R, trpC9830Δ(lacZYA), strA*	D. Gietz

* see table 3 for description of transformed plasmids.

2.3 Plasmids

A list of all the plasmids used in this study is provided in Table 3. YCpMS-Y and YCpMS-I (Figure 1) are pFL59+ derivative (Bonnaeud *et al.*, 1991), hybrid, yeast-bacterial shuttle vectors which can replicate autonomously in yeast and in *E. coli*. The vectors contain a replication origin (*ARS1*), a centromere sequence (*CEN6*) and a selectable marker (*HIS3*) from yeast and the replication origin (*REP*) and the ampicillin resistance determinant (AMP^R) i.e., the β-lactamase gene (*bla*), from the bacterial plasmid pBR322. The *CEN6* sequence allows YCpMS-Y and YCpMS-I to be maintained predominantly at a copy number of one in haploid yeast cells (Newlon 1988). In addition, both vectors carry the *SUP4*-o gene, an ochre suppressor allele of the yeast tyrosine tRNA gene. The replication origin (*M13 Ori*) 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 these vectors. The orientation of the *SUP4*-o gene on YCpMS-Y is such that the *SUP4*-o gene is transcribed counterclockwise relative to *M13 ori*. In YCpMS-I, the orientation of the *SUP4*-o gene is reversed relative to that in YCpMS-Y (hence the designation "I" for inverse orientation). These plasmids were provided by Dr. Michael Simon (Institut Curie, Cedex, France). Upon testing these two plasmids, I found that YCpMS-Y behaved abnormally i.e., the suppression was very unstable even in the wild type background. I attributed this to a defective *SUP4*-o gene on the plasmid. So I constructed YCpKR-Y (Figure 1) by replacing the *Hind* III- *Sal* I, M13-*SUP4*-o, fragment in YCpMS-Y with that from YCpJA1 (Figure 2). The new plasmid

Table 3. Plasmids Used in the Study

Plasmid	Genotype	Reference
YCpMP2	*ARS, CEN4, URA3, SUP4*-o *(Y), bla, REP, M13Ori*	Pierce *et al.* (1987)
YCpJA1	*ARS, CEN4, URA3, SUP4*-o *(I), bla, REP, M13Ori*	J. Armstrong
YCpMS-Y	*ARS, CEN6, HIS3, SUP4*-o *(Y), bla, REP, M13Ori*	M. Simon
YCpMS-I	*ARS, CEN6, HIS3, SUP4*-o *(I), bla, REP, M13Ori*	M. Simon
YCpKR-Y	*ARS, CEN6, HIS3, SUP4*-o *(Y), bla, REP, M13Ori*	This Study
YCpLK3EB	*ARS, CEN4, URA3, SUP4*-o *(Y), bla, REP, M13Ori*	L. Kohalmi
YCp50-4DA	*ARS, CEN4, URA3, pol3-4DA, bla, REP*	This Study
YCpKR-4DA	*ARS, CEN6, HIS3, pol3-4DA, bla, REP*	This Study
YIpJB1	*pol2* replacement vector	Morrison *et al.* (1991)
pWBK4	*pms1* disruption vector	Kramer *et al.* (1989b)

Y- *SUP4*-o transcription direction is counter clock wise; I- *SUP4*-o transcription direction is clock wise

termed YCpKR-Y was consistent in behaviour with YCpMS-I and was used in the study in the place of YCpMS-Y.

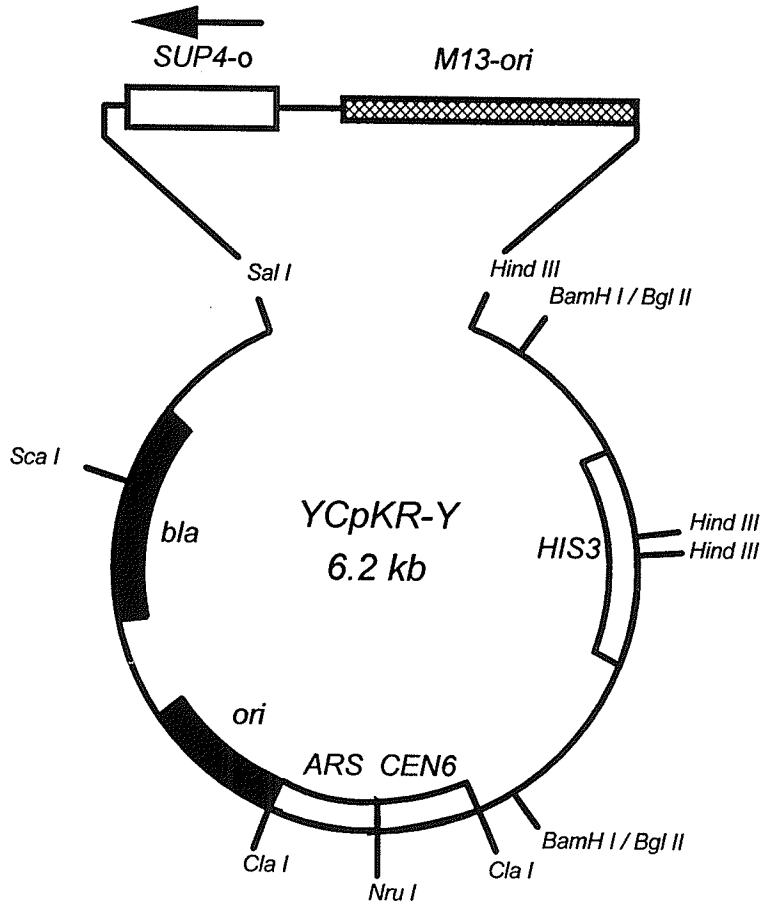
YCpMP2 (Figure 2) (Pierce *et al.* 1987) is similar to YCpKR-Y. The major differences are that this vector carries the *CEN4* centromere sequence and the selectable marker (*URA3*). YCpJA1 (Figure 2) (Armstrong and Kunz 1995) is almost identical to YCpMP2 but has *SUP4*-o in the I-orientation.

YCpLK3EB is a YCpJA1 derivative constructed by L. Kohalmi using site-specific mutagenesis to eliminate all of the *Bam* HI and *Eco* RI sites in the vector except those immediately flanking the yeast DNA insert carrying the *SUP4*-o gene.

Other plasmids were used for construction of yeast strains and included YIpJB1 (Morrison *et al.*, 1991), pWBK4 (Kramer *et al.* 1989b), YIpLac211-CAN (Roche *et al.* 1995), YCp50-4DA (Simon *et al.*, 1991), and YCpKR-4DA (Yang *et al.*, 1996). Additional details of these plasmids are provided in the relevant results section.

Figure 1. Plasmids YCpKR-Y and YCpMS-I

The arrows correspond to the direction of transcription of the *SUP4*-o gene.


SUP4-o
M13-ori
Sal I
Hind III
BamH I / Bgl II
Sca I
bla
YCpKR-Y
6.2 kb
HIS3
Hind III
Hind III
ori
ARS CEN6
BamH I / Bgl II
Cla I
Nru I
Cla I

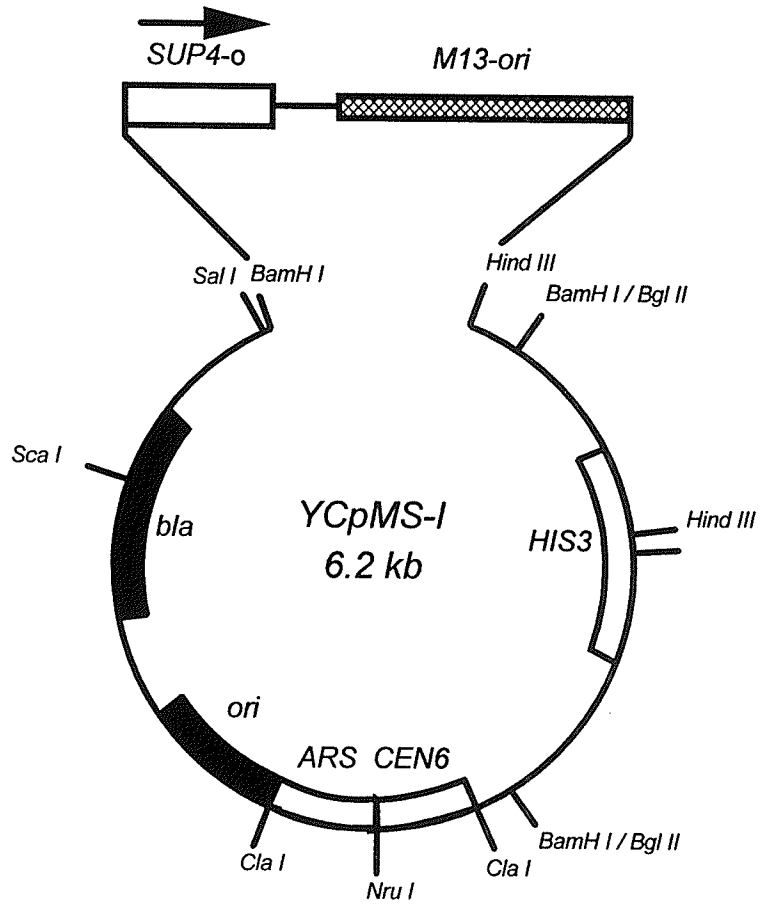
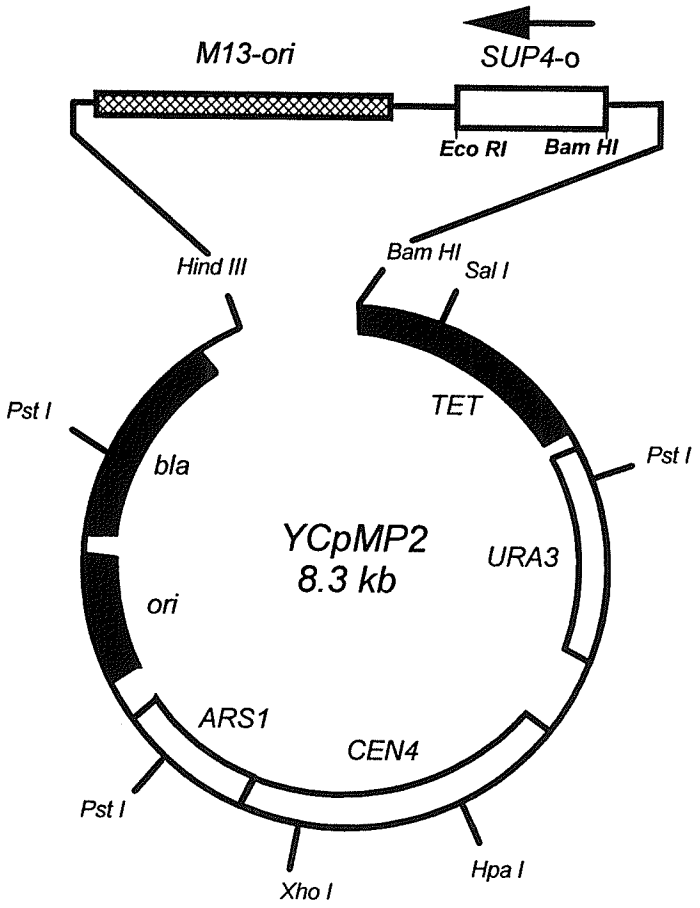
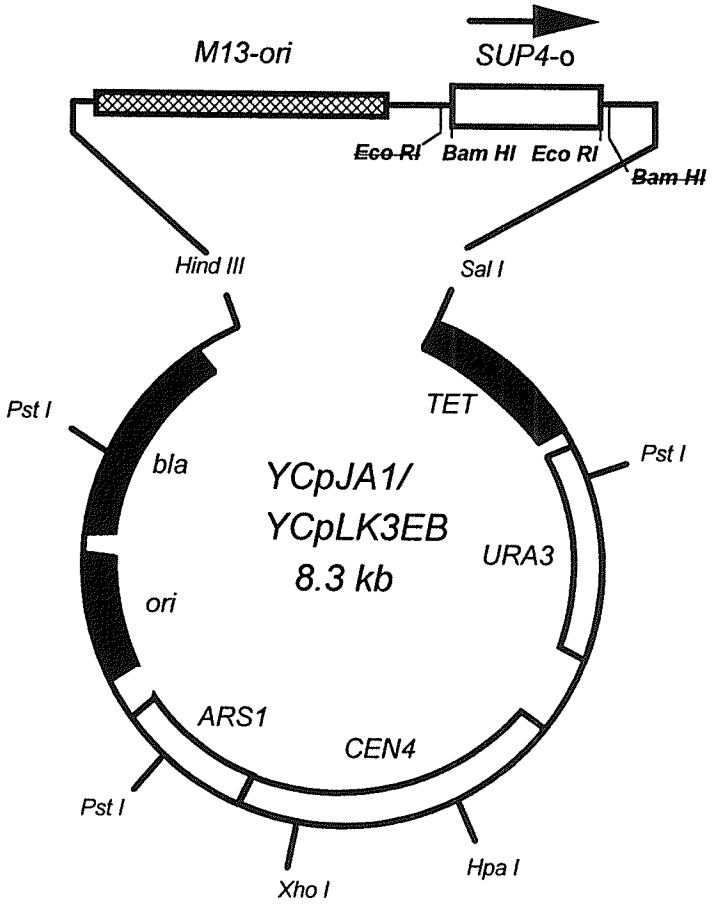

SUP4-o
M13-ori
Sal I BamH I
Hind III
BamH I / Bgl II
Sca I
bla
YCpMS-I
6.2 kb
HIS3
Hind III
ori
ARS CEN6
BamH I / Bgl II
Cla I
Nru I
Cla I

Figure 2. Plasmids YCpMP2 and YCpLK3EB/YCpJA1

Plasmids YCpJA1 and YCpLK3EB are identical except for two restriction enzyme sites. YCpLK3EB was constructed by removing the *Eco* RI and *Bam* HI sites of YCpJA1 (striked out in the figure). The arrows correspond to the direction of transcription of the *SUP4*-o gene.


M13-ori
SUP4-o
Eco RI
Bam HI
Hind III
Bam HI
Sal I
Pst I
TET
Pst I
bla
YCpMP2
8.3 kb
URA3
ori
ARS1
CEN4
Pst I
Xho I
Hpa I


M13-ori
SUP4-o
Eco RI
Bam HI
Eco RI
Bam HI
Hind III
Sal I
Pst I
TET
Pst I
bla
YCpJA1/
YCpLK3EB
8.3 kb
URA3
ori
ARS1
CEN4
Pst I
Xho I
Hpa I

2.4 Media

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

per litre: 40 g Dextrose

6.7 g Bacto yeast nitrogen base w/o amino acids (Difco)

Required amino acids (AA) 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 usual concentration. SD medium supplemented with lysine (lys), adenine (ade), leucine (leu), tryptophan (trp), uracil (ura) and histidine (his) is required by the wild type MKP-o strain and was designated SD+All medium. Omission medium lacking one or more AA were designated SD-"appropriate AA" medium. When canavanine (60 mg/ml) was added to the medium, it was designated +CAN medium.

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

per litre: 20 g Dextrose

20 g Bacto peptone (Difco)

10 g Bacto yeast extract (Difco)

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

C. YT (Miller 1972):

per litre: 8 g Bacto tryptone

5 g Bacto yeast extract

5 g sodium chloride

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

D. M9 (Miller 1972):

per litre: 500 mg thiamine

500 mg sodium chloride

246 mg magnesium sulphate

200 mg glucose

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 to make M9 +amp.

E. KC:

M9 medium supplemented with leucine, uracil, tryptophan, methionine and ampicillin and was designated KC medium.

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

per litre: 16.7 g Bacto agar

1.7 g Bacto yeast nitrogen base w/o amino acids

5 g $(NH_4)_2SO_4$

1 g 5-fluoro-orotic acid (FOA) (Sigma)

20 g glucose

Required amino acids were added at the concentrations suggested by Sherman *et al.* (1983). Bacto yeast nitrogen base w/o amino acids, $(NH_4)_2SO_4$, FOA, glucose and all required amino acids were added in a total volume of 500 ml, heated to 60° C to

dissolve and filter-sterilised (Nalgene™ Disposable Filterware, 115 ml). Simultaneously, 16.7 g agar in 500 ml H_2O was autoclaved and then cooled to 60° C. The two solutions were mixed just prior to pouring the plates.

F. Solid media: 20 g/l Bacto agar was added, except for FOA medium where only 16.7 g was added.

2.5 Detection of Mutation

2.5.1 Detection of *SUP4*-o Forward Mutation

The *SUP*-o system used in this study has been described (Pierce *et al.* 1987). Briefly, forward mutations in the *SUP4*-o gene were detected by scoring for reduced suppression of three ochre markers. The haploid yeast strains used in this study carry three ochre alleles which confer either resistance to the arginine analogue 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 a plasmid carrying *SUP4*-o are canavanine-sensitive and form white, lysine-independent colonies. Mutants lacking suppressor activity were selected as canavanine-resistant (CAN^R), red or pink colonies unable to grow when replicated to medium lacking lysine (Lys- colonies). Selection for reduced suppression of all three ochre markers detects at least a 30% decrease in the production of functional suppressor tRNA (Wang and Hopper 1988), and is unlikely to bias mutant recovery significantly for several reasons. First, mutations that prevent suppression of only two of the ochre mutations are rare (<1% of mutations analysed) at the

chromosomal *SUP4*-o locus (Kurjan and Hall 1982). Second, sequence alterations have not been detected in the plasmid copy of *SUP4*-o from mutants isolated using less stringent selection methods (Kunz *et al.* 1987). Third, 178 different substitutions can be recovered at 68 of the 75 exon sites and at 2 of the 14 intron positions in the gene, and a wide range of mutational classes has been identified, using the selection protocol employed here (Kunz *et al.* 1990; Kohalmi and Kunz 1992).

2.5.2 Detection of *lys2-1* and *ade2-1* Reversion and Forward Mutation at *CAN1*

Reversion of *lys2-1* was detected by selecting for lysine prototrophs (Lys^+). Since all of the strains used in this study also carry the *ade2-1* allele, *lys2-1* intragenic locus reversion results in the emergence of red, Lys^+ colonies whereas extragenic ochre suppression of *lys2-1* gives rise to white, Lys^+ colonies. Reversion of ade*2-1* was detected by selecting for adenine prototrophs (Ade^+), but *ade2-1* revertants were not categorised into locus revertants and suppressor mutations, only total reversion was measured. Forward mutation at the *CAN1* locus was detected by selecting for canavanine resistance.

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

To isolate spontaneous *SUP4*-o mutants, yeast strains were grown from low titre inocula (33 cells/ml) to stationary phase (ca. 1-2 x 10^7 cells/ml, as determined by a Coulter counter) in medium selective for the plasmid(s) (SD-His for wild type, *pol2* and *pms1* strains or SD-Ura-His for *pol3* strains). Cell suspensions were diluted and plated on the

same medium to determine plasmid retention and on SD+All plates to measure viability. To select for red, canavanine-resistant colonies, the cell suspensions were plated on plasmid selection medium containing 60 mg/ml canavanine. The plates were scored after 6 days incubation at 30° C. Red colonies that emerged on canavanine-containing medium were transferred to plasmid selection plates, grown for 2-3 days at 30° C, and replicated to plasmid selection medium lacking lysine, and the plates were then incubated at 30° C for 2-3 days. Lysine auxotrophs were scored as *SUP4*-o mutants.

To isolate *lys2-1* revertant colonies, yeast strains were inoculated (33 cells/ ml) in SD+All medium and grown at 30° C with shaking to stationary phase (ca. 1-2 x 10^7 cells/ml, as determined by a Coulter counter). Cell suspensions (25 ml) were centrifuged (3,020 x g, 5 min, 4° C) to pellet the cells which were then resuspended in 2.5 ml sterile water. These cell suspensions were diluted when necessary and plated SD+All and SD-Lys medium to assess viability and select Lys^+ colonies, respectively.

To isolate *ade2-1* reverent colonies, yeast strains were inoculated (33 cells/ ml) in supplemented minimal medium and grown at 30° C with shaking to stationary phase (ca. 1-2 X 10^7 cells/ml, as determined by a Coulter counter). Cell suspensions (25 ml) were centrifuged (3,020 x g, 5 min, 4° C) to pellet the cells which were then resuspended in 2.5 ml sterile water. These cell suspensions were diluted when necessary and plated SD+All and SD-Ade medium to assess viability and select Ade^+ colonies, respectively.

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

A. Plasmid Retention:

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

Where, P = plasmid retention

N_- = number of cells able to grow plasmid selection medium

N_+ = number of cells able to grow on SD+All

B. Mutation Frequency:

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

Where, f = mutation frequency

N = number of mutants

N_c = number of viable cells plated to select mutants

C. Mutation Rate

(Drake 1991):

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

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

C = reciprocal of efficiency of base-pair substitution (BPS) detection, calculated as {no. of non-BPS detected + [no. of

BPS detected × (no. of possible BPS / no. of detectable BPS)]} / no. of mutations sequenced [of 267 possible BPS in *SUP4*-o, 178 are detectable with the genetic screen used in this study (Kohalmi and Kunz 1992)].

f_m = the median mutation frequency

N_m = the median population size at the time of sampling including residual growth on the plates (3 generations for all yeast strains on canavanine-containing medium, determined by microscopic inspection)

2.7 DNA Isolation

2.7.1 Large Scale Yeast DNA Preparation

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

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

TE* buffer: 50 mM Tris, pH 7.4
20 mM Na_2EDTA, pH 7.4

TE 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 ml) were stored at -20° C.

Potassium acetate: 5 M potassium acetate was dissolved in double-distilled H_2O.

Total yeast DNA used for hybridisation was isolated by a modification of a procedure described by Sherman *et al.* (1983). Yeast was grown in 12 ml YPDA to stationary phase (ca. 1-2 x 10^8 cells/ml, as determined by a Coulter counter) and the cell suspension was centrifuged (3,020 x g, 5 min, 4° C) to pellet the cells. The supernatant was aspirated and the cells were resuspended in 500 µl of SCE buffer and transferred to a microfuge tube. The cells were pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 1 min, room temperature), washed with 500 µl SCE buffer, resuspended in 300 µl zymolyase, and then the cell suspension was incubated at 37° C for 1 h. The resulting spheroplasts were pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 10 s, room temperature) and resuspended in 357 µl TE* buffer. Sodium dodecyl sulphate (SDS, 36 µl,

10%, w/v) was added, and the contents were mixed gently by inversion and incubated for 30 min at 65° C. Potassium acetate (107 µl, 5 M) was added and the suspension was chilled for 15 min on ice. The precipitate was pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 15 min, 4° C), the supernatant transferred to a fresh tube and 1 µl of ice-cold ethanol (95%) added. The precipitated nucleic acids were pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 5 s, room temperature) and the pellet dried and dissolved in 400 µl TE buffer. RNase (6 µl) was added and the suspension was incubated for 30 min at 37° C. Then, 400 µl isopropanol (room temperature) was added, the solution mixed by inversion and the DNA pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 1 min, room temperature). The DNA pellet was washed with 70% ice-cold ethanol, dried and dissolved in 80 µl TE buffer and stored at -20° C.

2.7.2 Glass-Bead Prep for Yeast DNA Isolation

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

STET solution:

0 8% (w/v) sucrose

50 mM Tris, pH 8.0

50 mM Na_2EDTA, pH 8.0

5% (v/v) Triton X-100

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

TE buffer: see section 2.7.1

The glass-bead technique (modified from Robzyk and Kassir 1992) was used for fast processing of a larger number of samples for bacterial transformation. Putative *SUP4*-o mutants were grown to stationary phase at 30° C with shaking in 1.5 ml uracil omission medium. Next, the cell cultures were transferred to a microfuge tube and the cells were pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 1 min, room temperature). The cells were resuspended in 200 µl STET solution and 200 mg acid-washed glass beads was added to the tube which was mixed vigorously for 5 min using a vortex mixer. The tube was then incubated briefly on ice and the beads and cellular debris pelleted by centrifugation in a microfuge (Brinkman 5415C, 12,000 x g, 10 min, 4° C). Then, 100 µl of the supernatant was transferred to a tube containing 50 µl ammonium acetate, mixed well, and the tube was incubated on ice for 30 min. After the mixture was centrifuged (Brinkman 5415C, 12,000 x g, 10 min, 4° C), 100 µl of the supernatant was transferred to a fresh tube and the nucleic acids were precipitated by adding 200 µl of 95% ice-cold ethanol. The tube was then centrifuged (Brinkman 5415C, 12,000 x g, 10 min, 4° C) to pellet the DNA, the supernatant aspirated, and the DNA pellet was washed with 70% ethanol, dried by aspiration and dissolved in 20 µl TE buffer. DNA samples were stored at -20° C.

2.7.3 Rapid Alkaline Procedure for Plasmid DNA Isolation

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

To isolate a large number of plasmid DNA samples for DNA sequencing, a procedure modified from Morelle (1989) was used. Bacteria were grown overnight in 5 ml YT + amp at 37° C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 1 ml of GTE buffer and transferred to a microfuge tube. Cells were pelleted (Brinkman 5415C, 12,000 x g, 30 s, room temperature), the supernatant was removed by aspiration, and the cells were resuspended in 190 µl GTE buffer. Next, the tube was transferred to ice, 400 µl NaOH/SDS was added drop-wise and

the mixture was incubated on ice for 5 min. Ammonium acetate (300 µl) was added drop-wise and the mixture was incubated on ice for 10 min. The precipitate was pelleted by centrifugation (Brinkman 5415C, 12000 x g, 15 min, ^{4}o C) 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 incubated at room temperature for 10 min. Following centrifugation (Brinkman 5415C, 12,000 x g, 2 min, room temperature), the nucleic acid pellet was washed with 70% ice-cold ethanol, dried by aspiration and dissolved in 50 ml TE buffer. DNA samples were stored at -20° C.

2.7.4 Large Scale Preparation of Plasmid DNA

STE buffer:	100 mM NaCl 10 mM Tris HCl, pH 8.0 1 mM Na_2EDTA, pH 8.0
GTE buffer:	see section 2.7.3
Lysozyme:	Lysozyme (Boehringer Mannheim) (10 mg/ml) was dissolved in GTE buffer.
NaOH/SDS:	see section 2.7.3

Potassium acetate:	3 M potassium acetate was adjusted to pH 4.8 with glacial acetic acid.
Ammonium acetate	10 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65° C to achieve a pH of 7.8.
PEG:	13% (w/v) polyethylene $glycol_{8000}$ (Sigma) was dissolved in 1.6 M sodium chloride solution and filter-sterilised just prior to use.
TE buffer:	see section 2.7.1
RNase:	see section 2.7.1

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

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

one-fourth the volume of ammonium acetate and twice the volume of 95% ice-cold ethanol were added, the contents were mixed by inversion and the precipitate was pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 5 min, 4° C). The pellet was washed with 1 ml 70% ice-cold ethanol, dried by aspiration and dissolved in 500 µl TE buffer. DNA samples were stored at -20° C.

2.7.5 Preparation of Bacterial RNA

STE buffer:	See section 2.7.4
NaOH/SDS:	See section 2.7.3
Potassium acetate:	See section 2.7.4

Bacterial nucleic acid (primarily RNA), which was used for yeast transformation as a plasmid carrier, was prepared by modifying the latter half of the previous protocol (2.7.4), beginning after the first isopropanol precipitation and wash with 70% ethanol. JF1754 was used for RNA extraction. Following the wash, the pellet was dried, dissolved in 6 ml TE buffer and transferred by pipette to a 30 ml sterile Corex tube (Corex no. 8433). The nucleic acids were then extracted once with 6 ml TE-saturated phenol, once with 6 ml TE-saturated phenol:chloroform (1:1) and once with 6 ml TE-saturated chloroform. After each extraction, the samples were centrifuged (6,780 x g, 10 min, 4° C) and approximately 5 ml of the top aqueous layer was transferred to a sterile Corex tube. Next, 6 ml of isopropanol was added to precipitate the nucleic acids, the contents of the tube were mixed well by inversion and the tube was allowed to stand at room temperature for 10 min. After incubation, the nucleic

acids were pelleted by centrifugation (12,100 x g, 15 min, 4° C), washed with 1 ml ice-cold 70% ethanol, dried by aspiration, dissolved in 200 ml TE buffer, and then stored at -20° C.

2.8 Preparation of Nicked Heteroduplex Plasmid DNA

Heteroduplex DNA used in the mismatch correction experiments was prepared by a process involving denaturation followed by slow renaturation of a mixture of circular single-stranded and linear double-stranded DNA.

2.8.1 Preparation of Single-Stranded Plasmid DNA

A. Preparation of phage M13K07

The procedure described by Vieira and Messing (1987) was used. *E. coli* JF1754F$^+$ was grown overnight in 5 ml YT + tet at 37° C with shaking. Then, the overnight culture was diluted 5-fold by adding 1 ml of overnight culture to 4 ml YT + tet. The diluted JF1754F$^+$ cell suspension (200 µl) was mixed with 100 µl of phage M13K07 (10^7 - 10^8 pfu/ml), and incubated at room temperature for 20 min. An aliquot (50 µl) of the mixture was then plated onto a YT + tet + kan plate, and the plate was incubated at 37° C overnight. A single colony that emerged on the YT + tet + kan plate was picked, inoculated into 30 ml of 2x YT + tet + kan and the culture was grown for 24 h at 37° C with shaking. The cells were removed by centrifuging twice (17,300 x g, 15 min, 4° C), and the phage in the supernatant was used for the production of single-stranded plasmid DNA.

B. Production of single-stranded plasmid DNA

Formation of single-stranded plasmid DNA was induced by infection of NR3837 carrying YCpMP2, or YCpMP2 derivatives, with M13K07 helper phage according to the procedure of Vieira and Messing (1987). Plasmid-containing NR3837 cells were grown overnight in M9 + amp at 37° C with shaking. Then, 1 ml of overnight culture was transferred into 100 ml M9 + amp and incubated at 37° C with shaking (New Brunswick Gyrotory Water Bath Shaker G76, 220 rpm) for 30 min. The cells were then infected with 600 µl of freshly-prepared phage (7×10^{10} pfu) and incubated at a lower shaking speed (60 rpm) for 1 h. Next, 1 ml of kanamycin (7 mg/ml) was added and the culture was incubated at 37° C with shaking (220 rpm) for 24 h. The cells were removed by centrifugation (9,150 x g, 30 min, 4° C) and the supernatant was kept at 4° C for purification of single-stranded plasmid DNA.

C. Purification of single-stranded plasmid DNA

TES:	20 mM Tris, pH 7.5 10 mM NaCl 0.1 mM Na_2EDTA, pH 8.0
PEG:	20% (w/v) polyethylene $glycol_{8000}$ was dissolved in 2.5 M NaCl solution and filter-sterilised just prior to use.
Sodium acetate:	3 M sodium acetate was dissolved in glacial acetic acid while being heated to 65° C to achieve a pH of 4.8.

TE buffer: see section 2.7.1

Loening's buffer: 400 mM Tris

200 mM sodium acetate

10 mM Na_2EDTA

Adjusted to pH 8.0 with glacial acetic acid and stored at room temperature.

An aliquot (24 ml) of the supernatant containing the single-stranded plasmid DNA was transferred to a sterile Corex tube (Corex no. 8433), centrifuged (6,780 x g, 30 min, 4° C) and transferred to a fresh sterile Corex tube. PEG (4.2 ml) was added to the tube, the contents were mixed well and the tube was incubated at room temperature for 20 min before being centrifuged (6,780 x g, 30 min, 4° C). The supernatant was discarded, the pellet was dissolved in 250 µl TES buffer and transferred to a microfuge tube. Two lots of dissolved pellet (total = 500 µl) were transferred to each tube. The solution was extracted once with 500 µl TE-saturated phenol and once with 500 µl TE-saturated chloroform (1:24). For each extraction the contents of the tube were mixed vigorously for 30 s using a vortex mixer, the tube was centrifuged (Brinkman 5415C, 12,000 x g, 4 min, room temperature), the upper aqueous layer was transferred to a fresh microfuge tube, and 36 µl sodium acetate and 1 ml ice-cold 95% ethanol were added. The contents were mixed well and the tube was held at -20° C for 15 min. The DNA was pelleted by centrifugation (Brinkman 5415C, 12,000 x g,

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

2.8.2 Linearization of Plasmid DNA for Heteroduplex Construction

TE buffer:	see section 2.7.1
Digest mix:	500 µl plasmid DNA (100 mg DNA in TE buffer)
	58 µl 10x reaction buffer 2 from BRL
	20 µl *Xho*I (15 U/ml) 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,

12,000 x g, 15 min, 4° C), washed with 70% ice-cold ethanol, dried by aspiration, and dissolved in 60 ml TE buffer. The concentration of DNA was determined by A_{260} and the DNA was stored at -20° C.

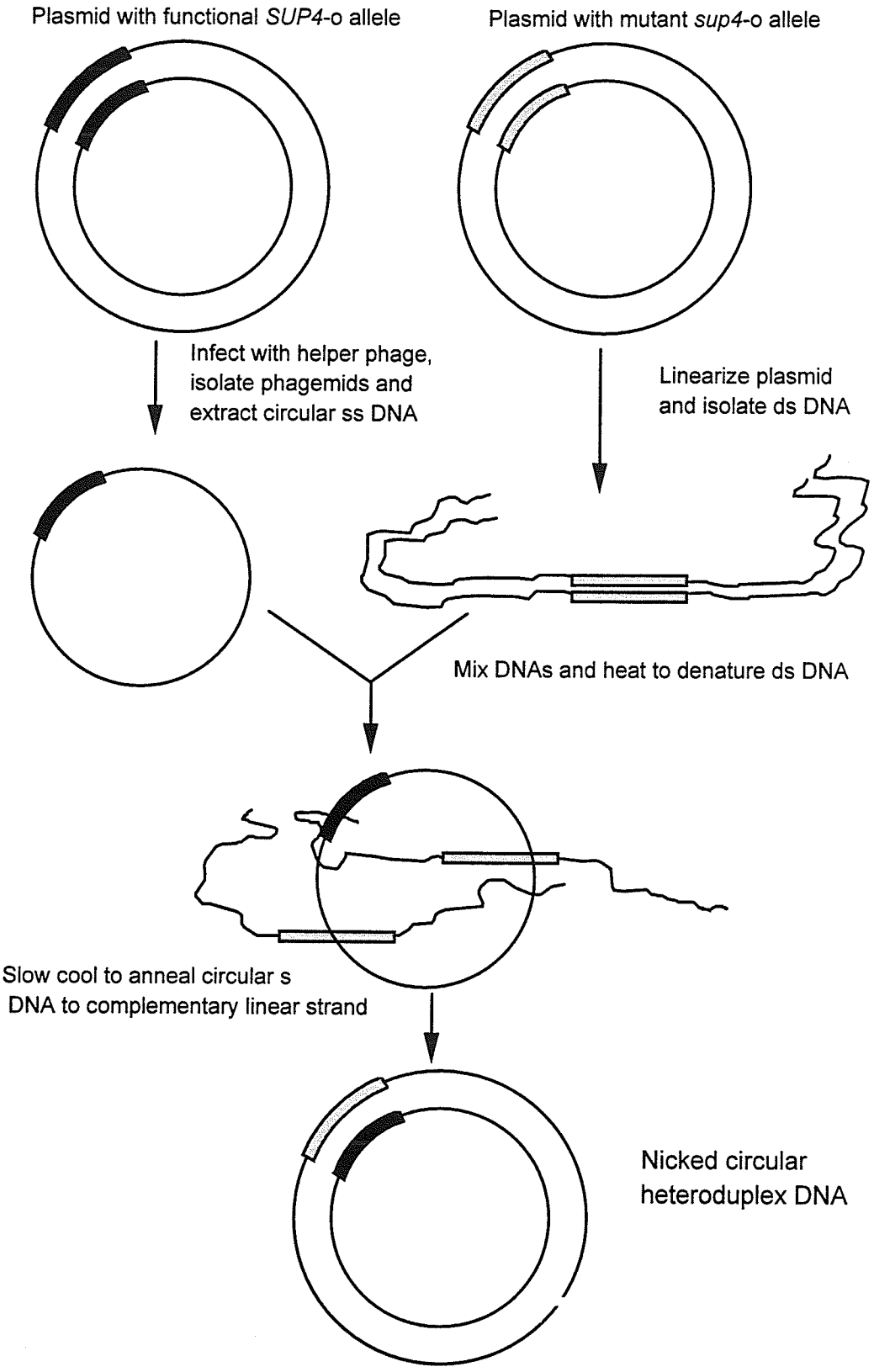
2.8.3 Construction and Purification of Nicked Heteroduplex Plasmid DNA

TE buffer:	see section 2.7.1
20x SSC	3 M NaCl 0.3 M sodium citrate Adjusted to pH 7.0 with 10 N NaOH.
Glycine-NaOH buffer:	1 M glycine was adjusted to pH 9.4 with NaOH and filter-sterilised.
Annealing mix:	30 μg linearized DNA in 48 ml TE buffer 60 μg single-stranded DNA in 80 ml TE buffer 92 μl 2.5x SSC buffer (diluted from 20x SSC buffer) 736 μl sterile double-distilled H_2O

10x Endonuclease V reaction buffer:	66.7 mM glycine-NaOH buffer, pH 9.4 5 mM $MgCl_2$ 8.3 mM 2-mercaptoethanol 0.5 mM ATP
Endonuclease V digest mix:	10 µl 10x endonuclease V reaction buffer 89 µl DNA in TE buffer (ca. 2.8 µg DNA) 1µl 3.5 U/µl *Micrococcus luteus* endonuclease V (United States Biochemical) Incubated at 37° C for 30 min.
Loening's buffer:	see section 2.8.1

Nicked heteroduplex plasmid DNA was constructed from combinations of linear double-stranded and circular single-stranded DNA by using a modification of a thermal denaturation and renaturation procedure (Kramer *et al.* 1989a). Linearized plasmid DNA (30 µg) was added to single-stranded circular DNA (ca. 60 µg of plasmid DNA plus M13K07 DNA) in a final volume of 956 µl SSC buffer. Aliquots (60 µl) of the mixture were dispensed into screw cap microfuge tubes. The tubes were incubated at 100° C for 4 min, transferred to a small 75° C bath, cooled slowly to 65° C, incubated at 65° C for 15

Figure 3. Construction of heteroduplex DNA


Plasmid with functional SUP4-o allele
Plasmid with mutant sup4-o allele
Infect with helper phage, isolate phagemids and extract circular ss DNA
Linearize plasmid and isolate ds DNA
Mix DNAs and heat to denature ds DNA
Slow cool to anneal circular s DNA to complementary linear strand
Nicked circular heteroduplex DNA

min, removed from the bath, and then incubated at room temperature for 30-45 min. The mixture was then separated by agarose gel (0.7% w/v in Loening's buffer) electrophoresis at 2 volt/cm overnight. Then the gel was stained with ethidium bromide solution (0.5 μg/ml) for 20 min, followed by rinsing with tap water for 10 min and two quick rinses with double-distilled H_2O. The position of the heteroduplex-containing band on the gel was visualized on a UV transilluminator. Ten heteroduplex-containing gel segments were excised and each piece was put into a separate microfuge tube. The heteroduplex DNA was recovered from the agarose by modification of a freeze-extraction procedure (Thuring *et al.* 1975). Each gel slice was frozen in a microfuge tube at -20° C overnight and the tube was centrifuged at maximum speed in a microfuge (Brinkman 5415C, 12,000 x g, 1.5 h, 4° C). The supernatant was then transferred to a sterile 6" glass tubes (Kimax) so that each tube contained about 4 ml of supernatant. Then, 2 volumes of butanol were added to remove ca. 50% of the H_2O from each tube, the tubes were inverted several times, and the aqueous layers were transferred to microfuge tubes, each tube receiving 500 μl. Following extraction of the supernatant with TE-saturated chloroform, and ethanol precipitation, the DNA was dissolved in TE buffer. Then, linear duplex DNA was eliminated from the heteroduplex preparation by digestion with *Micrococcus luteus* endonuclease V at 37° C for 30 min to 1 h. The remaining DNA was precipitated with 95% ice-cold ethanol, washed with 70% ice-cold ethanol, and dissolved in 50 μl TE buffer. Agarose gel electrophoresis (see section 2.8.1) of samples from these preparations indicated that the heteroduplex DNA was not detectably contaminated with single-stranded circular DNA or linearized DNA. Yields were determined by comparing the intensities of ethidium bromide-stained nicked heteroduplex

DNA bands with the intensities of DNA bands of known quantity. The nick and the mismatch sites were at least 4 kb apart.

2.9 Transformation Procedures

2.9.1 Bacterial Transformation

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

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

Bacterial cells were transformed using a modification of the calcium chloride procedure of Pierce *et al.* (1987). *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 ml aliquot of the cell suspension was transferred to a sterile microfuge tube containing yeast DNA (10 ml) that was isolated previously. The tube was chilled on ice for 1 h, heated at 42° C for 2 min and then chilled on ice for 2 min, and 2x YT (200 ml) 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.

Since the *pol3* strains carried two plasmids (YCp50-4DA plus YCpKR-Y/ YCpMS-I), a modification of the transformation procedure was used in order to determine the identity of the rescued plasmid. *E. coli* strain KC8 was used instead of the usual JF1754. This strain carries the *hisB436* mutation that conferrs histidine auxotrophy. This mutation however, can be complemented by the yeast *HIS3* gene (Dr. Gietz, personal communications). This feature of the strain was used to distinguish between the *SUP4*-o plasmid (YCpKR-Y or YCpMS-I) carrying the *HIS3* gene and the *pol3* plasmid (YCp50-4DA). Essentially, the yeast DNA isolated from *SUP4*-o mutants was transformed into KC8. The cells were then plated on YT+AMP medium and incubated for 18 hrs. Positive transformants were transferred to YT+AMP plates and incubated over night. The next day, the plates were replicated on to histidine omission (KC) plates. Histidine prototrophs were designated as those carrying only the *SUP4*-o plasmid. Histidine auxotrophs were designated as those carrying only the *pol3* plasmid and discarded. A similar situation was hypothesized for transformation into *E. coli* cells and all histidine prototrophs were regarded to possess only the *SUP4*-o plasmid. Sequencing analysis performed subsequently showed that it was the case.

2.9.2 Yeast Transformation

TE buffer: see section 2.7.1

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

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

Yeast cells were transformed using the lithium acetate procedure described by Ito *et al.* (1983) as modified by Schiestl and Gietz (1989) and Gietz *et al.* (1992). YPDA or appropriate minimal medium (100 ml) was inoculated with yeast cells and the culture was incubated with shaking at 30° C overnight until the cell titre reached 1-3 x 10^7 cells/ml as determined by a Coulter counter. The culture was centrifuged (4,080 x g, 5 min, room temperature) to pellet the cells which were washed twice in 5 ml TE buffer and resuspended in 1 ml lithium acetate. The suspension was then incubated at room temperature for 5 min without shaking. For each transformation, a 100 µl aliquot of the cell suspension was transferred to a sterile 13 mm tube (Sarstedt). DNA to be transformed was added followed by bacterial RNA (20 µl, 3.4 mg/ml) (see section 2.7.5) as carrier for the DNA. (For gene disruptions or replacements, 1-2 mg of the isolated DNA fragment was used). To introduce plasmids into yeast cells, 0.1-0.3 mg plasmid DNA was used. To assay mismatch correction, 0.01-0.05 mg heteroduplex DNA was used.) PEG (440 ml) was then added and the tube was incubated for an additional 30 min without shaking at room temperature.

DMSO (final concentration: 10% v/v) was added to the tube, followed by heating at 42° C for 15 min. Cells were then pelleted by centrifugation (1,850 x g, 5 min, room temperature), and washed twice with, and resuspended in, 1 ml sterile double-distilled H_2O. Aliquots (0.2 ml) of the cell suspension, or 10-fold dilutions of the cell suspension, were plated on appropriately supplemented minimal medium to select transformants, and the plates were incubated at 30° C for 5 days. For transformation with heteroduplex DNA, the proportions of red, white and red/white sectored transformants that emerged were determined. For other transformations, selected transformants were purified by streaking onto the same type of medium used to select the transformants, and incubating the plates for 2-3 days at 30° C. The genotypes of the transformants were then characterised by testing for growth on appropriately supplemented medium.

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.

Primer: 0.1 A_{260} U/ml M13/pUC forward sequencing primer (BRL). This 17 bp primer has the sequence 5'-d(GTAAAACGA-CGGCCAGT)-3'. Stored at -20° C.

[^{32}P] dATP: 3000 Ci/mmol α-[^{32}P] dATP (DuPont). Stored at -60° C.

Klenow: 1 U/μl DNA polymerase I Klenow fragment from *E. coli* (BRL). Stored at -20° C.

Hin buffer: 240 mM dithiothreitol
60 mM Tris, pH 7.5
60 mM NaCl
60 mM $MgCl_2$
1 ml aliquots were stored at room temperature.

Termination mixes: All dNTPs and ddNTPs were purchased from Pharmacia. All four mixtures were stored at -20° C.

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

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

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

Stop buffer:

10 mM Na_2EDTA, pH 8.0

95% (v/v) deionized formamide

0.1% (w/v) xylene cyanol FF

0.1% (w/v) bromophenol blue

10x TEB buffer:

20 mM Na_2EDTA, pH 8.0

1 M Tris

0.9 M boric acid

Stored at room temperature.

Insta-gel:

7.6% (w/v) acrylamide

0.4% (w/v) bis-acrylamide

7 M urea

1x TEB buffer

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

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

A modification of the dideoxynucleotide chain termination sequencing procedure (Sanger *et al.* 1977) as described by Korneluk *et al.* (1985) was used. To prepare double-stranded plasmid DNA for sequencing, the DNA was first linearized by mixing 1.2 µl of 10x buffer 3, 1.0 µl RNase, 1.0 µl of primer, 8 µl plasmid DNA (0.5-1 µg) and 1.8 µl *Bam*HI in a screw cap microfuge tube and incubating the tube for 30 min at 37° C. The tube was heated at 100° C for 3 min and immediately transferred to ice-water and allowed to cool for 2 min before proceeding. Successively, 1 µl dithiothreitol (100 mM), 1 µl Klenow and 1 ml [^{32}P] dATP were added and the mixture was stirred with the pipetman tip. An aliquot (2 µl) of this mixture was added to the side of each of 4 microfuge tubes containing 2 µl of either the ddA, ddG, ddC, or ddT termination mixture and the tubes were centrifuged (Brinkman 5415C, 12,000 x g, 2 s, room temperature) to mix both solutions. These microfuge tubes were then transferred to a 46° C waterbath and incubated for 20 min, and the reactions were terminated by adding 2.4 µl stop buffer. Finally, the microfuge tubes were heated for 3 min at 100° C, transferred immediately to ice, and 2.5 µl of each reaction mixture was electrophoresed on a sequencing gel (electrolyte: 1x TEB buffer) at constant power, to heat the gel to 50° C, for about 3.5 h. The gel was then vacuum-dried at 80° C for

1 h and exposed to Kodak XAR-5 film at room temperature (the exposure time was usually overnight, but sometimes varied according to the radioactive intensity of the dried gel).

2.11 Preparation of DNA Fragments for Strain Construction

2.11.1 Restriction Digests

Enzyme digest:	1x reaction buffer (10x buffer supplied by BRL)
	1 μg DNA
	5 units of enzyme (BRL) per mg of DNA
Stop buffer:	50 mM Na_2EDTA
	50% (w/v) sucrose
	0.1% (w/v) bromophenol blue
	Stored at 4°C.
λ DNA:	*Hind*III digested λ DNA, 500 mg/ml (BRL). The DNA was stored at 4°C and heated at 65°C for 5 min before use.
Loening's buffer:	See section 2.8.1

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

terminate the reaction, and the resulting DNA fragments were separated by agarose gel electrophoresis (0.7% w/v agarose dissolved in Loening's buffer), for 18 h at 1 volt/cm. A λ DNA size standard accompanied the DNA samples each time. The gel was stained with ethidium bromide (0.5 mg/ml) and destained for 20 min in double-distilled H_2O. The DNA fragments in the gel were visualised on a 302 nm UVtransilluminator and photographed using black and white film in a polaroid camera.

2.11.2 Isolation of DNA Fragments

TE buffer	See section 2.7.1
Silanized glass-wool:	Glass-wool was silanized with dimethyldichlorosilane in a 500 ml glass beaker by shaking a few times and rinsing thoroughly with sterile double-distilled H_2O. The silanized glass-wool was stored at room temperature in sterile double-distilled H_2O.
Ammonium acetate:	See section 2.7.4

DNA fragments were isolated from agarose gels by a silanized glass-wool method (Heery *et al.,* 1990). The caps of two 1.5 ml microfuge tubes were removed. A small hole was made in the base of a 0.6 ml microfuge tube with a syringe needle. A small amount of

silanized glass wool was cut and packed in the bottom of the 0.6 ml tube (3-4 mm in depth), which was then placed inside the 1.5 ml tube. The 1.5 ml tube was centrifuged (Brinkman 5415C, 12,000 x g, 20 min, room temperature) to remove any excess dimethyldichlorosilane from the glass wool. After centrifugation, the 1.5 ml outer tube was discarded and replaced with a fresh tube. The band containing the DNA fragment was excised from the agarose gel and trimmed. The trimmed agarose slice was placed in the 0.6 ml tube, which was then placed inside the 1.5 ml tube, and the tubes were centrifuged (Brinkman 5415C, 12,000 x g, 20 min, room temperature) until all the liquid had been forced out of the agarose gel slice. After centrifugation, the 0.6 ml tube was discarded and the supernatant in the 1.5 ml outer tube was then transferred to a fresh 1.5 ml tube without transferring any of the pellet. One-third the volume of ammonium acetate and equal volumes of isopropanol were added to the eluate. The contents of the tube were mixed well by inversion and the tube was incubated at room temperature for 10 min. After incubation, the nucleic acids were pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 15 min, RT), washed with 0.5 ml ice-cold 70% ethanol, dried by aspiration, dissolved in 10-20 µl TE buffer, and then stored at -20°C.

2.12 Hybridization Analysis

2.12.1 Agarose Gel Electrophoresis

Enzyme digest: 1x reaction buffer 3 or 1x reaction buffer 10 (10x buffer supplied by BRL)

5 mg DNA

	50 units of high concentration restriction enzyme (BRL) per μg of DNA
Stop buffer:	See section 2.11.1
λ DNA:	See section 2.11.1
Loening's buffer:	See section 2.8.1

Total yeast DNA (5 mg) was digested with the appropriate restriction enzyme overnight, Stop buffer (1/4 of the total reaction volume) was added to terminate the reaction and the resulting DNA fragments were separated by agarose (0.7% w/v, dissolved in Loening's buffer containing 0.5 mg/ml ethidium bromide) gel electrophoresis, for 18 h at 1 volt/cm (a l DNA standard was included). A picture of the gel was taken on polaroid film using a 302 nm UV transilluminator for later sizing of the bands, and the gel was soaked for 45 min in 0.5 M NaOH and then for 45 min in 1 M Tris (pH 8.0) at room temperature with slow shaking. Next, the gel was sandwiched between 3MM 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 between sheets of paper in a thick bound book.

2.12.2 Random Primers DNA Labelling System

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

Random Primers Buffer Mixture:

670 mM HEPES

170 mM Tris, pH 7.2

17 mM $MgCl_2$

33 mM 2-mercaptoethanol

1.3 mg/ml bovine serum albumin

18 OD_{260} units/ml oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8

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

dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA

dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA

dTTP solution:	0.5 mM dTTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA
Reaction mix:	5 ml Random Primers Buffer Mixture 20 mM dCTP 20 mM dGTP 20 mM dTTP 50 mCi [^{32}P]dATP
Stop solution:	200 mM Na_2EDTA, pH 7.5
DNA polymerase:	6 U/µl *E. coli* DNA polymerase I Klenow fragment (BRL). Stored at -20°C.
Yeast tRNA:	Yeast tRNA (Boehringer Mannheim) was dissolved in sterile double-distilled H_2O (20 mg/ml) and stored at -20°C.
Sodium acetate:	See section 2.8.1

[^{32}P]-labelled probes for hybridization were prepared using the BRL Random Primers DNA Labelling kit according to a modification of the protocol supplied by the manufacturer. DNA (25 ng), dissolved in 5-20 µl of distilled water in a sterile screw cap

microfuge tube, was denatured by heating in a boiling water bath for 5 min and then immediately cooled the tube on ice. The reaction mixture was added to the tube on ice and the final volume of 49 µl was made by adding sterile double-distilled H_2O. Next, DNA polymerase (1 µl) was added and the tube was incubated at 25°C for 2 hours. The reaction was stopped by adding 5 µl stop buffer. Successively, 5 µl yeast tRNA, 7 µl $MgCl_2$ (100 mM), 7 µl sodium acetate and 200 µl ice-cold ethanol (95%) were added. The tube was then held at -60°C for 1 h and the precipitate was pelleted by centrifugation (Brinkman 5415C, 12,000 x g, 15 min, 4°C). The supernatant was removed with a Pasteur pipette. Then, the pellet was washed with 1 ml ice-cold ethanol (70%), dried and dissolved in 100 µl sterile double-distilled H_2O. The tube was heated at 100°C for 5 min and then rapidly transferred to ice-water.

2.12.3 Hybridization Procedure

20x SSC buffer: see section 2.8.3

Salmom sperm DNA: Salmon sperm DNA (Sigma) was dissolved in sterile double-distilled H_2O (10 mg/ml) and then passed through a needle (22 gauge) several times to shear the DNA. Aliquots were stored at -20°C.

50x Denhardt's solution: 10 mg/ml ficoll

10 mg/ml polyvinylpyrolidone

10 mg/ml bovine serum albumin (BRL)

Stored at -20°C.

Prehybridization solution: 6x SSC

5x Denhardt's solution

0.5% (w/v) SDS

0.2 mg/ml salmon sperm DNA

Prepared just prior to use and heated to 65°C.

Hybridization solution:	10 mM Na_2EDTA
	6x SSC
	5x Denhardt's solution
	0.5% (w/v) SDS
	Prepared just prior to use and heated to 65°C.
Solution I:	2x SSC
	0.5% (w/v) SDS
Solution II:	2x SSC
	0.1% (w/v) SDS
Solution III:	0.1x SSC
	0.5% (w/v) SDS

Direct DNA hybridization in agarose gels was carried out as described by Tsao *et al.*, (1983) and Maniatis *et al.*, (1989). The dried gel was floated paper-side down on 2x SSC for 30 s and gently peeled off from the paper backing. The gel was then rolled into a tight spiral, using a nylon mesh to prevent gel-gel overlap, and placed in a 35 mm x 150 mm glass hybridization bottle (BIO/CAN Scientific). Prehybridization solution (20 ml) was added and the bottle was sealed and incubated for 10 min at 65°C in a BIO/CAN Scientific Turbo Speed Rotary Hybridization Oven. After 10 min, the bottle top was opened to release

the pressure build-up, refastened, and the bottle was further incubated for 2-4 h at 65°C in the oven. The prehybridization solution was removed and replaced with 20 ml of hybridization solution, the labelled DNA probe was added, and the bottle was sealed and incubated overnight at 65°C. The gel was then removed carefully from the bottle and the following washes were performed: a. 50 ml of solution I for 5 min at room temperature (twice); b. 50 ml of solution II for 15 min at room temperature (twice); c. 50 ml of solution III for 2 h, 1 h and 0.5 h at 65°C. The gel was then placed on 3MM Chr Whatman Chromatography paper, air-dried, covered with a single layer of Saran Wrap and exposed to Kodak XAR-5 film with an intensifying screen at -60°C (the exposure time varied according to the radioactive intensity of the dried gel).

2.13 Mapping Replication Fork Movement

The movement of a plasmid DNA replication fork was mapped by 2-dimensional gel electrophoresis using a modification of a procedure described by Huberman *et al.* (1987).

Nuclear Isolation Buffer (NIB):

17%[v/v] glycerol

50 mM MOPS

150 mM potassium acetate

2 mM $MgCl_2$

500 μM spermidine

150 μM spermine

Adjusted to pH 7.2

TEN1: 50 mM Tris

50 mM Na_2EDTA

100 mM NaCl

Adjusted to pH 8.0

TEN2: 10 mM Tris

1 mM Na_2EDTA

200 mM NaCl

Adjusted to pH 8.0

TEN3: 10 mM Tris

1 mM Na_2EDTA

800 mM NaCl

Adjusted to pH 8.0

TE Buffer: See section 2.7.1

Adjusted to pH 8.0

BND cellulose: From Serva

Caffeine buffer:	10 mM Tris
	1 mM EDTA
	1 M NaCl
	1.8% caffeine
	Adjusted to pH 8.0
Hoechst 33258 dye:	5 mg/ml in sterile double distilled water
TAE buffer:	40 mM Tris-acetate
	2 mM Na_2EDTA
Alkaline electrophoresis solution:	
	40 mM NaOH
Enzymes	Proteinase K (Boehringer Mannheim)
	Nru I (Gibco BRL)

A. Cell Growth

The *S. cerevisiae* strain WT-Y containing the centromeric plasmid YCpKR-Y was inoculated into 20 ml SD-his to maintain selection for the plasmid and grown overnight at 30°C with shaking. Thirty two-liter flasks each containing one liter of SD-his medium were inoculated with 10^5 cells/ml from the overnight culture and grown at 30°C with shaking until the cell titre reached 5 x 10^6 cells/ml (determined using a Coulter counter).

The cells were then harvested by centrifugation in sterile 500 ml plastic centrifuge bottles (Sorval SR5, 8,500 x g, 10 min, 4° C).

B. DNA Purification

The harvested cells were washed twice with ice-cold TE buffer, and were resuspended in 7.5 ml of ice-cold NIB. The cell suspension was mixed with an equal volume of acid washed glass beads and was vigorously swirled for 30 sec using a vortex mixer and then cooled for 30 sec on ice. The procedure was carried out in a cold room, and the mixing and cooling steps were repeated a total of 50 times. The liquid phase and 5 NIB washes (1 ml each) of the glass beads were pooled together and centrifuged (Sorval SR5, 5,000 x g, 10 min, 4° C) to pellet cell ghosts and nuclei. The pellet was resuspended 7.5 ml of TEN1. Sarkosyl was added to a final concentration of 1.5% and Proteinase K was added to a final concentration of 300 μg/ml. The sample was incubated for 1 hr at 37°C, and then centrifuged (Sorval SR5, 5,000 x g, 10 min, 4° C) to pellet the cell ghosts. The pellet was resuspended in 6 ml of TEN1. CsCl was added at a concentration of 1.05 g/ml. followed by 0.025 volumes Hoechst 33258 dye. The resulting solution was transferred to a disposable ultracentrifuge tube and the tube was topped with mineral oil and heat-sealed. The tube was centrifuged in an ultracentrifuge (Beckman L3-50 using a 50T1 rotor 100,000 x g, 48 hrs. at 20°C).

The bright blue band (plus the slightly lower fainter band of rDNA) containing chromosomal and plasmid DNA was identified under 360 nm UV light. The bands were collected using a syringe, equipped with a 23 gauge needle, by piercing the side of the tube and then dialyzed overnight against TEN2 in a glass beaker at 4° C. The dialyzed

material was transferred to a corex tube and equal volumes of n-butanol was added. The Hoechst 33258 dye was removed by n-butanol extraction from the dialyzed material by covering the tube with parafilm and inverting once and then aspirating off the n-butanol. DNA was precipitated as a fibrous mass with two volumes of 95% ethanol. The DNA was picked up using forceps, rinsed in 70% ethanol, dried and dissolved in 1 ml of TE buffer. This procedure yields yeast chromosomal DNA plus plasmid DNA of high purity.

C. BND Cellulose Fractionation

Non-replicating or completely replicated DNA were separated from replicating molecules (molecules of DNA containing a replication fork) by selective adsorption of the partially single-stranded DNA to BND-cellulose. Equal weights (the amount depends on the DNA concentration as well as the amount of replicting DNA required) of TEN3 equilibriated BND cellulose (BND cellulose washed several times with TEN3 and centrifuged at 12,000 x g, 1 min 4° C) and DNA (in the same buffer) were thoroughly mixed in a 1.5 ml microfuge tube by tapping the tube several times. After 10 sec. of equilibration, the tube was centrifuged (12,000 x g, 1 min 4° C). The supernatant was saved, and the pellet was washed five times with double pellet volumes of the same buffer. The combined supernatant and washes were designated the "flow-through" fraction. The pellet was then washed six times with equal volumes of caffeine buffer. The combined washes were designated the "Caffeine wash" fraction. DNA was recovered from the flow-through and caffeine wash fractions by precipitation with equal volumes of isopropanol. The tubes were microfuged (12,000 x g, 1 min 4° C). The pellets were dried and dissolved in 250 µl of TE buffer, reprecipitated with 500 µl of ethanol, microfuged

(12,000 x g, 1 min 4° C) and finally redissolved in 100 µl of TE buffer. This quick, simple procedure provided a 20-50 fold enrichment for replicating DNA with overall recovery of better than 50%. The concentration of DNA was measured using a spectrometer.

D. Two-dimensional gel electrophoresis

Three Caffeine washed DNA samples (2.5 µg aliquots) were digested to completion with *Nru I.* These samples were then loaded into wells of standard horizontal submerged 0.4% agarose gels (8x10 cms) made up in standard TAE buffer containing ethidium bromide (0.1µg/ml). Marker DNA (lamda *Hind* III fragments) was loaded and the gel was electrophoresed at 50 volts for 8 hrs. The gels were then photographed under long wave UV light. The portion of each of the three lanes containing the DNA of interest were cut out (> 5.5 kb). These gel fragments were rotated by 90° and placed in an end to end fashion with a two cm gap between each other on the top of a 20x20 cm agarose gel tray and embedded in 1.2% agarose. This new gel was soaked with gentle agitation in alkaline electrophoresis solution for 90 min. A hot spatula was used to form wells near the edge of the gel and marker DNA (lamda *Hind* III) was added. The gel was electrophoresed in alkaline buffer at 12 volts for 24 hrs. The gel was then neutralized in 1M Tris (pH8.0). The gel was then sliced length- wise to separate the three original lanes and dried under vacuum at 60° C for 2.5 hrs. The dried gel was stored between sheets of paper until hybridization.

2.14 Statistical Analysis

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

3 Results

3.1 Characterization of *pol3* Proofreading-Deficient Mutator Strains

In order to investigate the specificity of the mutator effect conferred by a proofreading defect in pol δ, strains isogenic to MKP-o, but, with a mutation in the *pol3* gene, resulting in the disruption of the proofreading function of pol δ, was required. A mutation on the *pol3* gene which reduces the proofreading activity below detection levels while maintaining wild type levels of polymerizing activity would be ideal. A strain harboring such a mutation (designated SMpol3) with a sequence alteration (*pol3-4DA*) in the exo II domain of *POL3* was constructed and provided by Dr. Simon (Institut Curie, Paris, France). The construction and characterization of the *pol3-4DA* mutation has been published (Simon *et al.* 1991). However, a brief description is provided here for the benefit of the reader.

3.1.1 Construction of a *pol3* mutant strain

3.1.1.1 Construction of a pol3-4DA exonuclease deficient (exo-) strain (by Dr. Simon)

Briefly, the *pol3-4DA* missense mutation (Aspartic acid (D) residue at position 407 on the Exo II domain substituted with Alanine (A) [Simon *et al.* 1991]) was introduced into a cloned copy of the *POL3* gene by site-directed mutagenesis. The mutated copy was then cloned as a 4.1 kb *Hind* III- *Sal* I fragment into the yeast centromeric vector, YCp50 (Rose *et al.* 1987) to generate a 11.4 kb plasmid designated YCp50-4DA. This plasmid was then transformed into MKP-o. After transformation, most of the open reading frame (ORF) of the chromosomal *POL3* gene was replaced with a

TRP1 marker. The resulting strain designated SMpol3 had wild type polymerizing activity and no detectable *POL3* linked proofreading activity. The procedure for disrupting the chromosomal copy of *POL3,* which is an essential gene, while maintaining a plasmid borne mutant *pol3* copy has been described (Simon *et al.* 1991).

3.1.1.2 Construction of pol3-Y and pol3-I strains

SMpol3 was transformed with YCpKR-Y or YCpMS-I to obtain two strains pol3-Y and pol3-I, which are isogenic to the wild type strains WT-Y and WT-I (see section 2.3), respectively. These two mutant strains now carried two centromeric plasmids, i.e., a *SUP4*-o plasmid, either YCpKR-Y or YCpMS-I and YCP50-4DA.

3.1.2 Identity of the replication fork that duplicates the SUP4-*o* gene

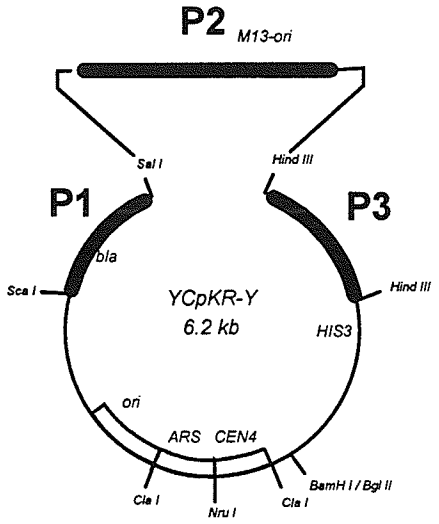
The uniqueness of the mutational spectrum for each of the two *pol3* strains studied seemed to suggest that strand identity could modulate the specificity of the *pol3* mutator. Although there was no significant difference in the mutation rate or fraction of individual class of mutations between WT-Y and WT-I, there was a significant difference in the distribution pattern of the two spectrum (see section 3.1.3.5). In plasmids YCpKR-Y and YCpMS-I (Figure 1), bidirectional replication begins at the *ARS1* sequence and proceeds clockwise and counterclockwise towards the *SUP4*-o gene. It was initially hypothesized that the clockwise replication fork (CRF) would move past the *SUP4*-o gene. This hypothesis was based on the knowledge that *CEN* sequences exhibit DNA-protein interaction during the S-phase and significantly delay replication fork movement (Greenfeder and Newlon 1992). Since the *SUP4*-o gene is located equidistant

Figure 4 A. Mapping the movement of the replication fork

A1. Position of the probes used in the hybridization procedure. Probe P1 is a 820 bp *Sca* I - *Sal* I fragment containing part of the *bla* sequence. Probe P2 is a 1.1 kb *Sal* I - *Hind* III fragment containing the *SUP4*-o gene and M13-ori sequence. probe P3 is a 1 kb *Hind* III fragment containing part of the *HIS3* gene.

A2. Hypothetical pattern of movement of the left replication fork in the process of duplicating the *SUP4*-o gene. The parental strands are shown in dotted lines while the newly synthesized daughter strands in shown in solid lines.

A1. Position of the probes



A2. Pattern of movement of the left replication fork

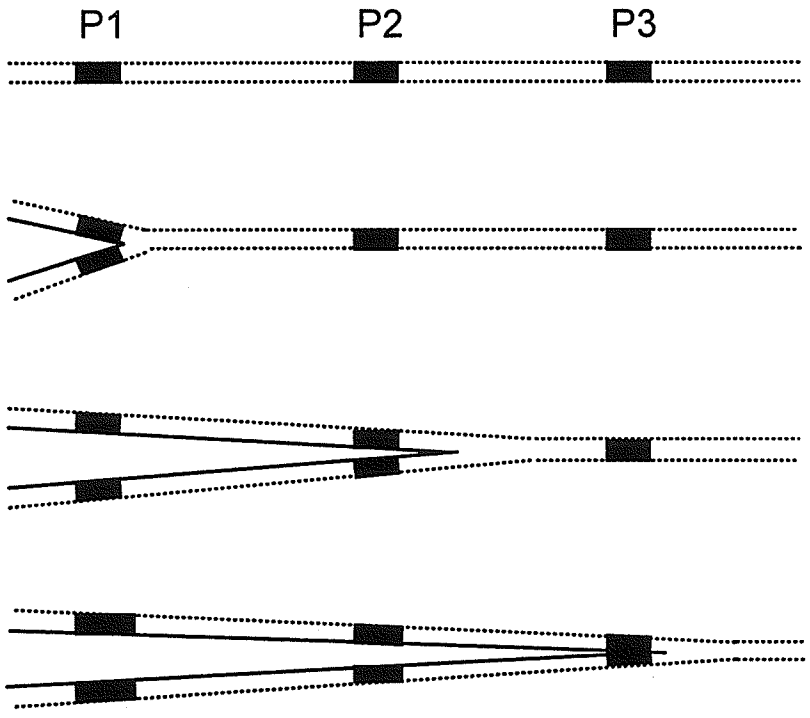
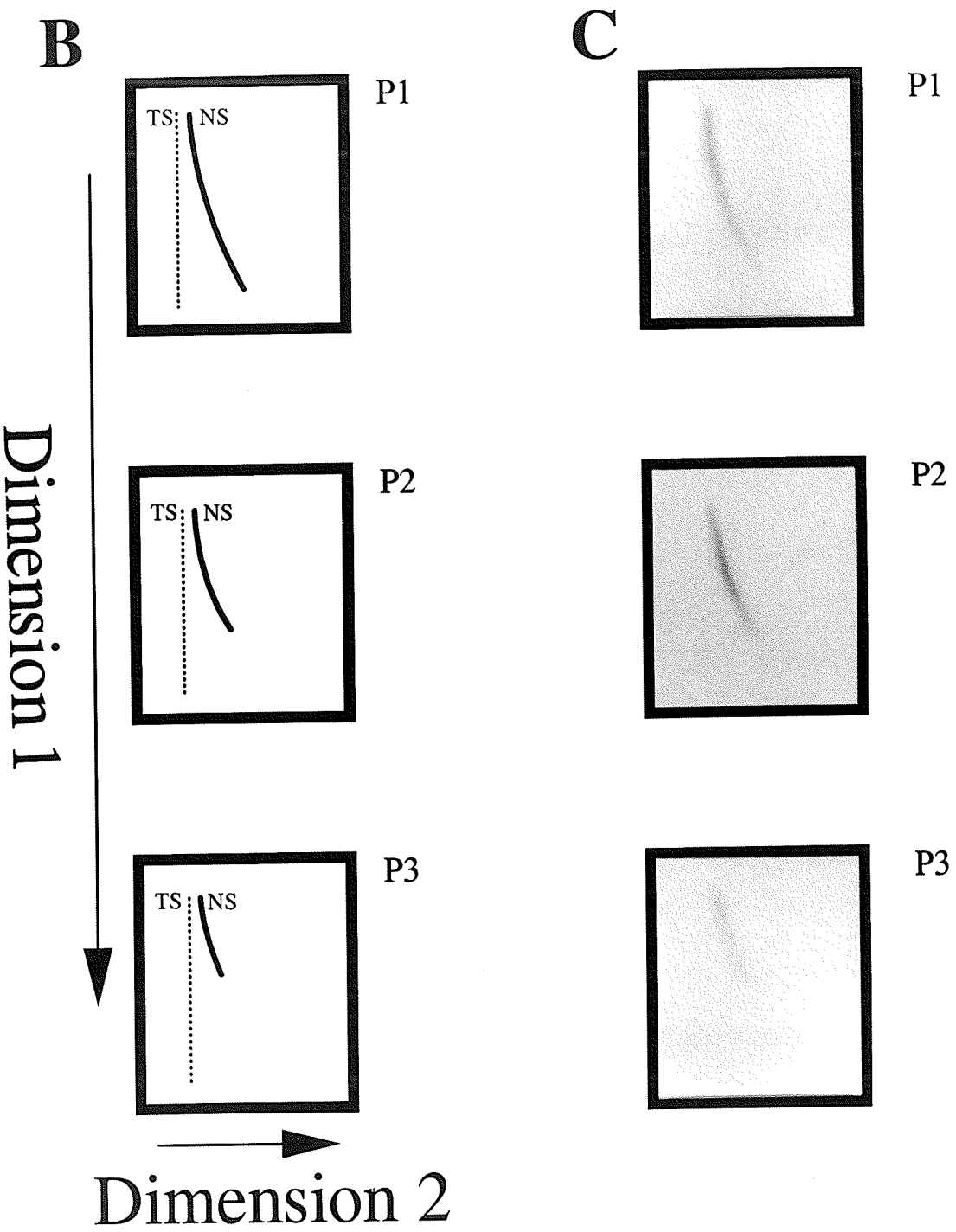


Figure 4 B and C. Hybridization of the 2 dimensional gel using probes P1, P2 and P3

B. Hypothetical hybridization patters of the second dimensional gel using probes P1, P2 and P3, assuming that the left replication fork replicates at least to the end of P3. TS indicates the position of the template strands and NS indicates the position of the hybridized daughter-strands for each probe.

C. Actual autoradiogram obtained by probing the second dimension gel with the three probes. The region of the gel containing the parental strands was removed before exposing it to an X-ray film.


B
C
P1
P2
P3
TS
NS
Dimension 1
Dimension 2

from the *ARS1* in the clockwise and counterclockwise directions, and the *CEN6* sequence is located immediately counterclockwise to the *ARS1* sequence, it seemed that the counterclockwise fork (CCRF) might be delayed sufficiently to allow the CRF to replicate past the *SUP4*-o gene. This scenario had to be verified in order to determine which of the *SUP4*-o gene strands is the leading strand template and which is the lagging strand template in the Y and I orientations. To accomplish this, I used 2-dimensional gel electrophoresis to map the movement of the replication fork in the plasmid YCpKR-Y. Since plasmids YCpKR-Y and YCpMS-I are identical except for the orientation of the *SUP4*-o gene, it was assumed that the pattern of movement of the replication fork would be identical for the two plasmids.

Plasmid as well as genomic DNA molecules in various stages of replication were isolated from exponentially growing non-synchronized WT-Y cells. A sample was enriched for replication fork containing molecules by BND-cellulose fractionation. This sample was linearised with *Nru* I which cuts between the *ARS1* and *CEN6* sequences. The DNA had to be linearized in order to aid separation in a gel. Equal amounts of DNA was then loaded into three wells and electrophoresed in a standard agarose gel to separate out the DNA fragments according to mass. For the plasmid molecules, the separation is such that the completely replicated molecules would travel the furthest distance while the molecules in the final stages of replication i.e., those with the longest daughter-strand, would travel the shortest. After this first dimension electrophoresis, a section of each of the lanes containing the fragments of interest was excised, rotated 90° and placed on the top of another gel in a linear order (tail of first piece towards the head of the next) with a

two cm gap between adjacent pieces. The gel pieces were fused with the main gel using melted agarose. This setup was then soaked in alkali to denature the DNA sample. Then, the gel was electrophoresed in the second dimension in alkaline buffer. In this case, the separation of the molecules is based on size. The parental strands would form a straight line, parallel to the gel slice while the daughter-strands would form an arc with the smallest fragments at the furthermost point on the arc (Figure 4B). The gel was cut in order to separate out the three sections corresponding to the three gel pieces placed on the top before electrophoresis, neutralized and dried under vacuum.

Each of the dried gel slices was hybridized using P1, P2 or P3 as probes (Figure 4A1). With respect to the *ARS1* sequence, assuming that the CRF replicates most of the plasmid, P1 lies before the *SUP4*-o gene, while P2 includes the gene and P3 lies past the gene (Figure 4A1). If the CRF duplicated at least till the end of P3 (Figure 4A2), P1 would bind the entire arc of daughter-strand while P2 would bind to most of the arc from the larger end, missing the tip which would correspond to the region between the *ARS1* sequence and the beginning of P2. P3, on the other hand, would bind only the top most part of the arc from the larger end, missing the part that corresponds to the region between the ARS1 sequence and the beginning of P3 (Figure 4B).

After hybridization, the gel was exposed to an x-ray film. When the film was developed, it was noticed that the parental strand masked the daughter-strand arc due to a relatively high concentration of completely replicated molecules in the preparation. After initial exposure to the X-ray film, the position of parental strand line (Figure 4B) was identified on the dry gel and the portion of the gel from the bottom of the parental strand

to the top of the gel was cut off. This effectively removes all the parental strands and the gel was exposed again to X-ray film for 48 to 72 hrs.

The hybridization results were similar to the scenario discussed above (Figure 4C). Probe P1 bound to the entire daughter-strand arc, P2 bound to most of the arc excluding the tip portion while P3 bound only to the top portion of the arc. This showed that it was in fact the CRF that replicated the *SUP4*-o gene. It was also clear that the CRF proceeded at least 1 kb past the end of the *SUP4*-o gene before encountering the CCRF since the end of P3 is located about 1 kb past the *SUP4*-o gene. There is no significant difference in the size between YCpKR-Y and YCpMS-I (Figure 1) and it is most likely that the pattern of movement of the replication fork is similar in the two plasmids. It should however be noted that *SUP4*-o gene in the two plasmids are in opposite orientations. In otherwords, with respect to the CRF, the direction of transcription of the *SUP4*-o gene is counterclockwise in YCPKR-Y while it is clockwise in YCpMS-I, as shown in Figure 1. From the results obtained from the 2 dimensional gel experiment, it was concluded that in YCpKR-Y, the transcribed strand is the lagging strand template and the direction of transcription is counter clockwise while the direction of replication fork is clockwise. In YCpMS-I, the transcribed strand is the leading strand template and the direction of transcription as well as replication fork is clockwise.

3.1.3 Specificity of the *pol3-4DA* mutator

The specificity of the *pol3-4DA* mutation was determined by DNA sequence characterization of spontaneous mutations arising in the *SUP4*-o gene in *pol3-4DA*

backgrounds (pol3-Y or pol3-I) and comparing it to the results obtained from similar experiments in the corresponding wild type backgrounds (WT-Y or WT-I). These four strains were categorized into four pairs for comparison purposes. The four pairs comprised, pol3-Y vs. WT-Y forming the Y-pair, pol3-I vs. WT-I forming the I-pair, pol3-I vs. pol3-Y forming the pol3-pair and WT-I vs. WT-Y forming the WT-pair.

3.1.3.1 Plasmid retention and SUP4-o mutation rate

The relative differences between a wild type and its corresponding *pol3* mutant should reflect the property of the individual mutator since the two strains under consideration are isogenic except for the *pol3* locus. Similarly, a comparison between these two *pol3* strains should reveal the *SUP4*-o orientation effect on the *pol3* mutator as the only difference between the strains is the orientation of the *SUP4*-o gene.

Cells were grown to stationary phase from a low inoculum (ca. 33 cells/ml) in appropriate media. The plasmid retention values for the *SUP4*-o plasmid were calculated by comparing the number of colonies that emerged on medium selective for the plasmid with the corresponding number for non-selective medium. The data for 30 independent cultures `were pooled to determine the percent plasmid retention data (Table 4).There was no significant difference between the plasmid retention values for the four strains, which ranged between 86.1% and 88.7%. Therefore, proofreading-deficiency does not affect plasmid maintenance and plasmid loss will not interfere in the recovery of *SUP4*-o mutants.

Table 4. Plasmid Retention

Strain	Colonies on selective medium	Colonies on non-selective medium	Percent plasmid retention
WT-Y	17,165	19,567	87.7
pol3-Y	6,266	7,355	85.2
WT-I	21,182	23,886	88.7
pol3-I	13,266	15,412	86.1

Colony counts are pooled values from 30 independent cultures

Table 5. Spontaneous *SUP4*-o Mutation Frequency and Rate

Strain	Mutation Frequency ($\times 10^{-6}$)	Mutation Rate ($\times 10^{-7}$)
WT-Y	0.58	2.0
pol3-Y	30.2	63.5
WT-I	0.58	1.9
pol3-I	114.6	213.2

Frequency values are the medians of 30 independent cultures

Relative to the wild type, the spontaneous *SUP4*-o mutation rate was increased in the *pol3* strain by 31.8 or 112.3 fold in the *pol3* strain in the Y- or I-pairs respectively. The rate was increased by 3.4 fold in the pol3-I strain in the *pol3*-pair while there was no difference between the two strains in the WT-pair (Table 5). The mutation rate increase in the *pol3* strains, in the Y- and I-pairs, reflect the *pol3* mutator effect suggesting the requirement of proofreading by pol δ for accurate replication. Additionally, the rate difference between the two *pol3* strains in the *pol3*-pair reflects a *SUP4*-o orientation effect on the *pol3* mutator since there is no difference in the mutation rate between the wild type strains (WT-set). This *SUP4*-o orientation effect seem to suggest that the same functional form of pol δ may not be replicating both the strands to the same extent, because, if it did, the over all rate in one orientation should be the net of errors made by pol δ while replicating both the strands and there should not be any change associated with switching the orientation of the *SUP4*-o gene.

3.1.3.2 Mutational classes detected

The different classes of sequence alterations and the proportions of each class detected for the four strains is provided in Table 6. SBPS, -1bp and +1bp were the predominant classes of mutations detected. Among these three classes, the rate of SBPS was the highest in each of the four strains followed by -1bp and +1bp mutations. This suggests that the most common replication errors are those that lead to SBPS. The mutation rates of these classes were increased in the *pol3* strains relative to the

Table 6. Sequence Alterations in *SUP4*-o Mutants

Alteration	WT-Y		pol3-Y		WT-I		pol3-I	
	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})
SBPS	173 (86.9)	17.4	156 (76.5)**#	486.9	161 (84.3)	16.1	171 (85.9)#	1832.3
NTBPS	None Detected		1 (0.5)	3.1	None Detected		None Detected	
1 bp deletion	15 (7.5)	1.5	28 (13.7)	87.2	17 (8.9)	1.7	22 (11.1)	235.7
>1 bp deletion	1 (0.5)	0.1	None Detected		3 (1.6)	0.3	None Detected	
1 bp Insertion	5 (2.5)	0.5	18 (8.8)**#	56.1	7 (3.7)	0.7	6 (3)#	64.3
Ty insertion	3 (1.5)	0.3	None Detected		None Detected		None Detected	
Duplication	1 (0.5)	0.1	None Detected		1 (0.5)	0.1	None Detected	
Complex change	None Detected		1 (0.5)	3.1	2 (1)	0.2	None Detected	
Total	199 (100)	19.9	204 (100)	635.4	191 (100)	19.1	199 (100)	2132.3

SBPS- single base pair substitution; NTBPS- non-tandem base pair substitution; Ty- yeast transposable element;Significance of difference: when compared to the corresponding wild type strain with similar *SUP4*-o orientation,* = $P < 0.05$, and ** = $P < 0.01$; when compared to the corresponding strain of same phenotype but opposite *SUP4*-o orientation, # = $P < 0.05$ amd ## = $P < 0.01$.

corresponding WT strain in both Y- and I-sets, suggesting that proofreading can correct replication errors that lead to SBPS as well as simple insertion and deletion mutations *in vivo*. When the magnitude of the rate increase in the *pol3* strains was considered, the maximum increase was for +1bp (111.8 fold) followed by -1bp (58 fold) and SBPS (28 fold) in the Y-pair, contrary to the situation in the I-pair where the maximum increase was for -1bp (139.5 fold) followed by SBPS (114.5 fold) and +1bp (92.5 fold). The rate of SBPS and -1bp events were higher for pol3-I in the pol3-pair while there was no difference in the rate of +1bp events. Additionally, while there was a significant difference in the fraction of the SBPS and +1bp ($P < 0.01$ in both cases) between the strains in Y- and pol3-sets ($P <$ at least 0.05), there was no such difference between the strains in I- and WT-pairs ($P > 0.05$). These results seem to suggest that changing the orientation of the *SUP4*-o gene, which would also change the identity of the two strands, modulates the specificity of the *pol3* mutator.

3.1.3.3 Single base-pair deletion/Insertion

The numbers and positions of the deletion and insertion events detected in the four strains are provided in Table 7. Most of the -1bp events detected in the four strains (73% of WT-Y, 64% of pol3-Y, 94% of WT-I and 100% of pol3-I) were in the run of 5 G·C pairs at 79 → 83. Additionally, all the +1bp events detected in the four strains were also in this position. This run of 5 G·C seems to be a one base-pair deletion/insertion hot spot for all the four strains. This suggests that the polymerase must have a low efficiency in accurately replicating simple repeat sequences probably due to a high degree of

Table 7. Multiple Mutations, Deletions and Insertions

Sites #	Mutation type ##	WT-Y*	ol3-Y*	T-I*	ol3-I*
- 40 → 508	548 bp deletion	-	-	1	-
6 or 7	SBP deletion	-	1	-	-
14 → 133	318 bp deletion	-	-	1	-
17 ↔ 18	Ty insertion	1	-	-	-
26 ↔ 27	10 bp duplicaton	-	-	1	-
26 ↔ 27	4 bp duplication	1	-	-	-
34	SBP deletion	-	2	-	-
34, 39	G → A, T → A	1	-	-	-
35 → 37	SBP deletion	1	-	-	-
37 ↔ 38	Ty insertion	2	-	-	-
51 → 58	8 bp deletion	-	-	1	-
52 → 54	SBP deletion	-	2	-	-
58 → 64	7 bp replaced with GGGCC	-	1	-	-
62	SBP deletion	2	-	-	-
64	SBP deletion	1	-	-	-
70 or 71	SBP deletion	-	-	-	-
79 → 83	SBP deletion	11	18	16	22
79 → 83	SBP insertion	5	18	7	6
79 → 83, 82 or 83	SBP deletion plus C→ A	-	-	1	-
84 → 86	SBP deletion	-	5	1	-
87, 89	T→ A, T→ C	-	1	-	-
89, 90 → 97	T → A plus 8 bp deletion	-	-	1	-
89 → 97	9 bp deletion	1	-	-	-

#= Sites for multiple deletions are estimates; ##= changes are given for the transcribed strand *= Number detected.

slippage by the DNA polymerase at nucleotide runs (Kunkel 1993; Strand *et al.* 1993). However, there was a significant difference only in the relative fraction of +1bp events between the strains in Y- and pol3-pairs ($P < 0.01$ in the case of the former and $P < 0.05$ in the case of the latter), probably because slippage events leading to insertion mutations could be a more common occurrence during replication by pol δ than those leading to deletion errors.

3.1.3.4 Single base-pair substitution

The fraction and rates of the six possible classes of SBPS detected in the four strains is provided in Table 8. All six classes were detected in each of the four strains. The rates of each class was higher for the *pol3* strain in Y and I-pairs and for the pol3-I strain in the pol3-pair. There was a significant increase only in the fraction of G·C → T·A, G·C → C·G and A·T → T·A transversions between the strains in the Y- and I-pairs. Additionally, there was a significant difference in the G·C → T·A, A·T → T·A, total transitions and total transversions between the strains in the pol3-pair (P at least < 0.05), while there was no significant difference for any class of SBPS events between the strains in the WT-pair ($P > 0.05$ in all cases). Among the three significant classes of transversions in the Y- and I-pairs, the fraction of A·T → T·A was increased in the *pol3* strain (7.3 and 4.4 fold in Y- and I-pairs, respectively) while the fraction of G·C → T·A and G·C → C·G were reduced ($P <$ at least 0.05). The rate increase was the largest for A·T → T·A transversions probably because these errors are normally proofread efficiently while the lowest rate increase was for G·C → C·G probably because the

Table 8. Single Base-Pair Substitutions

Alteration	WT-Y			pol3-Y			WT-I			pol3-I		
	No Detected	(%)	Rate (x 10^{-8})	No Detected	(%)	Rate (x 10^{-8})	No Detected	(%)	Rate (x 10^{-8})	No Detected	(%)	Rate (x 10^{-8})
G·C→ A·T(Tr)	42	(24.3)	4.2	24	(15.4)	74.7	52	(32.3)	5.2	42	(24.6)	50.0
A·T→ G·C(Tr)	18	(10.4)	1.8	16	(10.3)	49.8	14	(8.7)	1.4	24	(14.0)	257.2
Total Tr	60	(34.7)	6.0	40	(25.7)#	124.5	66	(41.0)	6.6	66	(38.6)#	707.2
G·C →T·A(Tv)	57	(32.9)	5.7	32	(20.5)*#	99.7	36	(22.4)	3.6	56	(32.7)*#	600.0
G·C →C·G(Tv)	31	(17.9)	3.1	2	(1.3)**	6.2	41	(25.5)	4.1	3	(1.8)**	32.1
A·T →C·G(Tv)	14	(8.1)	1.4	10	(6.4)	31.1	12	(7.4)	1.2	18	(10.5)	192.9
A·T → T·A(Tv)	11	(6.3)	1.1	72	(46.1)**##	224.1	6	(3.7)	0.6	28	(16.4)**##	300.1
Total Tv	113	(65.3)	11.3	116	(74.3)#	361.1	95	(59.0)	9.5	105	(61.4)#	1125.1
Total SBPS	173	(100)	17.4	156	(100)	486	161	(100)	16.0	171	(100)	1832.3

Tr = Transitions; Tv = Transversions; SBPS= single base pair substitution; Significance of difference: when compared to the corresponding wild type strain with similar *SUP4*-o orientation,* = $P < 0.05$, and ** = $P < 0.01$; when compared to the corresponding strain of same phenotype but opposite *SUP4*-o orientation, # = $P < 0.05$ amd ## = $P < 0.01$

Table 9. Distribution of Spontaneous Base Substitutions in the *SUP4*-o Gene

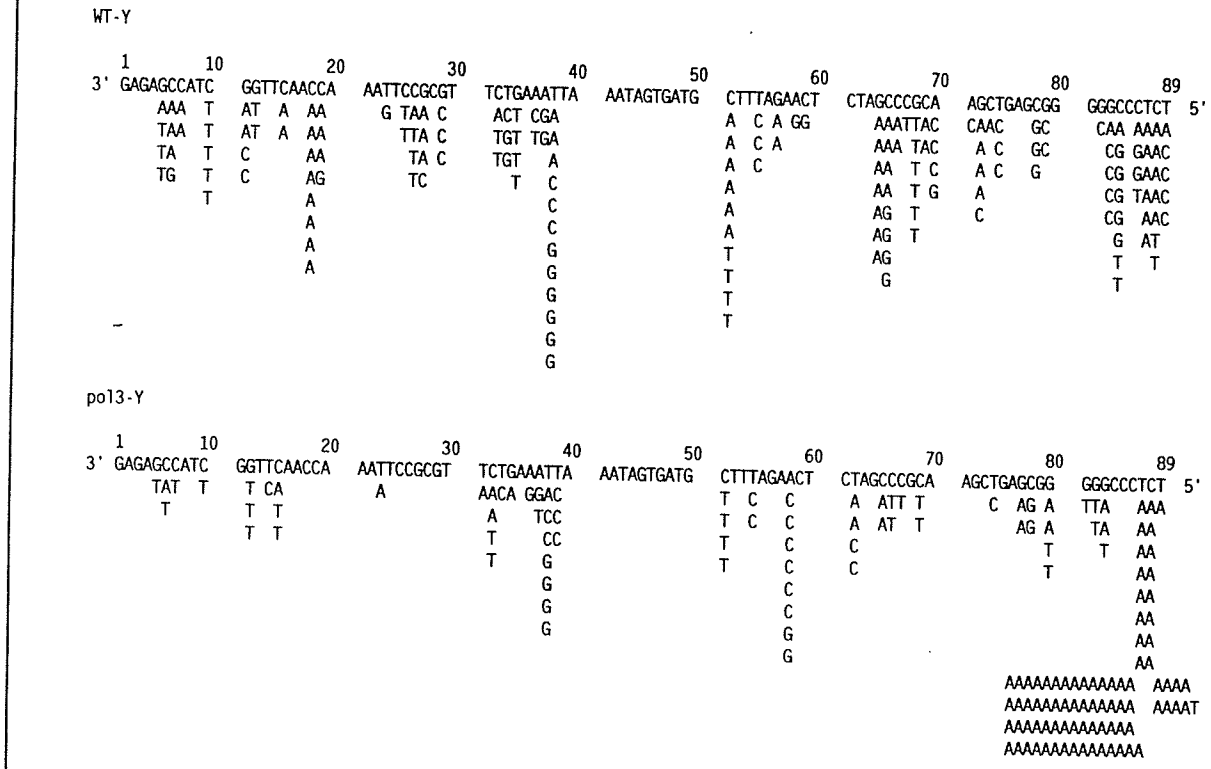
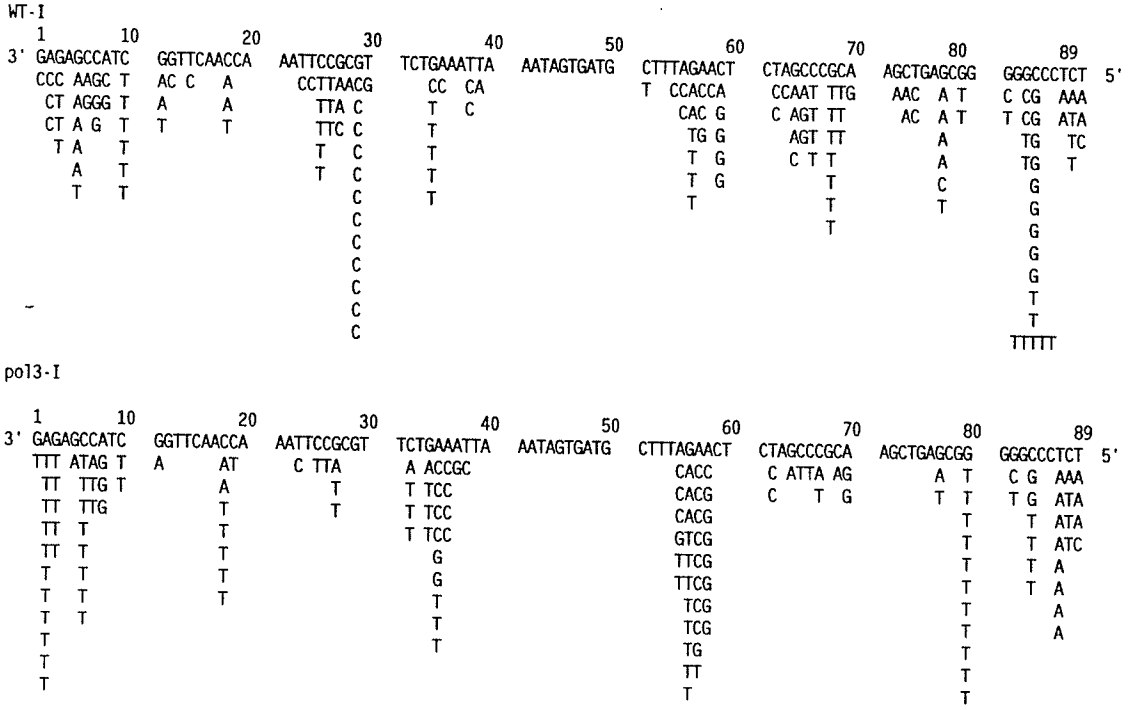


Table 10. Distribution of Spontaneous Base Substitutions in the *SUP4*-o Gene



polymerase makes very few replication errors that lead to such mutations or mismatch correction efficiently corrects such replication errors very efficiently.

3.1.3.5 Distribution of single base-pair substitution

The distribution of SBPS arising in the strains of the Y- and I-pairs are provided in Table 9 and 10, respectively. Substitutions were distributed throughout the *SUP4*-o gene with the exception of the 14 bp intron (sites 40 to 53) where changes occurred at only one position (site 51). No SBPS were detected in the regions immediately flanking the gene.

In the Y-pair, 52 sites were mutated of which, 18 were unique to WT-Y and 7 to pol3-Y while 27 sites were common to both the strains. In the I-pair, 57 sites were mutated of which, 18 were unique to WT-I, 7 to pol3-I and 32 sites were common to both the strains. Of these common sites, the mutation rate was increased at all the sites in the *pol3* background for either pairs. The rate increase ranged from 6.21 to 336 fold with an average value of 44.7 fold in the Y-pair while it ranged from from 18 to 970 fold with an average value of 134 fold. In the pol3-pair, a total 51 sites were mutated of which, 12 were unique to pol3-Y, 17 to pol3-I and 22 sites were common to both the strains. Of the common sites, the mutation rate was increased in all the sites in pol3-I with the exception of sites, 38, 87 and 88 where the rate was higher for pol3-Y. The rate increase ranged from 1.7 to 13.8 fold with an average value of 5.5 fold. The site wise mutation rate for all the four strains or the pairwise relative rate increase in the Y-, I- or pol3-pair was not uniform throughout the gene indicating that the *pol3* mutator had a much larger effect on

specific sites than can be expected based on the magnitude of increase in the overall mutation rate. Sites 58 (248 fold) and 87 (336 fold) had the largest increase in the Y-pairs, sites 35 (970 fold) and 58 (862.2 fold) displayed the largest increase in the I-pair and sites, 6, 34, 36 and 89 displayed the largest increase (13.8 fold) in the pol3-pair. This is probably because of the influence of sequence context/ neighbouring nucleotides on the polymerase during nucleotide incorporation.

In the WT-pair, a total of 61 sites were mutated of which, 11 were unique to WT-Y and 16 to WT-I while 34 sites were common to both the strains. Of these common sites, the mutation rate was greater at 18 sites for WT-Y and 13 sites for WT-I while there was no difference in 3 sites. The rate increase ranged from 1.1 to 5 fold with an average value of 1.2 fold. Similar to the situation in the other three pairs, the mutation rate was not uniform throughout the gene probably because of the effect of sequence context on the processes of replication and replication fidelity in general.

A statistical comparison (Adams and Skopek 1987) of the distributions of SBPS between the strains in the Y, I, *pol3* or WT-pairs indicated that the chance of random sampling error accounting for differences was less than 1 in 500 (with 1,700 simulated comparisons, the upper limit on the 95% confidence interval for the estimate of $P < 0.002$). So the differences or uniqueness of a spectrum should be due to the genotype and phenotype of a strain and not due to a sampling error or a chance occurrence.

3.1.4 Effect of SUP4-*o* orientation or strand identity on mismatch correction

The SBPS mutational spectrum of each of the four strains was unique with respect to the mutated sites and magnitude of mutation at the mutated sites (Tables 9 and 10). For instance, hot spots are at positions 29 and 84 for WT-Y, positions 2, 56 and 80 for pol3-Y, positions 38 and 51 for WT-I and positions 87 and 88 for pol3-I. Similar to WT-Y and WT-I, pol3-Y and pol3-I strains are identical to each other except for the orientation of the *SUP4*-o gene. So any difference in their mutational spectrum can be attributed to the *SUP4*-o orientation effect or in other words, the effect of DNA strand identity. This change associated with reversing the orientation of the gene could be due to differences in sequence context effect seen by the polymerase when it polymerizes the two strands.

There is also a possibility that it could be due to the specificity of mismatch correction in these sites when the orientation of the *SUP4*-o gene is reversed. In order to investigate this issue, the efficiency of mismatch correction in some of the hot spots positions in either orientation was assessed using heteroduplex DNA in wild type cells.

3.1.4.1 Construction of heteroduplex DNA

Three SBPS mutations, A → T at position 2 (2T), G → T at 56 (56T) and T → A at 87 (87A) were chosen for the heteroduplex study. Sites 2 and 56 were hot spots for

Table 11. Plasmids Used in the Construction of Heteroduplexes

Plasmid	Mutation	Mutation Position	Reference
YCpMP2	Wild type	-	Pierce *et al.* 1987
YCpMP2-M2T	A → T	2	Dr. Kunz*
YCpMP2-M56T	G → T	56	Dr. Kunz*
YCpMP2-M87A	T → A	87	Dr. Kunz*
YCpLK3EB	Wild type	-	L. Kohalmi
YCpLK3EB-M2T	A → T	2	Dr. Kunz*
YCpLK3EB-M56T	G → T	56	Dr. Kunz*
YCpLK3EB-M87A	T → A	87	Dr. Kunz*
YCpMP2-M18G	C → G	18	Dr. Kunz*
YCpMP2-M27A	G → A	27	Dr. Kunz*
YCpMP2-M51G	C → G	51	Dr. Kunz*
YCpMP2-M55T	A → T	55	Dr. Kunz*

* These mutant plasmids were from a plasmid library maintained in our lab

Table 12. Heteroduplex plasmids used in the study

Hetero-duplex	Mismatch	Gene Position	Mismatch Strand Identity	Mismatch Strand Identity
H1	T/T*^{n}	2	Transcribed	Lagging
H2	C/T*^{n}	56	Transcribed	Lagging
H3	A/A*^{n}	87	Transcribed	Lagging
H4	*A/A^{n}	2	Non-transcribed	Leading
H5	*A/G^{n}	56	Non-transcribed	Leading
H6	*T/T^{n}	87	Non-transcribed	Leading
H7	A/A*^{n}	2	Non-transcribed	Lagging
H8	G/A*^{n}	56	Non-transcribed	Lagging
H9	T/T*^{n}	87	Non-transcribed	Lagging
H10	*T/T^{n}	2	Transcribed	Leading
H11	*T/C^{n}	56	Transcribed	Leading
H12	*A/A^{n}	87	Transcribed	Leading
H13	^{n}*G/G	18	Transcribed	Lagging
H14	^{n}G/T*	27	Non-transcribed	Leading
H15	^{n}*A/C	27	Transcribed	Lagging
H16	^{n}*G/G	51	Transcribed	Lagging
H17	^{n}A/A*	55	Non-transcribed	Leading
H18	^{n}*T/T	55	Transcribed	Lagging

*- Mutant Allele; n- base on nicked strand

pol3-Y while they were cold spots for pol3-I. Similarly, site 87 was a hot spot for pol3-I while, relatively, a very few mutations were detected for pol3-Y (Tables 9 and 10).

A list of plasmids used in the construction of heteroduplexes is provided in Table 11. The plasmids, YCpMP2-M2T, YCpMP2-M56T and YCpMP2-M87A (M denotes that the plasmid carries a mutant allele of *SUP4*-o while the number and letter following the M denotes the position of the mutation and the base on the transcribed strand) carrying mutant *SUP4*-o alleles were obtained from the plasmid library maintained in our lab. The wild type plasmid YCpMP2 and these three mutant plasmids were used to construct 6 heteroduplexes (H1 to H6) for the Y-orientation (Table 12).

The plasmid, YCpLK-3EB-M2T was constructed by replacing the 260 bp *Eco* RI - *Bam* HI fragment in YCpLK3EB containing the wild type *SUP4*-o gene with the corresponding 260 bp *Eco* RI - *Bam* HI fragment from YCpMP2-M2 containing the mutant *SUP4*-o gene. The other two plasmids, YCpLK-3EB-M56T and YCpLK-3EB-M87A were constructed in a similar manner using mutant *SUP4*-o genes from YCpMP2-M56T and YCpMP2-M87A. The sequence alteration of the *SUP4*-o gene on the mutant plasmids, thus constructed, was verified by DNA sequencing. The wild type plasmid YCpLK3EB and its three derivative plasmids were used to construct 6 heteroduplexes (H7 to H12) for the I-orientation (Table 12).

Circular, single-stranded DNA was prepared from the wild type or corresponding derivatives carrying mutant *SUP4*-o alleles and annealed to linearized molecules of these derivatives or wild type plasmids, respectively (Figure 3). The resulting heteroduplexes carried a functional *SUP4*-o allele on one strand and a defective *SUP4*-o allele on the other.

These heteroduplex plasmids were then transformed into the wild type strains. Prior to the first round of DNA replication in the transformed cells, the mismatch can be corrected to the normal base-pair to give a functional *SUP4*-o allele. Alternatively, it might not be repaired. The *ade2-1* allele present in the yeast strains causes red colouring in the absence of a functional *SUP4*-o gene. Thus, under conditions selective for the plasmid, three types of transformant colony can emerge: 1. white (indicating correction to the normal base-pair); 2. red (indicating restoration to the incorrect base-pair); or 3. sectored red/white (indicating failure to repair the mismatch). The sectored colony appears in the absence of mismatch correction because one of the initial two daughter cells produced following the first round of DNA replication bears a plasmid with a functional *SUP4*-o allele while the other carries a plasmid with a defective copy. A sectored colony also might result from co-transformation of a cell with two heteroduplex plasmids, followed by correction and subsequent seggregation caused by the mutual instability of multiple copies of yeast centromeric plasmids in haploid yeast cells (Newlon 1988). By transforming strains with mixtures of YCpMP2 and mutant derivatives, it was demonstrated that less than 1% of the sectored transformants are due to co-transformation (Kunz *et al.* 1991). Furthermore, it was verified that colony colouring is associated with mismatch resolution by sequencing *SUP4*-o genes on plasmids derived from cells in red or white colonies or sectors (Kunz *et al.* 1991). In this system, the fraction of sectored colonies gives the relative efficiency of mismatch repair whereas the ratio of red to white colonies is a measure of the direction of mismatch correction.

Table 13. Analysis of strand specificity of mismatch correction

Hetero-duplex	Mismatch	Gene Position	No. of colonies	R:W[$]	Sectored colonies (%)
H1	$T/T^{*t, lg}$	2	3420	0.97	64
H10	$^{t, le}*T/T$	2	2968	1.19	59
H7	$A/A^{*nt, lg}$	2	3274	1.09	29
H4	$^{nt, le}*A/A$	2	3967	1.11	32
H2	$C/T^{*t, lg}$	56	2748	1.14	12
H11	$^{t, le}*T/C$	56	3467	1.21	16
H8	$G/A^{*nt, lg}$	56	3684	1.16	19
H5	$^{nt, le}*A/G$	56	2765	1.10	17
H3	$A/A^{*t, lg}$	87	3309	0.89	27
H12	$^{t, le}*A/A$	87	3659	0.93	29
H9	$T/T^{*nt, lg}$	87	3197	1.14	63
H6	$^{nt, le}*T/T$	87	3762	1.17	68

*- Mutant Allele. Identity of the strand carrying the incorrect base: t- transcribed strand, nt- non-transcribed strand, le- leading stand template, lg- lagging strand template. [$]- Non-sectored red : non-sectored white colonies

3.1.4.2 Specificity of Heteroduplex repair

The 12 heteroduplexes were transformed into MKP-o and the differences in the degree of mismatch correction were observed. The T/T mismatches were repaired much less efficiently (32 to 41% non-sectored or mismatch corrected colonies) than the other mismatches under study. The C/T mismatches (12 to 16% sectored colonies) were corrected most efficiently followed by G/A (81 to 88% non-sectored colonies) and A/A (68 to 73% non-sectored colonies) mispairs. Pair wise comparison of heteroduplex plamids with *SUP4*-o on either orientation (H1–H10, H4–H7, H2–H11, H5–H8, H3–H12 αvδ H6–H9) showed that there was no difference in the mismatch correction efficiency between the two plasmids in any of the pairs tested. Thus, reversing the orientation of the gene did not have any significant effect on mismatch correction efficiency in at least the three sites tested. In other words, strand identity, with respect to leading vs. lagging strands as well as transcribed vs. non-transcribed strands, did not have any significant effect on mismatch correction efficiency and the hot spots in the three sites tested must be due to the *pol3* mutator effect.

3.1.5 Effect of pol3-4DA mutation on mismatch correction

The spectrum of mutations in the *pol3* strain mainly reflects the base-pair errors that were not corrected by mismatch correction while the spectrum of mutations in the *pms1*Δ strain reflects the base-pair errors not corrected by proofreading. Although it has been determined that strand identity did not modulate mismatch correction (see section

3.1.4), it was not clear if the proofreading mutation affected the extent of mismatch correction. It has been shown that a proofreading defect in *E. coli* also reduces the efficiency of mismatch correction due to the saturation of the mismatch correction pathway with DNA replication errors (Schaaper 1988; Schaaper and Radman 1989). In order to determine the effect of the *pol3* exo- mutation on mismatch correction, the heteroduplex error correction efficiency was assessed in the pol3-4DA background and compared that in the wild type strain.

3.1.5.1 Construction of heteroduplex DNA

Six heteroduplexes, (H13 to H18) listed in Table 12, were used in the study. The heteroduplexes were constructed and provided by Yingying Yang and have been described (Yang *et al.* 1996). The heteroduplex DNA used in this experiment was constructed as described earlier. A list of plasmids used in the construction of the six heteroduplexes is provided in Table 11.

3.1.5.2 Construction of a pol3 *strain for mismatch correction assessment*

All the six heteroduplexes used in this study were YCpMP2 derivatives which had *URA3* as the selection marker. It would not be possible to maintain selection for these heteroduplex plasmids in SMpol3, as the strain is already Ura^+ by virtue of the *URA3* on its YCp50-4DA plasmid. To rectify this problem, the KRpol3 strain was constructed (Table 2). First, the *Hind* III- *Sal* I fragment on YCpKR-Y containing the M13- *SUP4*-o sequences was replaced by the 4.1 kb *Hind* III- *Sal* I fragment from YCp50-4DA

containing the *pol3-4DA* gene to create plasmid YCpKR-4DA. This new plasmid carrying the *pol3-4DA* gene has *HIS3* as the selectable marker and it can be maintained concurrently with the *URA3* based heteroduplex plasmids.

YCpKR-4DA was transformed into SMpol3. The resulting transformants were grown in YPDA and plated on FOA medium and incubated at 30° C for 48 hrs., to select for loss of the plasmid carrying the URA3 gene (Boeke *et al.* 1984). In uracil prototrophs, FOA is converted to 5-fluoro-orotidine-5'-monophosphate by the *URA5* gene product (orotate phosphoribosyl-transferase). The latter compound is decarboxylated to 5-fluoro-uridine-5'monophosphate (FUMP) by the *URA3* gene product (orotidine-5'-phosphate decarboxylase). FUM*P* is converted through a multi-enzyme pathway to 5'-fluoro-2'deoxy-5'-monophosphate (FdUMP) which is a potent inhibitor of dTM*P* synthetase which is required for the formation of dTTP. Thus, FOA inhibits DNA synthesis by preventing dTM*P* formation (Boeke *et al.* 1984). A microscopic observation of the cell doubling of Ura+ and Ura- strains plated on FOA plates revealed that Ura+ cells are killed before completion of one round of DNA replication. The, loss of a functional *URA5* or *URA3* gene would thus result in a FOA resistant (FOA^R) colony.

Several colonies were picked from the FOA plates and stored as potential isolates that have lost the YCp50-4DA plasmid. It is possible that some of the isolates were FOA^R due to a mutation on the *URA3* gene Additionally, some may carry both plasmids (YCpKR-4DA as well as YCp50-4DA with a mutated *URA3*). So, six isolates (KRpol3 1 to 6) were further tested by hybridization analysis to select an isolate that carried

Figure 5. Construction of KRpol3: hybridization analysis of Ura- isolates

Genomic DNA was isolated from six Ura- isolates. The DNA was digested to completion with *Eco* RI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 4.1 kb *Hind* III- *Sal* I fragment containing *pol3-4DA* from YCp50-4DA. Lane 1: KRpol3-1; Lane 2: KRpol3-2; Lane 3: KRpol3-3; Lane 4: KRpol3-4; Lane 5: KRpol3-5; Lane 6: KRpol3-6. The band sizes are in kb.

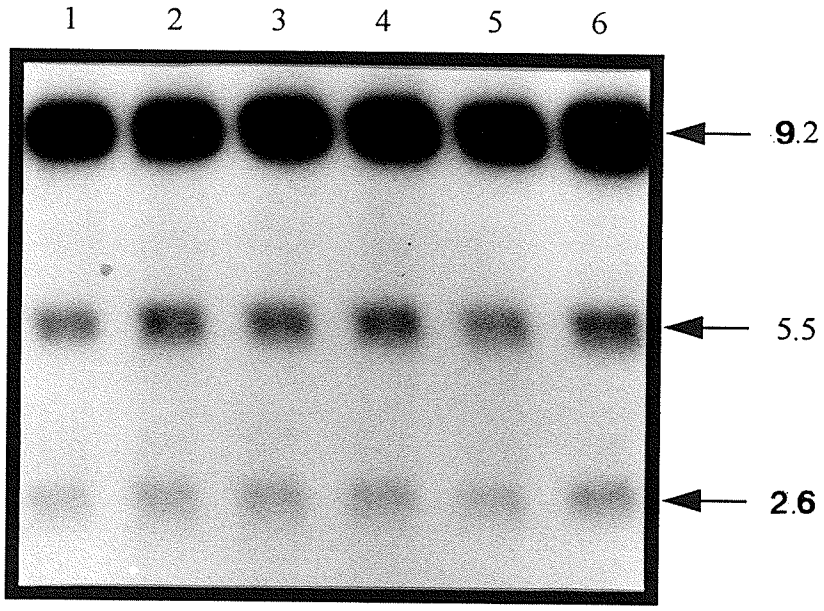

1
2
3
4
5
6
9.2
5.5
2.6

Table 14. Analysis of Mismatch Correction in Wild Type and *Pol3* Strains

Hetero-duplex	Mismatch	Gene Position	MKP-o			pol3-4DA		
			No. of colonies	R:W[$]	Sectored colonies (%)	No. of colonies	R:W[$]	Sectored colonies (%)
H13	[n]*G/G	18	2301	1.9	29	698	1.7	9
H14	[n]G/T*	27	1545	0.5	12	789	0.4	5
H15	[n]*A/C	27	1729	1.0	25	1469	0.9	10
H16	[n]*G/G	51	1708	1.1	23	639	0.9	8
H17	[n]A/A*	55	3142	0.7	26	1255	0.8	9
H18	[n]*T/T	55	1608	1.5	61	688	1.5	26

*- Mutant Allele; n- base on nicked strand; [$]- Non-sectored red : non-sectored white colonies.

YCpKR-4DA but had lost YCp50-4DA. Genomic DNA was isolated from the six KRpol3 isolates. The DNA was digested to completion with *Eco* RI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 4.1 kb *Hind* III- *Sal* I fragment containing *pol3-4DA* from YCp50-4DA. YCp50-4DA is 11.4 kb while YCpKR-4DA is 9.2 kb in size and both these plasmids are linearised with *Eco* RI. All the six isolates displayed only the 9.2 kb band (Figure 5). In addition, they also displayed two smaller bands (5.5 and 2.6 kb). These two bands were expected from the chromosomal disrupted *pol3* gene (Simon, personal communications) and were relatively lighter due to their relatively smaller sizes. The first isolate was designated KRpol3 and was used in the heteroduplex study.

3.1.5.3 Specificity of Heteroduplex repair

The heteroduplexes (H13 to H18) were transformed into KRpol3. The T/T mismatches were repaired much less efficiently (39% and 74% non-sectored colonies in the wild type and KRpol3, respectively) than the other four (G/G, G/T, A/C and A/A) mismatches under study. The G/T mismatches (78% and 95% non-sectored colonies in the wild type and KRpol3, respectively) were corrected most efficiently. In comparison to the wild type, the efficiency of mismatch repair was enhanced in KRpol3 for all the mismatches tested. The magnitude of enhancement was about 90% for T/T mismatches and between 20 and 28% for the other mismatches tested. This shows that in yeast, mismatch correction efficiency is actually increased in a proofreading-deficient strain.

3.2 Characterization of *pol2* proofreading-deficient mutator strains

In order to investigate the specificity of the mutator effect conferred by a proofreading defect in pol ε, I used a strain isogenic to MKP-o, but, with mutations in the *pol2* gene specifically disrupting only the proofreading function. The disruption vector used in the construction of the strain was provided by Dr. Morrison and has been described (Morrison *et al.* 1991). However, a brief description of the construction of the vector is provided in the following section for the benefit of the reader.

3.2.1 Construction of a *pol2* mutant strain

3.2.1.1 Construction of a pol2 exonuclease deficient mutant allele (By Dr. Morrison)

I used the mutation created by Morrison (Morrison *et al.* 1991) to characterize the specificity *pol2* exo- mutator. The mutation comprises a double missense mutation (AA Aspartic acid → Alanine at position 290 and Glutamic acid → Alanine at position 292) in the exo I domain (Morrison and Sugino 1993) of *POL2*. Yeast mutant strains carrying this mutation had wild type polymerizing activity while its proofreading activity was reduced below detectable levels. The mutant also had a mutator phenotype *in vivo* (Morrison *et al.* 1991). The mutation was constructed on a cloned copy of *POL2* using site directed mutagenesis. Subsequently, a 2.1 kb *Hpa I - EcoRI* fragment of the mutated *pol2* gene comprising the 3' end of the gene including the two missense mutations and the 3' flanking region was cloned into the *Nru* I - *Eco* RI interval of plasmid YIp5 and designated YIpJB1 (Figure 6A). This plasmid was constructed and provided by Dr. Morrison (National Institute of Environmental Health Sciences, Research Triangle Park,

North Carolina). The construction and verification of this plasmid is published elsewhere (Morrison *et al.* 1991).

3.2.1.2 Construction of a pol ε exonuclease deficient (exo-) strain

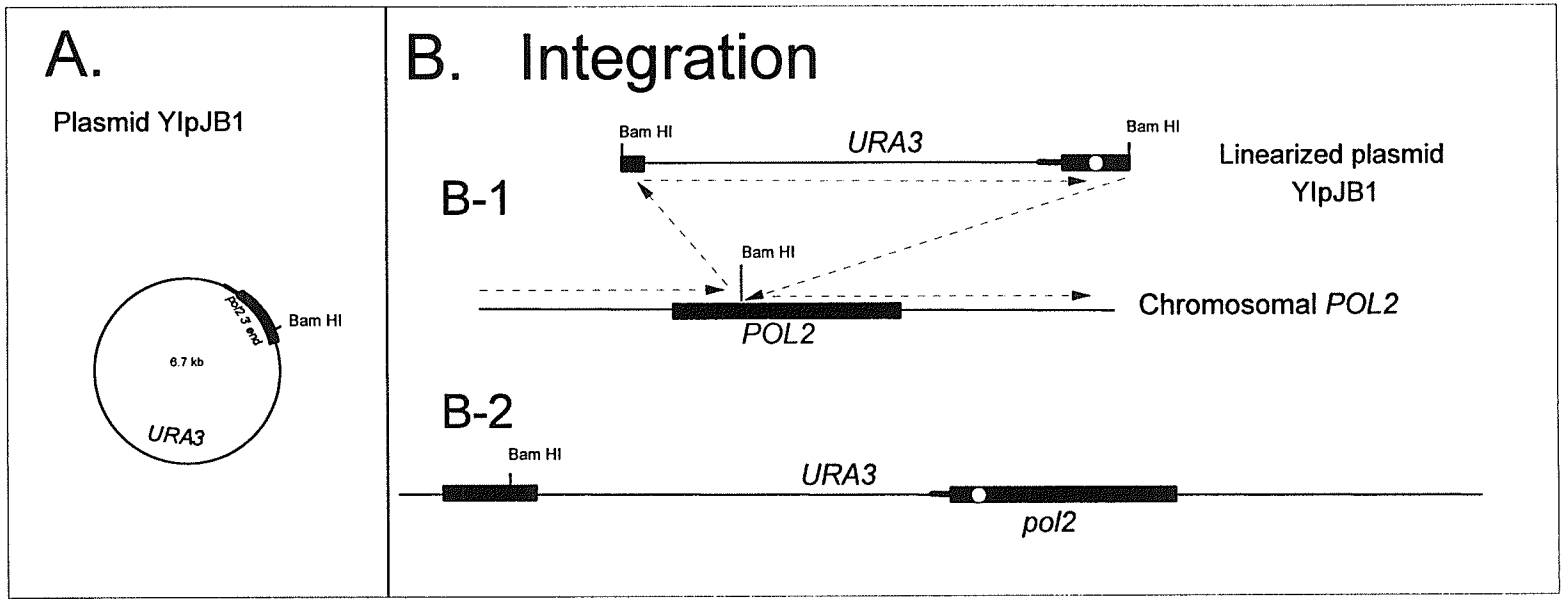
An isogenic *pol2* exo- derivative of MKP-o was constructed as shown in Figure 6. MKP-o was transformed with *Bam* HI-linearized plasmid YIpJB1. Linearization of YIpJB1 with *Bam* HI targets the integration of the plasmid into the *Bam* HI site of *POL2* via homologous recombination (Rothstein 1991). Integration of YIpJB1 into the site of the chromosomal *POL2* will result in the integration of the exo- mutation into the *pol2* gene and duplication of the 2.1 kb 3' end of the gene (Figure 6B). The plasmid-borne *URA3* gene would now be being flanked by the mutant *pol2* and the duplicated 3' end. Thus, integration of YCpJB1 would render the *pol2* gene deficient in proofreading and such a strain would exhibit a mutator phenotype (Morrison *et al.* 1991) (Figure 6B).

Since MKP-o is a uracil auxotroph (Table 2) the *URA3* gene in the plasmid allows integration of YIpJB1 to be detected by selecting for growth on medium lacking uracil. Several Ura+ transformants were picked for analysis. First, the transformants were tested for all the known markers (Ura+, Lys-, Ade-, Leu-, Trp-, His- and Can^R) by transferring some cells to the different AA omission medium and checking for growth after 48 hrs. incubation at 30^o C. The mutator phenotype of some of the isolates that tested positive for all the markers was verified by observing the lysine reversion frequency. This was achieved by plating stationary phase cultures grown in SD-Ura broth on to SD-Ura-Lys plates and comparing the number of emerging Lys+ colonies with appropriately grown

Figure 6. Construction of the KRpol2 strain

Step 1. Integration: *Bam* HI linearized YIpJB1 (A) was integrated into the *Bam* HI site of the chromosomal *POL2* gene as shown in (B).

Step 2. Recombination: The duplicated part of the *POL2* gene along with the plasmid sequence was excised by reciprocal intrachromosomal recombination between the repeated regions as shown in (C). The recombination event will generate either the desired *pol2* mutant as shown in (C2) or a *POL2* revertant as shown in (C3).


A.
Plasmid YIpJB1
pol2 3' end
Bam HI
6.7 kb
URA3
B. Integration
B-1
Bam HI
URA3
Bam HI
Linearized plasmid
YIpJB1
Bam HI
Chromosomal POL2
POL2
B-2
Bam HI
URA3
pol2

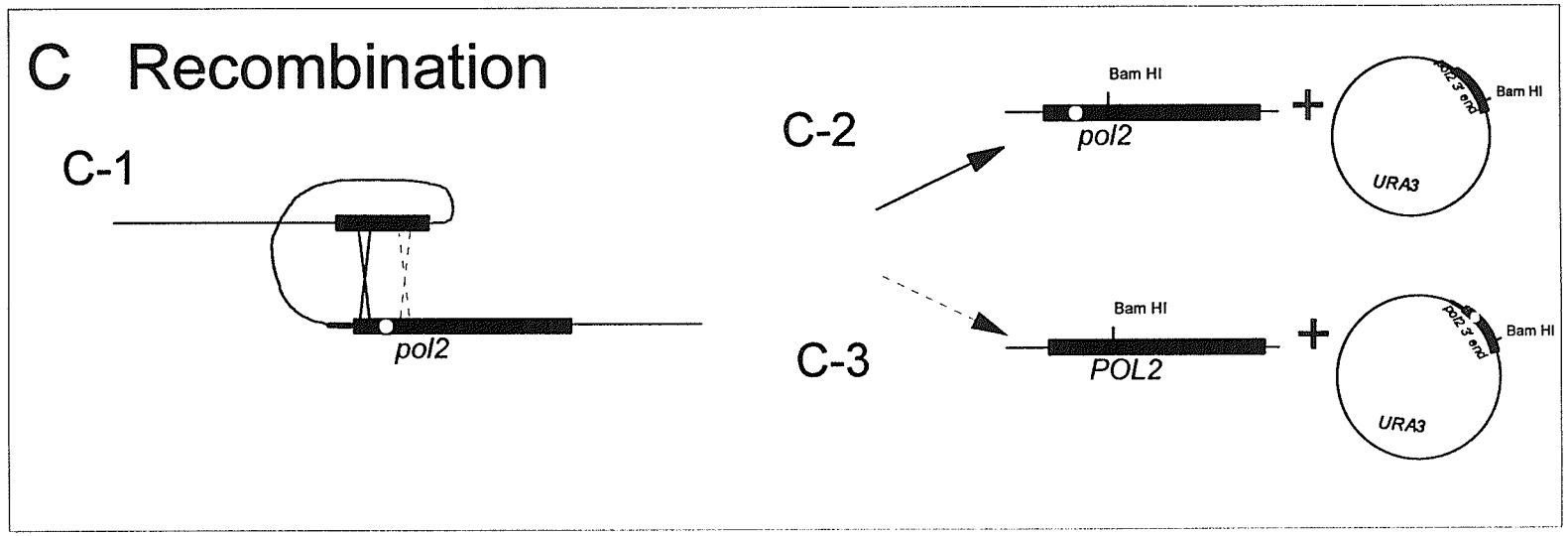
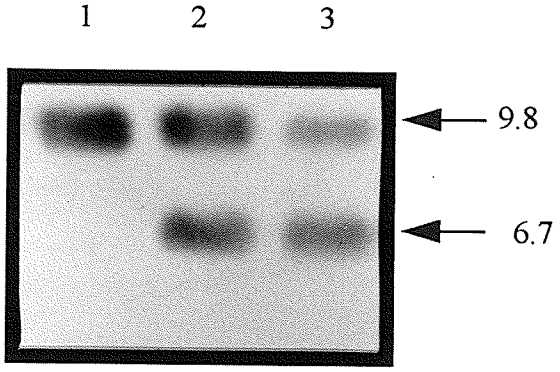

C Recombination
C-1
pol2
C-2
Bam HI
pol2
pol2 3' end
Bam HI
URA3
C-3
Bam HI
POL2
pol2 3' end
Bam HI
URA3

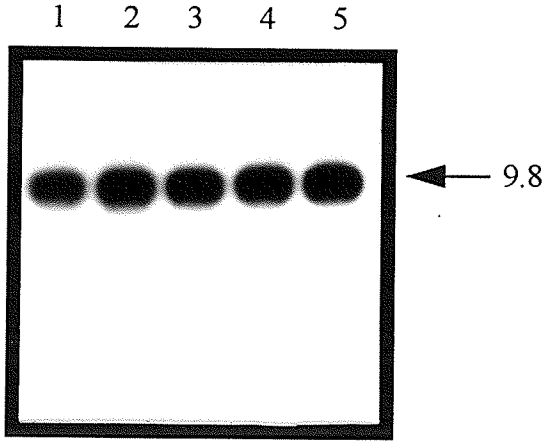
Figure 7. Construction of the KRpol2 strain: hybridization analysis of Ura+ transformants and Ura- isolates

Step 1. Integration: Genomic DNA was isolated from MKP-o and Ura+ transformants. The DNA was digested to completion with *Bam* HI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 0.8 kb *Bam* HI - *Sal* I fragment of *pol2* from YIpJB1. Lane 1: Mkp-o; Lane 2: ε1-1; Lane 3: ε1-2.

Step 2. Recombination: Genomic DNA was isolated from MKP-o and Ura- isolates. The DNA was digested to completion with *Bam* HI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the same probe used in the integration step. Lane 1: MKP-o; Lane 2. ε2–1; Lane 3. ε2–2; Lane 4. ε2–3; Lane 5. ε2–4. The band sizes are in kb.



A



B

BamHI and plated wild type cultures. Cultures displaying a >5 fold increase in the number of Lys+ colonies were designated as mutators. All the tested isolates displayed a mutator phenotype relative to the wild type. Genomic DNA was isolated from MKP-o and two potential *pol2* mutants (ε1-1 and ε1-2). The DNA was digested to completion with *Bam* HI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 0.8 kb *Bam* HI-*Sal* I fragment of *pol2* from YIpJB1. Integration of the plasmid in the desired location should yield a novel 6.7 kb band in addition to a wild type band of unknown size. The required 6.7 kb band as well as the wild type band (which measured 9.8 kb) was detected for all the transformants (Figure 7A). The mutant ε1-1 was used in the further construction steps.

Intrachromosomal recombination occurring between the repeated 2.1 kb *pol2* fragment would delete the *URA3* gene and, depending on the precise location of the recombination event, would either result in the restoration of a wild type gene or integration of the mutation into the gene (Figure 6C). If the recombination event occurs upstream to the mutation, it will result in a mutant allele and if it occurs downstream to the mutation, it will regenerate a wild type allele (Figure 6C). In any case, there should be a close to 50% occurrence for each type.

An overnight culture of ε1-1 grown in SD-Ura was subcultured into YPDA and was grown for 18 hrs. Dilutions of the culture were then plated in FOA medium and incubated at 30° C for 48 hrs., to select for loss of the *URA3* gene (Boeke *et al.* 1984). Several Ura-, FOA^R colonies were picked and tested for the known genomic markers as in the integration step. Ten isolates that tested positive for all the markers were tested for

mutator phenotype by determining the frequency of lysine reversion, as in the integration step, and comparing it to the corresponding values from ε1-1 and wild type. The isolates that resembled the ε1-1 values were designated as mutators. Four isolates ε2–1, ε2–2, ε2–3 and ε2–4 displayed a mutator relative mutator phenotype.

Genomic DNA was isolated from MKP-o and the four potential *pol2* mutants. The DNA was digested to completion with *Bam* HI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 0.8 kb *Bam* HI-*Sal* I fragment of *pol2* from YIpJB1. Recombination would result in the loss of the 6.7 kb seen after the integration step (Figure 6C). Thus, the mutants would have a band similar to the wild type. All the isolates tested displayed the desired banding pattern (Figure 7B). The first isolate ε-2-1 was designated KRpol2 and was used for further characterization.

KRpol2 was transformed with YCpKR-Y or YCpMS-I to obtain two strains pol2-Y and pol2-I, isogenic to WT-Y and WT-I (see section 2.3), respectively.

3.2.2 Specificity of the *pol2* mutator

3.2.2.1 Lysine and adenine reversion frequencies.

Reversion of *lys2-1* was detected by selecting for lysine prototrophs (Lys^+). Since all of the strains used in this study also carry the *ade2-1* allele, *lys2-1* intragenic locus reversion results in the emergence of red, Lys^+ colonies whereas extragenic ochre suppression of *lys2-1* gives rise to white, Lys^+ colonies (see section 2 for description of these alleles). Reversion of ade*2-1* was detected by selecting for adenine prototrophs

Table 15. Lys^- and Ade^- Reversion Frequency

Strain	$Lys^- \rightarrow Lys^+$ Reversion ($\times 10^{-8}$)	$Lys^- \rightarrow Lys^+$ Supression ($\times 10^{-7}$)	$Ade^- \rightarrow Ade^+$ ($\times 10^{8}$)	$Can^S \rightarrow Can^R$ ($\times 10^{-8}$)
WT-Y	0.2	3.4	0.52	--
pol2	4.2	18.2	9.15	--
KRCMKP-o	--	--	--	9.3
KRCpol2	--	--	--	80.91

Frequency values are the medians of 30 independent cultures

Table 16. Plasmid Retention

Strain	Colonies on selective medium	Colonies on non-selective medium	Percent plasmid retention
WT-Y	17,165	19,567	87.7
pol2-Y	19,673	22,453	87.6
WT-I	21,182	23,886	88.7
pol2-I	21,480	24,958	86.1

Colony counts are pooled values from 30 independent cultures

(Ade^+), but *ade2-1* revertants were not categorised into locus revertants and suppressor mutations, only total reversion was measured. The frequency of lysine reversion through locus reversion and suppression was increased by 21 and 5.4 fold, respectively in the *pol2* background when compared to the wild type. The frequency of adenine reversion was also increased by 17.6 fold in the *pol2* background. These increases reflect the *pol2* mutator phenotype (Table 15).

3.2.2.2 Plasmid retention

The relative differences between a wild type (Y or I) and its corresponding *pol2* mutant should reflect the property of the *pol2* mutator since the two strains under consideration are isogenic except for the *pol2* locus. There was no difference between the plasmid retention values for the four strains which ranged between 86.1% and 88.7%. This indicates that the *pol2* proofreading defect did not alter plasmid retention (Table 16).

3.2.2.3 Interference of the specificity of the pol2 mutator in the genetic screen for *SUP4-o mutants*

When the strains pol2-Y and pol2-I were plated on SD-His+Can to isolate *SUP4*-o mutants as canavanine-resistant red colonies, a very heavy background growth was observed. When cells were diluted (100 fold relative to wild type) and plated, a large number of small white colonies, too numerous to count, were observed (Figure 8.3 [pol3-Y] and 8.4 [pol3-I]). Initially, this situation was attributed to a defective genetic background or an experimental error during the strain construction. But, five independent

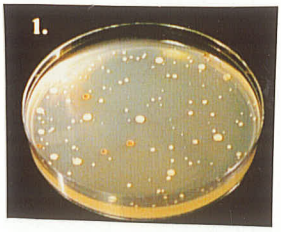
Figure 8. Comparison of the induction of CAN^R colonies by the wild type and *pol2* strains

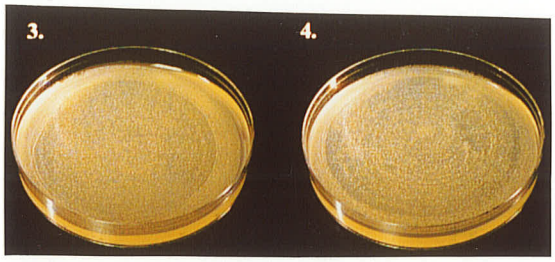
Plates 1 and 2: Wild type cells were plated (3 x 10^6 cells/plate) on SD -His +CAN plates and incubated for 6 days at 30° C. Plate 1 : WT-Y; Plate 2: WT-I.

Plates 3 and 4: *pol2* mutant cells were plated (3 x 10^4 cells/plate) on SD -His +CAN plates and incubated for 6 days at 30° C. Plate 1 : pol2-Y; Plate 2: pol2-I.

Plates 5: A group two Ura- isolate was plated (3 x 10^6 cells/plate) on SD -His +CAN plates and incubated for 6 days at 30° C.

Plate 6: A white CAN^R colony from Plate 3 was streaked on an SD+ALL plate and incubated for 3 days at 30° C.


1.


3.
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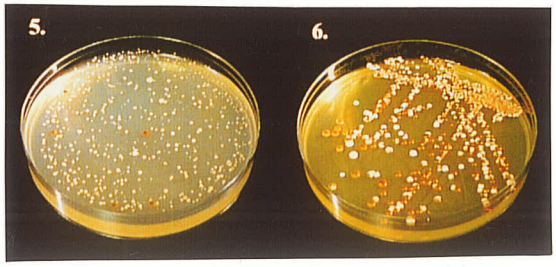

5.
6.

strain construction experiments using five different, tested, genetic stocks of the wild type yielded the same result. A construction was also attempted using WT-Y and WT-I as the genetic back ground without any success.

3.2.2.3.1 Determination of number of cell doubling before inhibition by canavanine

Since most of the *pol2* colonies coming up on the canavanine plates were very tiny and white, it could be possible that the *pol2* cells sustained a significant number of doublings on these plates before being inhibited. A microscopic observation of the cell growth of WT-Y and WT-I cells on canavanine plates revealed that these cells undergo up to three cell divisions before being inhibited by canavanine. A similar experiment with pol2-Y and pol2-I revealed that the average number of doublings for *pol2* cells was 3.2 before being inhibited, which is not different from the wild type. Thus, the induction of the tiny white colonies cannot be attributed to increased background growth before inhibition by canavanine.

3.2.2.3.2 Determination of CAN1 mutation rate

The colonies produced by the *pol2* strains on canavanine plates were white in color. It is possible that *pol2* mutator has a hot spot on the *CAN1* and generates a large number of unsuppressible mutations on the suppressed *can1-100* gene, thus forcing it to loose the suppressability and result in a large increase in canavanine resistant white colonies. To probe this possibility, I constructed wild type and *pol2* strains with functional *CAN1* gene instead of *can1-100* on the chromosome so that these strains can

be used to calculate the forward spontaneous mutation frequency of the *CAN1* locus *in vivo* .

A *CAN1* replacement vector, YIpLac211-CAN (Roche *et al.* 1995), was provided by Dr. Gietz (University of Manitoba, Canada). The plasmid was constructed by cloning a 1.8 kb *Bam* HI - *Sal* I yeast chromosomal DNA fragment carrying the *CAN1* gene into a derivative of the *URA3* containing plasmid, YIpLac211 (Gietz and Sugino 1988) lacking the *Eco* RI site in the polylinker region (Figure 9A). This plasmid was linearized with *Eco* RI and transformed into MKP-o and KRpol2. Linearization of the plasmid with *Eco* RI targets the integration of the plasmid into the *Eco* RI site of the chromosomal *can1-100* locus such that the *URA3* and bacterial DNA sequences of the plasmid are flanked on one end by the *can1-100* gene and by *CAN1* on the other (Figure 9B). Three potential integrants (MC1-1 [MKP-o derivative], KC1-1 and KC1-2 [KRpol2 derivatives]) were picked and analyzed for integration of the plasmid by DNA hybridization analysis. Genomic DNA was isolated from wild type MKP-o, and the three potential *CAN1* integrants. The DNA was digested to completion with *Eco* RI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 1.4 kb *Hind* III fragment containing *CAN1* from YIpLac211-CAN. Integration of the plasmid in the desired location should yield a novel 5.5 kb band in addition to a wild type band of unknown size (Figure 9B). The required 5.5 kb band as well as the wild type band (which measured 12.2 kb) was detected for all the transformants (Figure 10A). The isolates MC1-1 and KC1-1 were used in the further construction steps.

The two integrants were grown overnight in SD-Ura, subcultured into YPDA and plated on FOA plates (Boeke *et al.* 1984). FOA^R colonies were first screened for

Figure 9. Construction of KRCMKP-o and KRCpol2

Step 1. Integration: *Eco* RI linearized YIpLac211CAN (A) was integrated into the *Eco* RI site of the chromosomal *can1-100* gene as shown in (B).

Step 2. Recombination: One copy of the gene (*CAN1/can1-100*) along with the plasmid sequence was excised by reciprocal intrachromosomal recombination between the repeated regions as shown in (C). The recombination event will generate either the desired *CAN1* isolate as shown in (C2) or a *can1-100* revertant as shown in (C3).

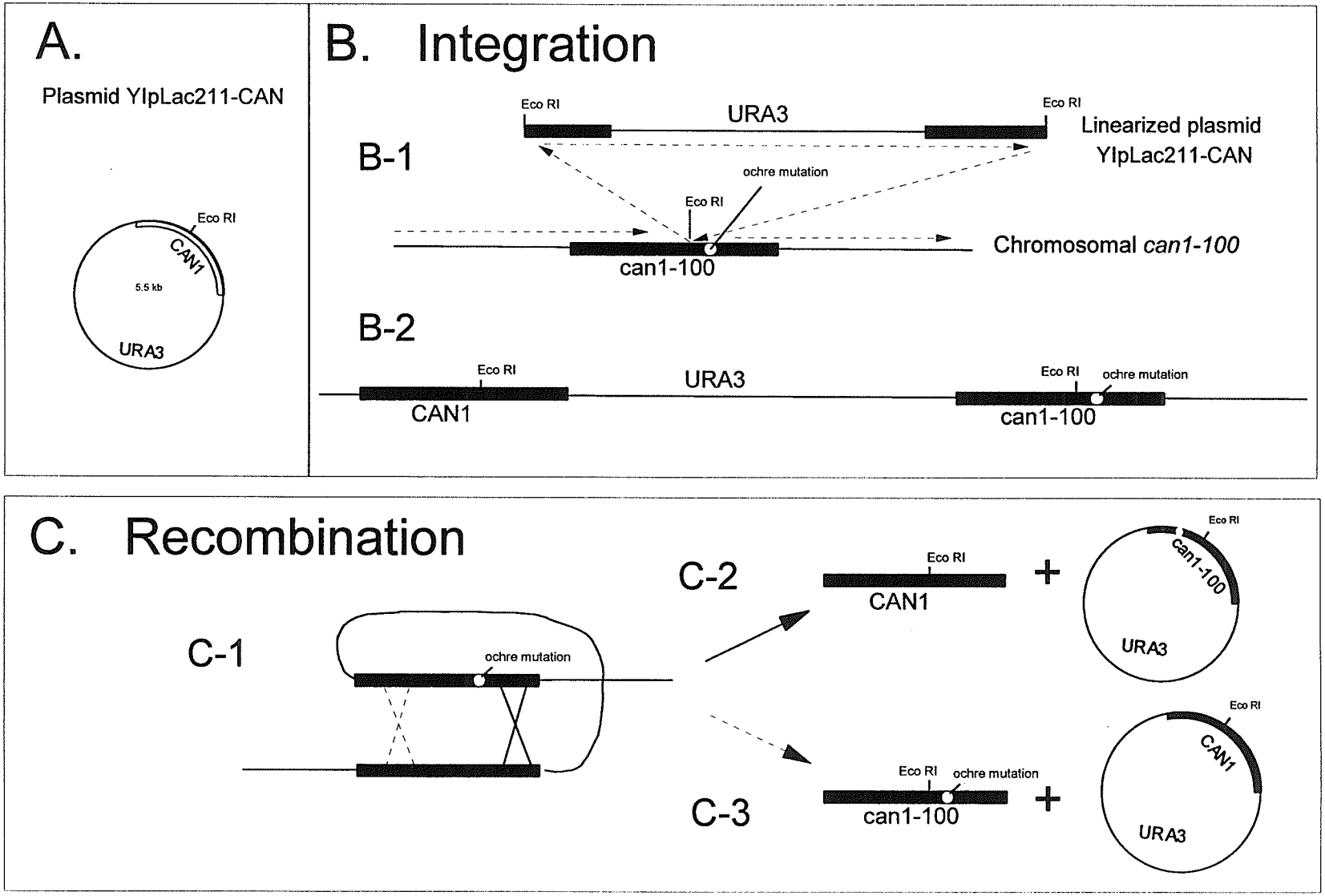
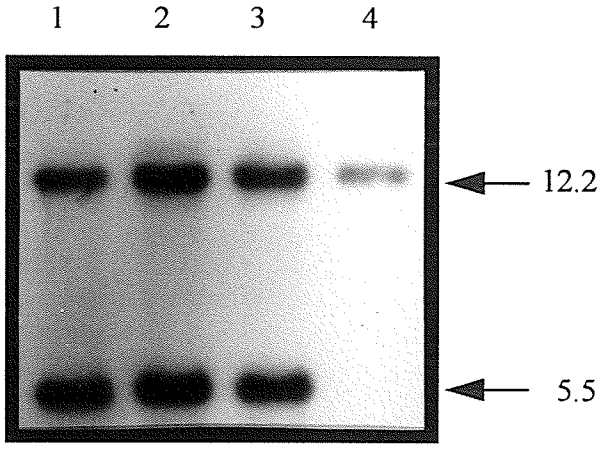

A.
Plasmid YIpLac211-CAN
Eco RI
CAN1
5.5 kb
URA3
B. Integration
B-1
Eco RI
URA3
Eco RI
Linearized plasmid
YIpLac211-CAN
ochre mutation
Eco RI
Chromosomal can1-100
can1-100
B-2
Eco RI
URA3
Eco RI
ochre mutation
CAN1
can1-100
C. Recombination
C-1
ochre mutation
C-2
Eco RI
CAN1
+
Eco RI
can1-100
URA3
C-3
Eco RI
ochre mutation
can1-100
+
Eco RI
CAN1
URA3

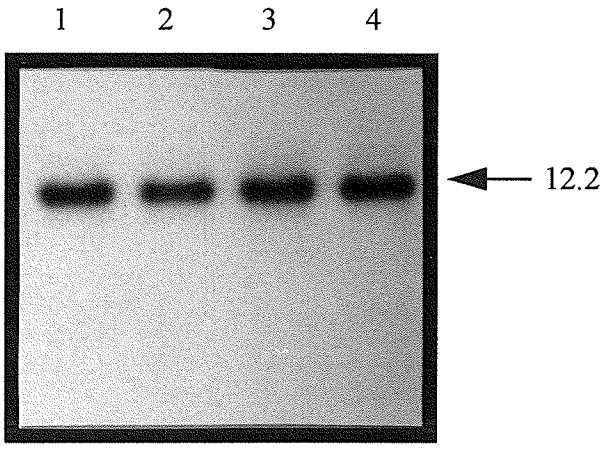
Figure 10. Construction of KRCMKP-o and KRCpol2: hybridization analysis of Ura+ transformants and Ura- isolates

Step 1. Integration: Genomic DNA was isolated from wild type MKP-o, and three potential *CAN1* integrants. The DNA was digested to completion with *Eco* RI and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the 1.4 kb *Hind* III fragment containing *CAN1* from YIpLac211-CAN. Lane 1: MC1-1; Lane 2: KC1-1; Lane 3: KC1–2; Lane 4: MKP–o. The band sizes are in kb.

Step 2. Recombination: Genomic DNA was isolated from MKP-o and three potential *CAN1* isolates. The DNA was digested to completion with *Eco RI* and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the same probe used in the integration step. Lane 1: MC2-1; Lane 2: KC2-1; Lane 3: KC2–2; Lane 4: MKP–o. The band sizes are in kb.



A



B

canavanine sensitivity by plating on canavanine medium. The CAN^S isolates were designated as potential *CAN1* integrants. One MC1-1 derivative designated MC2-1 and two KC1-1 derivatives designated KC2-1 and KC2-1 were further analyzed by hybridization. Intrachromosomal recombination occurring between the repeated sequences of *can1-100* and *CAN1* would delete the *URA3* gene and, depending on the precise location of the recombination event, would either result in the restoration of a wild type *CAN1* gene or a mutant *can1-100* gene (Figure 9C). If the recombination event occurs upstream to the mutation, it will result in a mutant allele and if it occurs downstream to the mutation, it will regenerate a wild type allele (Figure 9C). Although there should be a close to 50% occurrence for each type, all the isolates chosen for hybridization analysis were expected to be *CAN1* as they exhibited a CAN^S phenotype.

Genomic DNA was isolated from MKP-o and the three potential *CAN1* isolates. The DNA was digested to completion with *EcoRI* and electrophoresed in an agarose gel. The gel was then denatured, neutralized and dried. The dried gel was probed with the same probe used in the integration step. Recombination would result in the loss of the 5.5 kb seen after the integration step (Figure 9C). Thus, the mutants would have a band similar to the wild type. All the isolates tested displayed the desired banding pattern (Figure 10B). The MKP-o derivative (MC2-1) was designated KRCMKP-o and the KC2-1 was designated KRCpol2 and was used for further characterization.

The frequency of spontaneous forward mutation in the chromosomal *CAN1* locus was analyzed using these CAN^S strains. Relative to the KRCMKP-o (wild type), the KRCpol2 rate was increased 8.7 fold. This is consistent with the 5 fold increase observed

by other researchers (Morrison *et al.* 1991). The observed 8.7 fold increase is not sufficient to account for the heavy increase in CAN^R colonies observed in the pol2-Y and pol2-I strains.

3.2.2.4 Induction of antisuppressor mutations in pol2 strains

It would be possible that the *pol2* mutator had a hot spot for antisupressor mutations, that affects the functioning of the supressor gene in supressing the *can1-100* locus. An antisupressor mutation, a defect in any one of the several steps of t-RNA processing, would reduce the level of supressor activity available which in the case of our system could lead to increase in canavanine resistance of cells in spite of harboring a functional *SUP4*-o gene (Dr. Kunz, personal communication). In this situation, the extent of supression required by the *can1-100* locus is hypothesized to be relatively higher than that required for *ade2-1* to explain the white colored CAN^R mutant induction.

In order to determine whether the heavy induction of CAN^R colonies was due to antisuppressors and not due to loss of functional *SUP4*-o gene, plasmid DNA was isolated from several of the CAN^R colonies resulting from plating pol2-Y and pol2-I on SD-His+Can plates. The plasmids were then transformed into MKP-o. Upon testing the transformants, it was confirmed that the *SUP4*-o gene was functional as it successfully suppressed all the three suppressible markers of MKP-o. When plated on SD-His+Can, they resembled the WT-Y and WT-I strains (Figure 8.1 [WT-Y] and 8.2 [WT-I]). Some of the CAN^R white colonies that emerged when pol2-Y was plated on a SD-His+Can plate was streaked on an SD+ALL plate, which is non-selective for the plasmid. This resulted

in a collection of white, red and sectored colonies representing colonies formed by cells that still retain the plasmid, cells that have lost the plasmid and cells that have lost the plasmid after a few divisions, respectively (Figure 8.6). This is consistent with the normal behavior of strains carrying a centromeric *SUP4*-o plasmid, as seen, when plated on fully supplemented plates to assess the viability for determination of plasmid retention. This is consistant with the possibility that the induction of the potential antisupressor mutation reduced the supression of the *can1-100*, but, not the *ade2-1* locus.

In a final attempt to confirm the induction of antisupressor mutations in the *pol2* background, ε1-1 was transformed with YCpKR-Y or YCpMS-I and designated ε1-1Y and ε1-1I. These two strains were grown in SD-Ura-His medium in order to maintain selection for the plasmid as well as the stability of the YIpJB1 integration. The strains are now *pol2* mutators as the integration of YIpJB1 would result in *URA3* being flanked by a mutant *pol2* gene and a 2.1 kb duplicated 3' end of the gene. Independent cultures of either of these strains was plated on SD-Ura-Can. There was an increase in the number of white CAN^R colonies, too numerous to count even when plated to 100 fold dilutions (resembling pol2-Y and pol2-I plates shown in Figure 8.3 and 8.4) compared to the wild type which showed between 30 and 50 such colonies (Figure 8.1 [WT-Y] and 8.2 [WT-I]) (probably resulting from non-suppressible mutations on *can1-100*). Additionally, several independent cultures of ε1-1Y was grown in SD-His, a medium selective for the *SUP4*-o plasmid but not for YIpJB1 (therefore inducing the recombinational excision of the *URA3* and the duplicated *pol2* region) to stationary phase. These were then subcultured into SD-His broth (ca 33 cells per ml), grown to stationary phase and plated on SD-His+Can

medium after freezing a portion of the culture in 50% glycerol for future analysis. The cultures were divided into two groups based on the *CAN*^{R} colony count data: 1. group one which resembled the previous experiment results (too numerous to count even at 100 fold dilution relative to wild type [Figure 8.3 and 8.4]) and 2. group two with relatively fewer *CAN*^{R} colonies (Figure 8.5) (an average of 1.5 to 2 fold increase in white CAN^R colonies than wild type). The *SUP4*-o plasmid loss was induced in several of these isolates by growing on YPDA broth and plating on SD+ALL plates and were testing on appropriate AA omission medium. Red, His$^-$, Ade$^-$, Lys$^-$, *CAN*^{R} colonies were used for lysine reversion analysis. Lysine reversion analysis revealed that group one isolated showed a 10 to 15 fold increase in the overall frequency of lysine reversion (data not shown), while group two isolates displayed a wild type levels of reversion. Based on these observations, it was concluded that group one isolates are *pol2* (caused by a recombinational event upstream of the *pol2* mutation on YIpJB1) while group two isolates are *POL2* revertants (caused by a recombinational event downstream of the *pol2* mutation on YIpJB1). The small increase in the group two isolates is probably due to a few *pol2* cells in the initial inocula. So it is clear that the presence of the *pol2* phenotype results in the induction of elevated levels of antisupressor mutations that reduced the suppressability of the *can1-100* mutation in the *pol2* strains. This situation created a problem for the analysis of the specificity of the *pol2* mutator using our system because the selection of *SUP4*-o mutants was not possible. Accordingly, no further characterization of the *pol2* mutator was done.

3.3 Characterization of *pms1*Δ mismatch correction deficient mutator strains

In order to investigate the specificity of the mutator effect conferred by a mismatch correction defect, an MKP-o derivative, deficient in mismatch correction was required. There are at least three groups of mismatch genes identified to date in yeast, i.e., the *MSH* genes, the *PMS* genes and the *MLH* genes. It is believed that gene products from these three gene families are involved in the same mismatch correction pathway during the nuclear mismatch correction process (see introduction). Hence, deletion of any one of the genes would result in a deficiency in mismatch correction. It was decided to use a *pms1*Δ (*PMS1* deletion mutant) strain to characterize a defect in mismatch correction because a *PMS1* deletion vector that can be used to construct such a deletion strain was available in our laboratory. The *PMS1* gene of MKP-o was deleted to obtain an isogenic mismatch deficient strain.

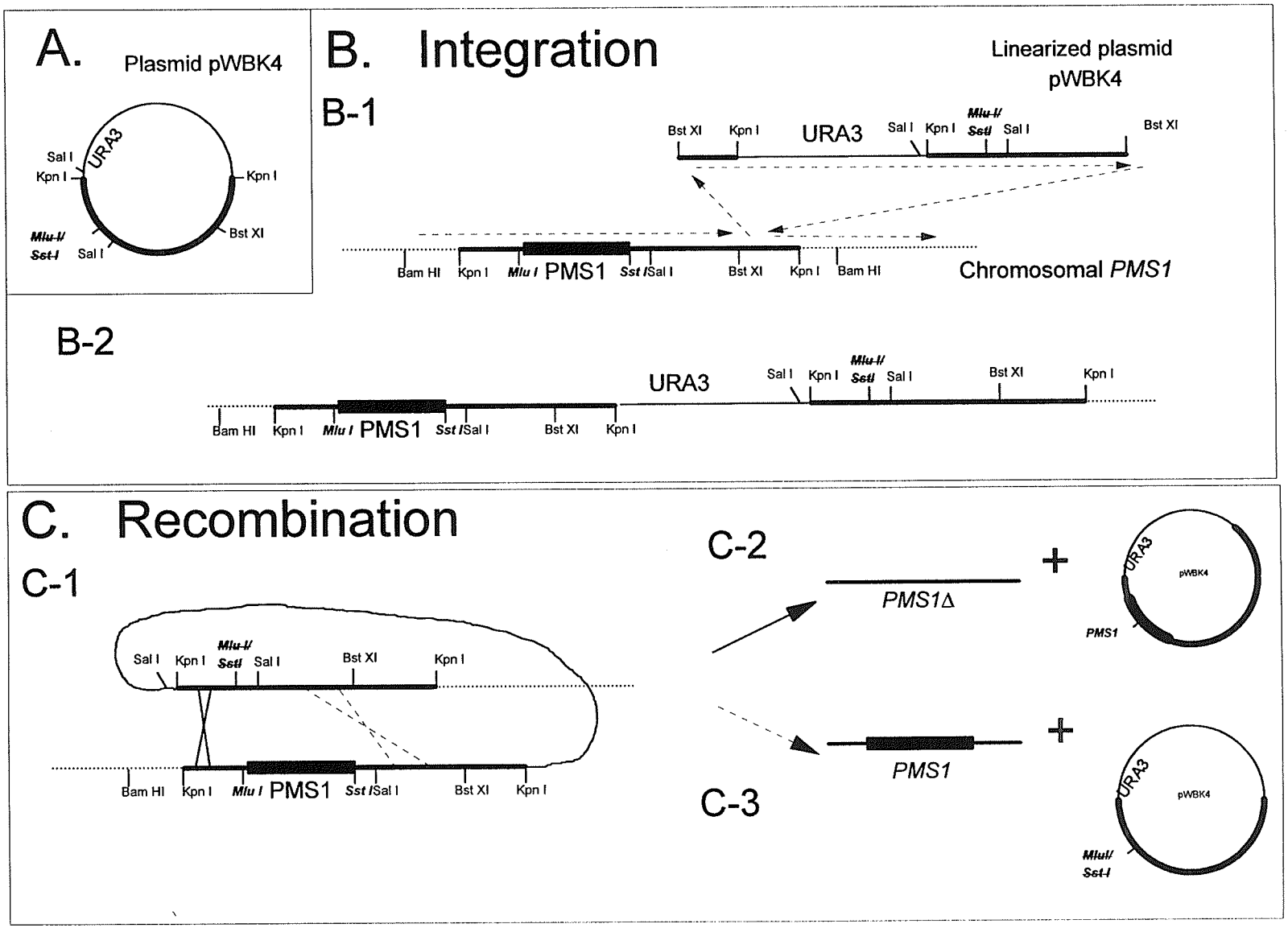
3.3.1 Construction of a *pms1* mutant Strain

KRpms1, an isogenic derivative of MKP-o having the entire ORF of the *PMS1* gene deleted, was constructed as diagrammed in Figure 11. The *PMS1* deletion plasmid pWBK4 (Figure 11A) used in the construction has been described (Kramer *et al.* 1989b). A description of the plasmid is provided here for the benefit of the reader. Plasmid pWBK4 is a yeast integrating plasmid containing *URA3* as a selectable marker in yeast. It also carries a 5.2 kb *Kpn* I yeast chromosomal DNA fragment encompassing the *PMS1* locus and flanking DNA, but, the 2.7 kb *Mlu* I - *Sst* I fragment carrying the entire ORF of the *PMS1* gene has been deleted. Transformation of yeast cells with *Bst* XI-linearized pWBK4 targets

Figure 11. Construction KRpms1

Step 1. Integration: *Bst* XI linearized pWBK4 (A) was integrated into the *Bst* XI site beside the *PMS1* gene in the chromosome, resulting in the duplication of its flanking region as shown in (B).

Step 2. Recombination: The duplicated sequence along with the plasmid sequence was excised by reciprocal intrachromosomal recombination between the repeated regions of flanking regions of the gene as shown in (C). The recombination event will generate either the desired *pms1*Δ isolate as shown in (C2) or a *PMS1* revertant as shown in (C3).



Disruption of *PMS1*

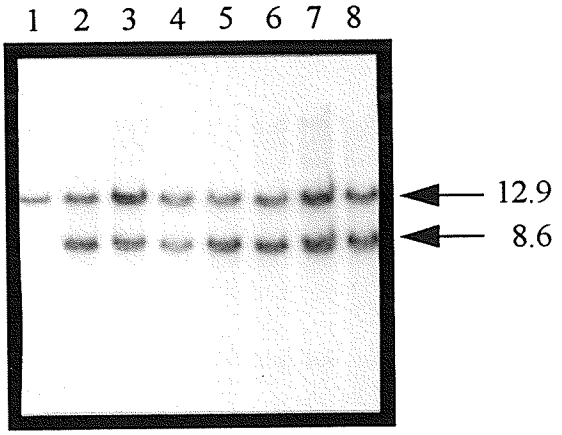
plasmid integration to the *Bst* XI site 3' of *PMS1* via homologous recombination (Rothstein 1991). Based on this principle, the plasmid was introduced into MKP-o (Figure 11B). Since MKP-o is a uracil auxotroph, (Table 2), the *URA3* gene in the plasmid allowed integration to be detected by selection for growth on medium lacking uracil. Integration of pWBK4 in Ura^+ transformants was confirmed by genomic DNA hybridization analysis. Genomic DNA from MKP-o as well as seven Ura^+ transformants, KRP1-1 to KRP1-7, were digested to completion with *Sal* I and electrophoresed on an agarose gel. The DNA in the gel was denatured, and the gel was dried. The dried gel was used for DNA hybridization analysis using the 1.3 kb *Bst XI* - *Kpn* I yeast DNA fragment from pWBK4 as a probe (Figure 11A). The integration of pWBK4 at the *Bst* XI site 3' to *PMS1* should result in the appearance of a novel 8.6 kb band in addition to the 12.9 kb band expected for genomic DNA from MKP-o. The 8.6 kb band was detected for all transformants (Figure 12A).

Intrachromosomal recombination between the repeated 1.3 kb *Kpn* I - *Mlu* I fragments in the construct deletes *PMS1* and *URA3* (Figure 11C). Thus, loss of *PMS1* can be monitored by scoring for elimination of *URA3*. by selecting for FOA^R colonies on FOA plates. Since the *Bam* HI chromosomal DNA fragment encompassing *PMS1* is 19.2 kb, and *PMS1* is approximately 2.7 kb, the recombination event that deletes most of the insert including *PMS1* reduces the 19.2 kb *Bam* HI fragment to 16.5 kb (Figure 11C). KRP1-1 was grown to stationary phase in SD-Ura medium, subcultured into YPDA, grown for 18 hrs. and then plated on FOA medium to select isolates that had lost the *URA3* marker (Boeke *at al.* 1984). Two Ura^+ isolates were tested for the change in band size, mentioned above, by DNA hybridization analysis. Genomic DNA from the two Ura^- isolates, KRP2-1

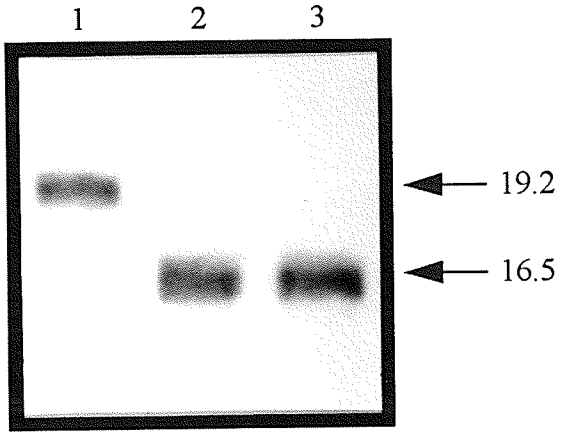
Figure 12. Construction KRpms1: hybridization analysis of Ura+ transformants and Ura- isolates

Step 1. Integration: Genomic DNA from MKP-o as well as seven Ura^+ transformants were digested to completion with *Sal* I and electrophoresed on an agarose gel. The DNA in the gel was denatured, and the gel was dried. The dried gel was used for DNA hybridisation analysis using the 1.3 kb *Bst XI* - *Kpn* I yeast DNA fragment from pWBK4 as a probe. Lane 1: MKP-o; Lane 2: KRP1-1; Lane 3: KRP1-2; Lane 4: KRP1-3; Lane 5: KRP1-4; Lane 6: KRP1-5; Lane 7: KRP1-; Lane 8: KRP1-7. The band sizes are in kb.

Step 2. Recombination: Genomic DNA from MKP-o as well as two Ura- isolates were digested to completion with *Bam* HI and electrophoresed on an agarose gel. The DNA in the gel was denatured and the gel was dried. Hybridisation analysis was performed on the gel using the same probe used in the integration step. Lane 1: MKP-o; Lane 2: KRP2-1; Lane 3: KRP2-2. The band sizes are in kb.



A



B

and KRP2-2, as well as MKP-o, was digested with *Bam* HI, and electrophoresed on an agarose gel. The DNA in the gel was denatured and the gel was dried. Hybridization analysis was performed on the gel using the same probe used in the integration step. Both the mutant isolates exhibited the expected change in fragment size from 19.2 kb to 16.5 kb (Figure 12B).

KRP2-1 was transformed with YCpKR-Y of YCpMS-I to create two strains designated pms1-Y and pms1-I which were used to assay the specificity of mismatch correction defect on the spontaneous mutation of *SUP4*-o in either orientation.

3.3.2 Specificity of the *pms1*Δ mutator

The specificity of the *pms1*Δ mutation was determined by DNA sequence characterization of spontaneous mutations arising in the *SUP4*-o gene in the *pms1*Δ background (pms1-Y or pms1-I) and comparing it to the results obtained from similar experiments in the corresponding wild type backgrounds (WT-Y or WT-I; these are the same set of data used as wild type for the *pol3* strains). The data were categorized into four pairs for comparison purposes as in the case of the *pol3* strains. The four pairs comprised, pms1-Y vs. WT-Y forming the Y-pair, pms1-I vs. WT-I forming the I-pair, pms1-I vs. pms1-Y forming the pms1-pair and WT-I vs. WT-Y forming the WT-pair. Since the WT-pair has been discussed previously along with the *pol3* , it will not be presented here. The different classes of mutations isolated from the wild type and mutant strains are provided in Table 20, 21 and 22.

3.3.2.1 Plasmid Retention and SUP4-o Mutation Rate

The relative differences between a wild type (Y or I) and its corresponding *pms1*Δ mutant should reflect the property of a *pms1*Δ mutator since the two strains under consideration are isogenic except for the *pms1*Δ locus. There was no difference between the plasmid retention values for the four strains which ranged between 87.6% and 88.7% (Table 17). This indicates that the *pms1*Δ induced mismatch correction defect did not alter plasmid retention and plasmid loss will not interfere in the recovery of *SUP4*-o mutants.

Relative to the wild type, the *pms1*Δ spontaneous *SUP4*-o mutation rate was increased by 10.6 and 12 fold in the Y- and I-pairs, respectively (Table 18). There was no difference in the mutation rate between pms1-Y and pms1-I (pms1-pair). The mutation rate difference between the wild type and corresponding *pms1*Δ strains reflect the *pms1*Δ mutator effect. Additionally, the finding that there was no significant difference in the mutation rate between pms1-Y and pms1-I strains suggests that the *pms1*Δ mutator is not modulated by the *SUP4*-o orientation i,e., strand identity of the *SUP4*-o gene.

3.3.2.2 Lysine and adenine reversion frequencies.

The frequencies of lysine locus reversion and lysine suppression reversion was increased by 26.5 and 4.5 fold, respectively in the *pms1*Δ background when compared to the wild type (Table 19). The frequency of adenine reversion was also increased by 14.8

Table 17. Plasmid Retention

Strain	Colonies on selective medium	Colonies on non-selective medium	Percent plasmid retention
WT-Y	17,615	19,567	87.7
pms1-Y	20,895	23,854	87.6
WT-I	21,182	23,886	88.7
pms1-I	21,040	23808	88.4

Colony counts are pooled values from 30 independent cultures

Table 18. Spontaneous *SUP4*-o Mutation Rate and Frequency

Strain	Mutation Frequency ($\times 10^{-6}$)	Mutation Rate ($\times 10^{-7}$)
WT-Y	0.58	2.0
pms1-Y	11.0	21.1
WT-I	0.58	1.9
pms1-I	11.9	22.7

Frequency values are the medians of 30 independent cultures

Table 19. Lys^- and Ade^- Reversion Frequency

Strain	$Lys^- \rightarrow Lys^+$		$Ade^- \rightarrow Ade^+$
	Reversion ($\times 10^{-8}$)	Supression ($\times 10^{-7}$)	($\times 10^{-8}$)
WT-Y	0.2	3.4	0.52
pms1	5.3	15.4	7.7

Frequency values are the medians of 30 independent cultures

fold in the *pms1*Δ background (Table 19). These increases reflect the *pms1*Δ mutator phenotype and suggest that *PMS1* is involved in correction of replication errors which if left uncorrected would eventually lead to mutations.

3.3.2.3 Mutational classes detected

The different classes of mutations isolated from the wild type and mutant strains are provided in Table 20. SBPS, -1bp and +1bp were the predominant events detected in all the cases. The relative rates of these classes were higher in the *pms1*Δ strains in both the Y- and I-pairs. The relative fraction of SBPS was significantly reduced the pms1 background ($P < 0.01$) while the fractions of -1bp and +1bp were increased ($P < 0.01$ in both cases). Additionally, in the case of the *pms1* strain, rates of SBPS, -1bp or +1bp were increased by 7, 37.8 or 61.1 fold in the Y-pair or by 8.9, 33.2 or 41.1 fold in the I-pair (Table 20). This suggests that the mismatch correction pathway corrects replication errors that lead to single base-pair deletion and insertion mutations much more efficiently than those that lead to SBPS. This observation is consistant with earlier findings from our lab (Yang 1995).

As in the case of the wild type and *pol3* strains, most of the -1bp and +1bp events detected in the pms1-Y and pms1-I were at the run of 5 G·C pairs at position 79 → 83. This adds evidence to the earlier suggestion that the polymerase must have a low efficiency in accurately replicating simple repeat sequences probably due to a high degree of slippage by the DNA polymerase at nucleotide runs (Kunkel 1993; Strand *et al.* 1993). The rate increase in the *pms1*Δ strains relative to the WT strains was highest for

Table 20. Sequence Alterations in the *SUP4*-o Gene

Alteration	WT-Y		pms1-Y		WT-I		pms1-I	
	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})
SBPS	173 (86.9)	17.4	124 (59.0)**	123.0	161 (84.3)	16.1	133 (62.4)**	142.0
NTBPS	None Detected		None Detected		None Detected		None Detected	
1 bp deletion	15 (7.5)	1.5	57 (26.9)**	56.7	17 (8.9)	1.7	53 (24.9)**	56.5
>1 bp deletion	1 (0.5)	0.1	None Detected		3 (1.6)	0.3	None Detected	
1 bp Insertion	5 (2.5)	0.5	31 (14.6)**	30.8	7 (3.7)	0.7	27 (12.7)**	28.8
Ty insertion	3 (1.5)	0.3	None Detected		None Detected		None Detected	
Duplication	1 (0.5)	0.1	None Detected		1 (0.5)	0.1	None Detected	
Complex change	None Detected		None Detected		2 (1)	0.2	None Detected	
Total	199 (100)	19.9	212 (100)	211.0	191 (100)	19.1	213 (100)	227.0

SBPS- single base pair substitution; NTBPS- non-tandem base pair substitution; Ty- Ty yeast transposable element; Significance of difference: when compared to the corresponding wild type strain with similar *SUP4*-o orientation,* = $P < 0.05$, and ** = $P < 0.01$; when compared to the corresponding strain of same phenotype, but, opposite *SUP4*-o orientation, # = $P < 0.05$ amd ## = $P < 0.01$.

Table 21. Multiple Mutations, Deletions and Inertions

Sites #	Mutation type ##	Wt-Y*	pms1-Y*	Wt-I*	pms1-I*
- 40 → 508	548 bp deletion	-	-	1	-
6 or 7	SBP deletion	-	-	-	-
14 → 133	318 bp deletion	-	-	1	-
17 ↔ 18	Ty insertion	1	-	-	-
26 ↔ 27	10 bp duplicaton	-	-	1	-
26 ↔ 27	4 bp duplication	1	-	-	-
34	SBP deletion	-	-	-	-
34, 39	G → A, T → A	1	-	-	-
35 → 37	SBP deletion	1	-	-	-
37 ↔ 38	Ty insertion	2	-	-	-
51 → 58	8 bp deletion	-	-	1	-
52 → 54	SBP deletion	-	-	-	-
58 → 64	7 bp replaced with GGGCC	-	-	-	-
62	SBP deletion	2	-	-	-
64	SBP deletion	1	-	-	-
70 or 71	SBP deletion	-	-	1	-
79 → 83	SBP deletion	11	55	16	52
79 → 83	SBP insertion	5	31	7	27
79 → 83, 82 or 83	SBP deletion plus C→ A	-	-	1	-
84 → 86	SBP deletion	-	2	1	1
87, 89	T→ A, T→ C	-	-	-	-
89, 90 → 97	T → A plus 8 bp deletion	-	-	1	-
89 → 97	9 bp deletion	1	-	-	-

- Sites for multiple deletions are estimates; ## - changes are given for the transcribed strand; * - Number detected.

+1bp events (61 fold and 41 fold for the Y- and I-pairs, respectively) followed by -1bp events (37.8 and 33.2 fold for the Y- and I-sets, respectively). This suggests that most of the slippage errors created by the polymerase were corrected efficiently by the mismatch correction system.

3.3.2.5 Single base-pair substitutions

All six possible SBPS were detected in both the strains. The rates of all the six SBPS events were higher in the *pms1*Δ background (Table 22) and the magnitude of increase ranged from 3.5 to 16 fold. There was however a significant difference only in the G·C → T·A transversions and only in the I-set. This difference is due to the relatively lower levels of these errors detected in WT-I probably due to the effect of strand identity on replication and not due to mismatch correction. Thus, generally, mismatch correction neither seemed to have any specificity in the correction of SBPS nor was it modulated by strand identity.

The distribution of SBPS arising in the two strains is provided in Table 23 and 24. Substitutions were distributed throughout the *SUP4*-o gene with the exception of the 14 bp intron (sites 40 to 53) where changes occurred at only one position (site 51). No SBPS were detected in the regions immediately flanking the gene (Table 23).

In the case of the Y-pair, 54 sites were mutated of which, 14 were unique to WT-Y and 9 to pms1-Y while 31 sites were common to both the strains. In the case of the I-pair, 56 sites were mutated of which, 15 were unique to WT-I and 6 to pms1-I while 35 sites were common to both the strains. Of these common sites, the mutation rate was

Table22. Single Base-Pair Substitutions

Alteration	WT-Y		pms1-Y		WT-I		pms1-I	
	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})	No Detected (%)	Rate (x 10^{-8})
G·C → A·T(Tr)	42 (24.3)	4.2	35 (28.2)	35.2	52 (32.3)	5.2	36 (27.0)	38.4
A·T → G·C(Tr)	18 (10.4)	1.8	9 (7.3)	9.1	14 (8.7)	1.4	10 (7.5)	10.7
Total Tr	60 (34.7)	6.0	44 (35.4)	44.3	66 (41.0)	6.6	46 (34.6)	49.0
G·C →T·A(Tv)	57 (32.9)	5.7	55 (44.4)	55.3	36 (22.4)	3.6	56 (42.1)**	62.9
G·C →C·G(Tv)	31 (17.9)	3.1	16 (12.9)	16.1	41 (25.5)	4.1	21 (15.8)	19.2
A·T →C·G(Tv)	14 (8.1)	1.4	5 (4.0)	5.0	12 (7.4)	1.2	4 (3.0)	4.3
A·T → T·A(Tv)	11 (6.3)	1.1	4 (3.2)	4.0	6 (3.7)	0.6	6 (4.5)	6.4
Total Tv	113 (65.3)	11.3	80 (64.5)	80.5	95 (59.0)	9.5	87 (65.4)	92.8
Total SBPS	173 (100)	17.4	124 (100)	125	161 (100)	16.0	133 (100)	142.6

Tr = Transitions; Tv = Transversions; SBPS= single base pair substitution; Significance of difference: when compared to the corresponding wild type strain with similar *SUP4*-o orientation,* = $P < 0.05$, ** = $p < 0.01$

Table 23. Distribution of Spontaneous Base Substitutions in the *SUP4*-o Gene

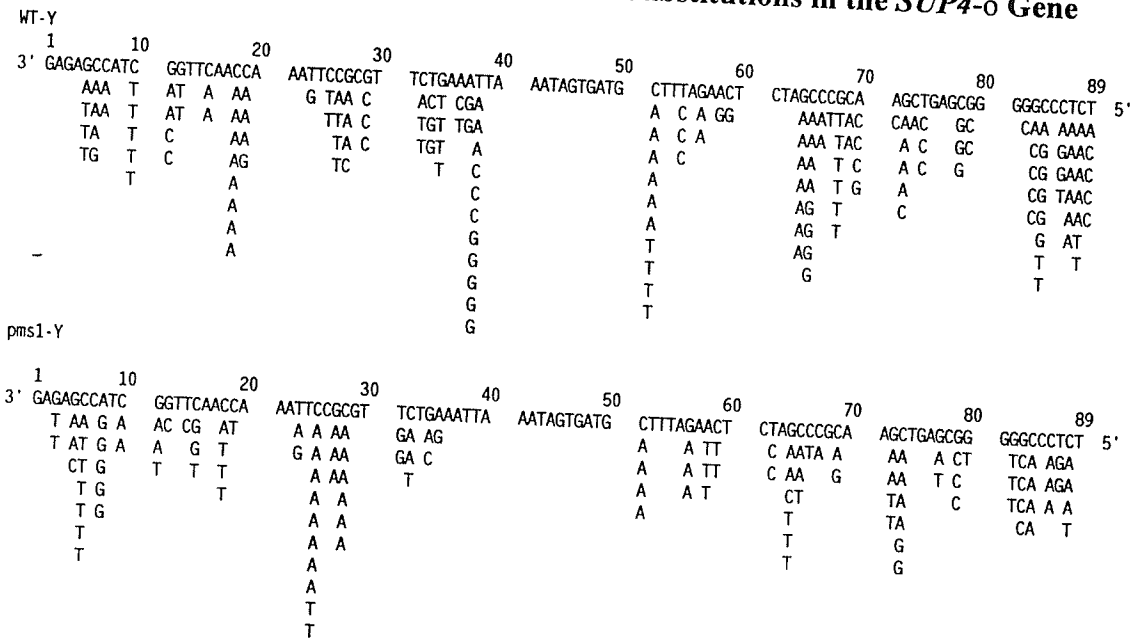
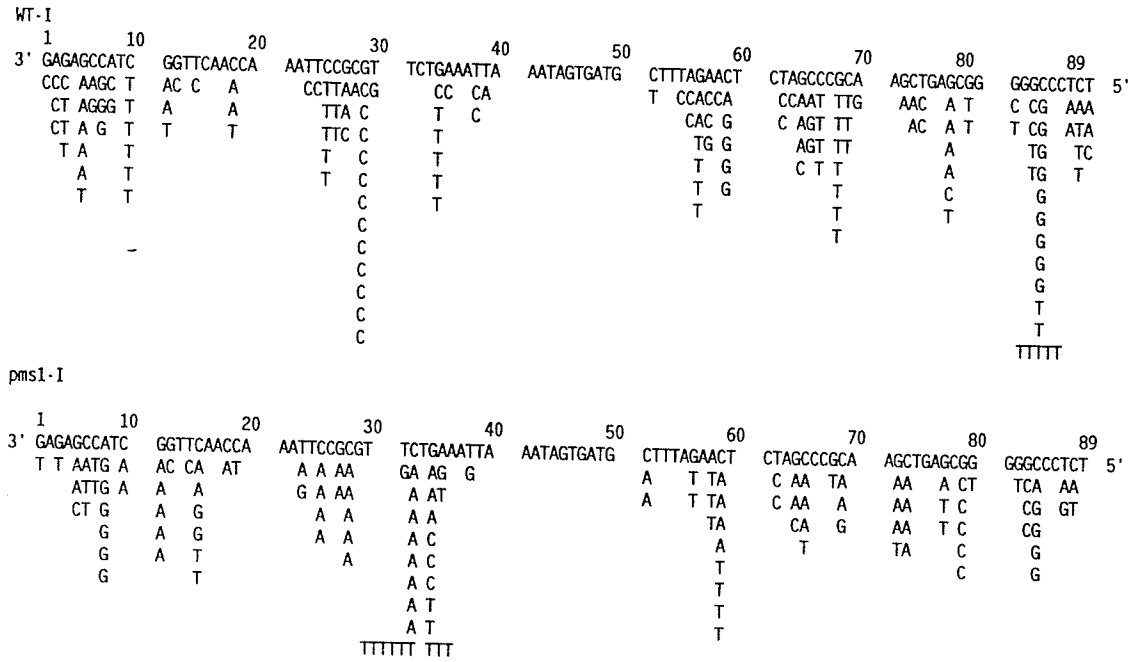


Table 24. Distribution of Spontaneous Base Substitutions in the *SUP4*-o Gene



increased at all the sites in the *pms1* background in both the Y- and I-pairs. The rate increase ranged from 2.5 to 60 fold with an average value of 9 fold for the Y-pair while it ranged from 1.5 to 42.8 fold with an average value of 9.9 fold for the I-pair. Sites 73 (60 fold) and 25 (50 fold) showed the largest increase in pms1-Y while sites 72 (42.9 fold), 58 (30.1 fold) and 8 (32.1 fold) showed the largest increase in pms1-I. The rate increase was not uniform indicating that the *pms1* mutator had a much larger effect on specific sites than can be expected based on the magnitude of increase in the overall mutation rate. This effect is similar to that seen in the proofreading deficient strain and is probably due to the influence of sequence context/ neighbouring nucleotides on the mismatch correction process.

A statistical comparison (Adams and Skopek 1987) of the distributions of SBPS between the strains in the Y, I, *pol3* or WT-pairs indicated that the chance of random sampling error accounting for differences was 1 in 500 (with 1,700 simulated comparisons, the upper limit on the 95% confidence interval for the estimate of $P < 0.002$). So the differences or uniqueness of a spectrum should be due to the genotype and phenotype of the strain and not due to a sampling error or a chance occurrence.

There was no significant difference between the two *pms1*Δ strains in the fraction or rate of any of the sequence alterations detected. A statistical comparison, as explained above, performed on the spectrum of SBPS obtained from pms1-Y against that from pms1-I indicated that the chance of random sampling error accounting for differences was 1 in 15 ($P > 0.6$). So the differences seen must be due to chance. This observation further emphasizes that the mismatch correction is not modulated by strand identity.

4 Discussion

The main objective of this study was to attempt to answer the following questions: 1. does proofreading and mismatch correction contribute significantly to DNA replication fidelity?; 2. what are the specificities of the proofreading or mismatch correction defects conferred by mutations in the 3' → 5' exonuclease domains of *POL3* (pol δ) or deletion of the *PMS1* genes, respectively?; 3. does a proofreading defect cause a saturation of the mismatch correction pathway?; 4. does strand identity affect the specificity of proofreading (and so provide evidence consistant with pol δ replicating only one strand) or mismatch correction?; and 5. does sequence context modulate the extent of proofreading or mismatch correction?. The following approaches were used to attempt to find answers for to these questions:

1. The identity of the transcribed strand with respect to the replication template was determined by identifying the replication fork that duplicated the *SUP4*-o gene using two dimensional gel electrophoresis technique.
2. The specificity of the *pol3* and *pms1*Δ mutators were determined by characterizing the mutations arising in a plasmid-borne copy of the yeast *SUP4-o* gene. The *pol2* mutator could not be characterized due to the interference of the mutator with the genetic screen used in the isolation of *SUP4*-o mutants.
3. The effect of strand identity was determined by characterizing the mutations arising in the *SUP4*-o gene in two orientations, in the wild type, *pol3* and *pms1*Δ backgrounds,

such that the transcribed strand would be the leading strand template in one orientation and the lagging strand template in the other orientation.

4. The effect of strand identity on mismatch correction was further characterized using pair-wise comparisons of the efficiency of repair of heteroduplex plasmid DNA containing defined mismatches in the *SUP4*-o gene placed in either orientations in a wild type background.

5. The influence of the proofreading defect conferred by *pol3* on mismatch correction was determined by assessing the efficiency of the repair of heteroduplex plasmid DNA containing defined mismatches in a *pol3* background.

The discussion section that follows is based on the following assumptions. Literature supporting these assumptions as well as their limitations, if any, are discussed in the relevant sections:

1. Deletion of *pms1* significantly reduces mismatch correction while the *pol3-4DA* mutation significantly reduces proofreading by pol δ:

   It has been shown that the mismatch correction levels in a *pms1*Δ background is reduced by about 75%. Additional mismatch repair pathways possibly exist in yeast and accounts for about 25% of mismatch correction (Bishop *et al.* 1989; Kramer *et al.* 1989; Yang 1995). It has also been shown that the proofreading activity in *pol3-4DA* strains is reduced to below detection levels, although it may not be completely eliminated (Simon *et al.* 1991). Thus, it should be noted that there is a possibility of

my results being obscured by interference from residual proofreading as well as mismatch correction by other pathways.

2. The spectrum of mutations detected in the *pms1*Δ background should predominantly be due to the mutational events caused during replication but not corrected by proofreading by pol δ. Similarly, the mutational events detected in the *pol3* strain should be mostly due to replication errors not corrected by mismatch correction:

   It should be noted that these estimates are lower limits since the mutational events detected in either case may also represent those due to other pathways such as DNA repair (Schaaper 1993). Pol δ as well as pol ε have been suggested to be involved in repair in addition to replication (Nishida *et al.* 1988; Tsurimoto *et al.* 1990; Morrison *et al.* 1991; Morrison *et al.* 1993; Wang *et al.* 1993; Morrison and Sugino 1994; Budd and Campbell 1995). Thus the *pol3* mutator effect may be the result of the absence of correction of errors formed during replication as well as repair synthesis.

3. Nucleotides misincorporated by a polymerase are corrected by the intrinsic proofreading activity of the polymerase and there is no competition for correction from other proofreading proficient polymerases or 3' → 5' exonucleases:

   This has been suggested to be the case at least for the relationship between yeast pol δ and pol ε although a competition by the two polymerases to correct the same pool of errors seems to exist (Morrison and Sugino 1994). This competition has been suggested to be only a potential one (Morrison and Sugino 1994). It has however, been shown that, *in vitro*, pol δ is capable of proofreading errors caused by pol α

(Perrino and Loeb 1990). So, one cannot rule out the possibility of other proofreading activities compensating, to some extent, for the loss of proofreading by pol δ in the *pol3* mutator strains. By the same arguement, it is also possibe the *pol3* mutation causes defects in other *POL3* linked pathways.

4. A yeast centromeric plasmid mimics a yeast chromosome with regard to replication and repair:

Although these plasmids maintain some chromatin structure and segregate like chromosomes, they are much smaller, circular rather than linear and may not be able to achieve the higher order chromatin structures achieved by *bone fide* yeast chromosomes. Therefore, replication and repair of a plasmid borne copy of a gene may not necessarily be similar to those of a chromosomal gene. Additionally, plasmid retention is seen only in about 80 percent of the cells in all the strains studied. However, the plasmid retention values is uniform among the strains studied, and cells that have lost the plasmid are eliminated from the population by growth on medium selective for the plasmid. The 15 to 20% plasmid loss would remove a small fraction of the mutant plasmid from the total population. Considering the mutation rate, this error would be negligible and would not be significant unless the plasmid loss in a strain is linked to a specific type of mutation, in which case, we would fail to detect a specificity factor of the polymerase.

4.1 Identity of the transcribed strand

Mapping the movement of the replication fork on plasmid YCpKR-Y demonstrated that the transcribed strand is the lagging strand template in the Y-

orientation while it was the leading strand template in the I-orientation. Therefore, switching the orientation of the *SUP4*-o gene essentially changed its strand identity. This experiment was performed with the intention that if during the course of the study, the identity of the polymerase(s) replicating each strand (provided, of course, that the widely prevalent hypothesis that different polymerases replicate each strand [Morrison *et al.* 1990; Tsurimoto *et al.* 1990; Burgers 1991; Sugino 1995] is true) were determined by one of the researchers working in that area, the strand identity of the *SUP4*-o gene would prove useful in further understanding the strand specificity of the polymerase mutators. However, since this information is not available to date, the strand identity data can only be used to make some speculations.

4.2 Effect of strand identity on proofreading and mismatch correction

Comparison of the two *pol3* mutational spectra (pol3-Y and pol3-I in Tables 9 and 10, respectively) suggests that switching the orientation of the gene, or in other words, changing the strand identity, had a significant effect on the spectrum of mutations detected. The mutational spectrum generated depends on the properties of the polymerase and the nucleotide sequences of the template DNA. If pol δ replicated only one strand, say the leading strand as suggested by Burgers (1991), the spectrum of mutations detected in the pol3-Y orientation would depend on the sequence of non-transcribed strand while the pol3-I spectrum would be dictated by that of the transcribed strand. In such a scenario, since the sequence context of the transcribed and non-transcribed strands is not the same, the pol3-Y and -I spectra would be significantly different. Although this

difference would be predominantly due to the specificity of the replicating polymerase, it should be noted that there would be some influence by other related pathways such as DNA repair and mismatch correction. If, on the other hand, pol δ replicated both the strands to the same extent, the spectrum detected in one orientation should be dictated by the sum total of the sequences of both the transcribed as well as the non-transcribed strands, which would essentially be the same for both the orientations of the *SUP4*-o gene. Therefore, switching the orientation of the gene should not have any significant effect on the spectrum of mutations detected. It should, however, be noted that the two spectra may not be identical due to the specificities of other related pathways such as proofreading by pol ε, replication errors made by pol α and the specificity of the mismatch correction system. Statistical comparisons showed that there was a significant difference ($P < 0.02$) in the distribution of SBPS between the pol3-Y and I spectra (Tables 9 and 10) and hence, it is likely that pol δ does not function on both the strands to the same extent.

The mutation rate was 3.5 fold higher in pol3-I relative to pol3-Y. In other words, the proofreading defect conferred a higher magnitude of mutational events when the transcribed strand was the leading strand template. If pol δ was the leading strand polymerase (Burgers 1991), this suggests that the efficiency of proofreading by pol δ may be significantly higher when it replicates the transcribed strand than when it replicates the non-transcribed strand. This would be similar to situation where preferential excision repair of the transcribed strand was seen in some yeast (Sweder and Hanawalt 1992) as well as *E. coli* genes (Isabel and Hanawalt 1989). However, if pol δ was the lagging

strand polymerase as suggested by Morrison (1990), the situation would be reversed, therefore a definitive conclusion cannot be reached until the nature of the eukaryotic DNA replication machinary is solved.

There was no significant difference in the distribution of substitutions between the spectra generated by pms1-Y and pms1-I ($P > 0.6$). By the same arguement, on the influence of the sequence context of the associated strand, used for proofreading, mismatch correction does not seem to be dependent on the strand identity. Additionally, pair-wise comparisons of mismatch correction of heteroduplex DNAs, with the same mismatch on the *SUP4*-o gene, in either orientation also suggests that there was no influence of strand identity on mismatch correction (Table 13). So in an overall picture, mismatch correction should be involved in the correction of replication errors left behind by the replication machinery of both the strands to the same extent. The efficiency of mismatch correction however, varied with the type of mismatch. This aspect is discussed in the following sections.

The distribution of SBPS detected in the two wild type strains (WT-Y and WT-I in tables 9 and 10, respectively) were significantly different ($P < 0.02$), suggesting, by the same argument as for the *pol3* spectra, that the form of the polymerase(s) replicating the leading strand is most probably different from that replicating the lagging strand. However, since there was no difference in the mutation rate between the two wild type strains, it is likely that both the leading and lagging strand replication machineries are equally accurate although each may have a different sequence context specificity.

4.3 Effect of sequence context on proofreading and mismatch correction

In the absence of a sequence context effect for both proofreading and mismatch correction, the rate increase in the *pol3* or *pms1*Δ mutator strain should be uniform for all the sites detected on the *SUP4*-o gene. However, this is not the case in the *pol3* or *pms1*Δ strains. For example, in the pol3-Y, the rate of A·T → T·A events was increased by 336.5 fold at site 87 and by only 31 fold at site 23 while in pms1-I strain, the G·C → C·G events at site 79 was increased by 27 fold while the same event was increased by only 8 fold at site 83. Additionally, unique hot and cold spots were identified for all the spectra under study. This suggests that the sequence context/ neighbouring nucleotides could modulate the efficiency of proofreading, mismatch correction and replication.

It should be noted that since the actual replication error that leads to a mutation cannot be accurately determined, care should be taken before making any definite conclusions on the effect of sequence context. For example the higher increase of A·T → T·A events at site 87 may be mainly due to T/T mismatches while the lower increase of the same mutational event at site 23 may be due to A/A mismatches or *vice versa*. Since both these mismatches would eventually lead to the same mutation, the difference in the rate seen could be due to the difference in the specificity of proofreading in correcting that particular mismatch rather than a sequence context effect. It is also possible that the difference in rates for the same mutation at different sites could be due to the influence of the proofreading specificity in a *pms1*Δ strain or mismatch correction specificity in a *pol3* strain.

It is interesting to note that a similar sequence context effect was seen in the two wild type strains too. For example, G·C → C·G events were 4 fold higher in WT-I relative to WT-Y at several sites. This might be due to the overall specificities of replication, proofreading, mismatch correction and DNA repair.

4.4 Effect of proofreading deficiency on mismatch correction

In *E. coli*, defects in proofreading causes a deficiency in mismatch correction due to a saturation of the mismatch correction pathway by the large number of replication errors left behind by the replication machinary due to a defective proofreading activity (Schaaper 1988; Schaaper and Radman 1989; Schaaper 1993). If this was the case in yeast, the mutations detected in the *pol3* background would reflect replication errors not corrected by mismatch correction due to its saturation rather than its natural specificity. Interestingly, comparison of heteroduplex repair in a wild type and *pol3* backgrounds (Table 14) suggested that the efficiency of mismatch correction is actually enhanced in the *pol3* background. This result is contradictory to that observed in *E. coli* suggesting that there might be inherent differences in the efficiency pattern of mismatch correction between prokaryotes and eukaryotes. This enhancement of mismatch correction in the *pol3* strains would obscure the *pol3* mutational spectra because, the spectrum of mutations seen in a *pol3* background would comprise errors left behind by *pol3* and not corrected by an enhanced action of mismatch correction. So the *pol3* spectra must be considered as lower limits of the mutator.

It is interesting to note that a similar enhancement of mismatch correction efficiency was noted in a *rad3-1* mutant strain (Yang *et al.* 1996; this work has been recently submitted for publication in Current Genetics). The yeast Rad3 protein is required in the excision step of nucleotide excision repair (Reynolds and Freidberg 1981: Wilcox and Prakash 1981). It is also required for initiation of transcription by the RNA polymerase II (Feaver *et al.* 1993). Analysis of the RAD3 protein revealed that it has a DNA-dependent ATPase/ helicase activity capable of unwinding DNA·DNA as well as DNA·RNA duplexes. An analysis of mRNA levels of genes required in mismatch correction in a *rad3-1* background showed that the enhancement of the mismatch correction in *rad3-1* strains is not due to a potential RNA polymerase II mediated increase in the transcript levels of the genes required for mismatch correction (Yang *et al.* 1996). It is suggested that the Rad3 protein could contribute to the DNA replication fidelity via its helicase activity (Yang *et al.* 1996). If that was the case, the *rad3-1* mutation, which is a sequence alteration in a consensus helicase motif (Song *et al.* 1990), possibly increases replication errors (Yang *et al.* 1996). It is possible that the cell responds to an induction of replication errors through an unknown pathway and results in the enhancement of the mismatch correction system (Yang *et al.* 1996). This is consistant with my results that show that mismatch correction is enhanced in a *pol3* strains which displays a significant increase in replication errors.

4.5 Specificity of proofreading and mismatch correction

4.5.1 Mutational classes detected

Among the different classes of mutations detected, the highest mutation rate was for SBPS, followed by -1bp and +1bp events in the wild type, *pol3* and *pms1*Δ strains studied. The rate of these three mutational events was significantly higher in the mutant strains relative to the corresponding wild type strain. This is probably because these three mutational events are the result of the most common types of replication errors which are normally corrected efficiently by proofreading or mismatch correction or both (Morrison *et al.* 1993). Individual types of mutations are discusssed in more detail in the following sections. Pol3-Y displayed significant increase in the fraction of SBPS and +1bp events relative to the wild type, while pol3-I showed no such specificity. This is probably due to the effect of strand identity on the *pol3* mutator. Taking the two *pol3* strains together, there does not seem to be any specificity pattern among the mutational classes detected. The rate increase in the *pol3* strains for SBPS, -1bp and +1bp events relative to the corresponding wild type strain are not uniform suggesting a specificity for *pol3*. A defenitive conclusion cannot however, be made because the mutational spectrum seen for a *pol3* strain might also be influenced by the specificity of mismatch correction and proofreading by pol ε. In general, the proofreading activity of pol δ must have a broad specificity and is likely involved in correcting most DNA replication errors. However, the largest rate was for SBPS in both the wild type and *pol3* strains, suggesting that the errors that lead to SBPS are the most predominant among replication errors and are corrected efficiently by proofreading.

In the *pms1*Δ strains, there was a significant increase in all the three classes of mutations in both orientations. Additionally, the rate increase as well as the percentage of mutants detected per class was the highest for +1bp events followed by -1bp and SBPS events. Mismatch correction must therefore be involved in correcting deletion and insertion events more efficiently than SBPS events. This is discussed in more detail in the following section.

Since the mutations seen in a proofreading-deficient strain are predominantly due to replication errors not corrected by mismatch correction and *vice versa*, in an over all picture, it is possible that, proofreading is mainly involved in correcting SBPS premutational events while mismatch correction is mainly involved in correcting those that result in simple deletion and insertion mutations. These observations are contradictory to the case in *E. coli* where proofreading is involved predominantly in the correction of premutational events that lead to transversions while mismatch mainly corrects those that lead to transition events (Schaaper and Dunn 1991; Schaaper 1993). It is possible that the specificity of proofreading and mismatch correction differ considerably in eukaryotes compared to prokaryotes and that the mismatch correction process evolved to repair lesions that are not proofread efficiently.

4.5.2 Single base pair deletion and addition events

There was a significant increase in the fractions of +1bp and -1bp events in the two *pms1*Δ strains relative to their corresponding wild type while there was no such consistant difference in the case of the two *pol3* strains. Among these two events, the

*pms1*Δ strains displayed the largest increase for +1bp mutations. Since the mutations detected in a *pms1*Δ strain are caused by replication errors not corrected by proofreading, it is likely that mismatch correction corrects replication errors that lead to single base-pair insertion and deletion mutations very efficiently and among these errors, it preferentially corrects those that would lead to +1b. This is consistent with results obtained by measuring the efficiency of mismatch correction of heteroduplex DNA containing defined single base-pair deletion and addition events (Yang 1995). It has been suggested that DNA polymerases often produce -1bp and +1bp events while replicating simple repeats due to template loop formation and nascent-strand slippage (Streisinger *et al.* 1966). Consistent with this, most of the single base pair deletion and addition events detected in the wild type, *pol3* and *pms1*Δ strains were at a run of 5 G·C pairs at position 79 → 83 and a run of 3 C·G pairs at 84 → 86. As the frequency of deletion and insertion errors increases with the length of the repeat sequence, it is possible that the occurrence of strand slippage by polymerases is also increased with the length of the repeat sequence. Primer-strand slippage by polymerases, if left uncorrected will lead to genome expansion caused by the insertion events. It has been shown that such simple genome expansion is associated with genetic disorders such as Huntington's disease and human non-polyposis colon cancer (HNPCC) (reviewed in Loeb 1994). It has also been shown that HNPCC is linked to deficiency in mismatch correction (Fischel *et al.* 1993; Parson *et al.* 1993). These genetic disorders indicate the significance of maintaining genetic stability. Taking my data collectively, it is possible that mismatch correction is involved

primarily in the correction of simple insertion events, thus maintaining genome stability and preventing genetic disorders that arise through genome expansion.

4.5.3 Single base pair substitutions

Among the different classes of SBPS detected in the two *pms1*Δ strains, there was a significant increase in the fraction of G·C →T·A transversions in the pms1-I strain. A comparison of the two *pms1*Δ strains and the two wild type strains showed that this increase was due to a small reduction in the G·C → T·A events in the WT-I strain and most probably not due the specificity of the *pms1*Δ mutator. The relative rate increase for the classes of SBPS in the *pms1*Δ strains ranged between 3.5 and 16 fold. This can be considered moderate and uniform compared to a 2 to 500 fold increase seen in the *pol3* strains. This suggests that replication errors that lead to SBPS are very efficiently proofread *in vivo*.

Based on the fractions of the individual classes of SBPS detected, mismatch correction did not show any specificity while the *pol3* strain showed a significant difference in the fraction of G·C → T·A, G·C → C·G and A·T → T·A events. Among these three classes, the fraction of A·T → T·A transversions was increased in the *pol3* strain relative to the wild type in both orientation (Table 8). Since mismatch correction repairs T/T mismatches (which would eventually lead to A·T → T·A transversion) inefficiently, it is likely that proofreading corrects it very efficiently. It is interesting to note that in the *pol3* background, the fractions of G·C → C·G transversions is reduced in both orientations or Y-orientation. This is most probably because of an increase in the

efficiency of mismatch correction in the *pol3* background (see section 4.4). This view is supported by the observation from heteroduplex correction experiments that show that G/G mismatches (which would lead to G·C → C·G events) are corrected more efficiently in the *pol3* background than wild type background (Table 14). Additionally, G/A and C/T mismatches (which would lead to G·C → T·A events) are corrected much more efficiently than A/A and T/T mismatches (which would lead to A·T → T·A events) (Table 13). Considering all the data together, proofreading corrects all the SBPS errors with a potential increase in efficiency in the correction of A·T → T·A events. Mismatch correction on the other hand does not have any significant specificity in correction mismatches that lead to SBPS. However, in the event of excessive error production as seen in the *pol3* strain, the efficiency of mismatch correction can be enhanced significantly and in such an event, it may correct SBPS errors more efficiently.

The *pol3* mutator displays the least rate increase for G·C → C·G mutations (2 or 8 fold for the Y or I orientations, respectively) while the largest rate increase was for A·T → T·A events (203 or 503 fold for the Y or I orientations, respectively). The heteroduplex correction data (Table 13 and 14) show that mismatch correction corrects T/T mismatches which would lead to A·T → T·A mutations very inefficiently. Additionally, it has been shown through similar heteroduplex correction studies that C/C mismatches which would lead to G·C → C·G events are also corrected very poorly by mismatch correction. The low rate increase for the G·C → C·G events suggests that the polymerase makes very few C/C mismatches. The very high rate of A·T → T·A suggests

that the polymerase makes a lot of T/T mismatches but, they are however, efficiently proofread.

4.6 Interferance of the specificity of the *pol2* mutator in the genetic screen used to isolate *SUP4*-o mutations

The *pol2* mutator caused a significant increase in the spontaneous reversion of *lys2-1* and *ade2-1* mutations as well as the spontaneous forward mutation in the *CAN1* locus. The increase in reversion frequencies in the *pol2* strains were comparable to that seen in the *pms1*Δ strains. This suggests that proofreading by pol ε may be involved in error correction. Pol ε has been suggested to be involved in repair (Nishida *et al.* 1988; Tsurimoto *et al.* 1990; Wang *et al.* 1993; Budd and Campbell 1995). However, since the cells were not subjected to any external mutagenic agents, the increase in the mutation frequencies could not be only due to a lack of accurate DNA repair synthesis of exogenous mutations. It is likely that the increase in mutation rate is a combined effect of inaccurate replication and repair. The 5 to 20 fold frequency increase seen in the *pol2* strain for the various markers tested is comparable to the results of other researchers (Morrison *et al.* 1991; Morrison and Sugino 1994).

The specificity of the *pol2* mutator could not be determined due to an excessive generation of antisupressor mutations. The *pol2* mutator apparently has a hotspot in an unidentified antisupressor locus. In other words, the *pol2* mutator causes a reduction in the production of functional supressor tRNA. This reduction affects the suppression of the *can1-100* locus but not the *ade2-1* or *lys2-1* since most of the CAN^R colonies detected

in the pol2-Y and -I strains were white indicative of a suppressed *ade2-1* locus and lys^+ indicative of a suppressed *lys2-1* locus. This is probably because the degree of supression required by the *can1-100* locus might be more than that required by the *lys2-1* and *ade2-1* loci. The genetic screen used is highly sensitive and can detect a 30% reduction in ochre supression as CAN^R colonies (Dr. Kunz personal communication). So, the *pol2* mutator must be causing a defect in one or more of the tRNA processing steps resulting in a 30% or higher reduction in functional *SUP4*-o tRNA. As this situation made it impossible to collect *SUP4*-o mutants, the *pol2* mutator was not characterized.

It is tempting to speculate that my results suggest that proofreading is involved in the correction of premutational events leading to SBPS while mismatch correction mainly corrects those leading to deletion and insertion events in yeast. However, caution has to be taken before making any specific conclusions because there are several unresolved issues surrounding eukaryotic DNA replication and DNA replication fidelity including the identity and properties of the replication machinary.

5 Future Work

An actual comparison of the relative contribution of mismatch correction (*pms1*Δ) and proofreading (*pol3* or *pol2*) would be more meaningful if the specificity of these single mutants are compared to the wild type as well as double mutants and triple mutants. In a given orientation of the gene, if the wild type, *pms1*Δ-*pol3*-*pol2* triple mutant, *pol3*- *pol2* double mutant as well as the *pol3*, *pol2* and *pms1*Δ single mutants can be characterized, the specificity of the contribution by proofreading and mismatch

correction towards the accuracy of DNA replication can be mapped using the following scenario: The wild type strain would reflect the contribution by proofreading (by pol δ and pol ε), mismatch correction and other mechanism such as DNA repair and base selection by polymerases. The difference between the triple mutant and *pms1*Δ single mutant would reflect the contribution by proofreading (pol δ and pol ε). The difference between the triple mutant and *pol3-pol2* double mutant would reflect the contribution by mismatch correction. The difference between the double mutant and *pol3* or *pol2* would reflect the contribution of proofreading by pol ε or pol δ, respectively. Finally, the difference between the wild type and the triple mutant would reflect the contribution of the residual proofreading and mismatch correction as well as other mechanisms such as DNA repair and base selection. I could not perform such a study due to the lethality of *pol3-pms1*Δ, and *pol3-pol2* double mutants (Dr. Simon and Dr. Morrison personal communications; Morrison *et al.* 1993; Morrison and Sugino 1994). The double mutant is lethal due to the multiplicative relationship between these two mutators, leading to an enormous increase in genetic errors in vital genes (Morrison *et al.* 1993). However, diploids of these double mutants are viable probably because the presence of two copies of all the genes compensates the high error production to some extent (Morrison *et al.* 1993).

A diploid of MKP-o can be used as the wild type genetic background to construct *pol3*, *pol2*, *pms1*Δ, as well as the required double and triple mutant strains discussed above. The plasmid shuffle technique (Simon *et al.* 1991; Morrison and Sugino 1994) can be used to construct these mutants. A single copy of the *SUP4*-o gene can be integrated

adjacent to an origin of replication to serve as the reporter gene. The *SUP4*-o gene can be inserted in either orientations to determine the effect of strand identity of the mutators. The movement of replication fork duplicating the gene can be mapped easily using the two dimensional gel electrophoresis technique and the strand identity of the *SUP4*-o gene can be resolved. It should be noted that some of the *SUP4*-o mutants detected in each strain could be due to the recombinational deletion of the single copy *SUP4*-o gene. If this were the case, the frequency of the deletion event must be used as a correction factor in determining the mutation rate of each strain. Although this is a potential drawback of the system, it can also be viewed as a tool to measure the effect of each of the mutator on genetic recombination. The cells that have lost the *SUP4*-o gene can be eliminated from the population by flanking the gene with two markers and maintaining selection for them. The strains thus constructed could be used to determine the mutational specificity of the individual as well as combination of the mutators as discussed above. Observation of the specificity of *pol3* and *pol2* mutators with *SUP4*-o on either orientation could provide insights into the functional role of pol δ and pol ε in replication.

The *pol2* mutator was incompatible with our system due to a potential increase in the induction of antisupressor mutations that affected the suppression of the *can1-100* locus. It is possible that this incompatibility could be specific to the *can1-100* locus. The system could be re-engineered to include additional suppressible loci, loss of suppression of which could be detected through forward mutagenesis (for example URA3 on FOA medium)

It would also be interesting to investigate the specificity of other genes involved in mismatch repair, such as genes belonging to the MSH and MLH family (New *et al.* 1993; Prolla *et al.* 1994). A recent study in our laboratory showed that mismatch correction is enhanced in the *rad3-1* background (Yang *et al.* 1996). The Rad3 protein has been suggested to be contributing to DNA replication fidelity (Montelone *et al.* 1988; Song *et al.* 1990; Yang *et al.* 1996). A study on mismatch correction in *RAD3* and *rad3-1* strains showed that *RAD3* may not be directly functioning in mismatch correction (Yang *et al.* 1996). It has been suggested that the *rad3-1* causes a reduction in the replication fidelity and hence an increase in replication errors. The cells could respond to this increase in replication errors by an enhancement of the mismatch correction pathway (Yang *et al.* 1996). This scenario could also be the case in the *pol3* strains which display excessive replication errors. It would be interesting to determine the pathway of the regulation of mismatch correction in these mutator strains.

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