Influence of DNA Precursor Levels

on Spontaneous and Induced Mutagenesis

in *Saccharomyces cerevisiae*

by

Susanne Elisabeth Kohalmi

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ABSTRACT

1 ABSTRACT

During this study, spontaneous or induced mutations were characterized in *SUP4*-o, a suppressor tRNA gene of the yeast *Saccharomyces cerevisiae*. The first stage of this work focussed on characterizing the mutator phenotypes associated with dNTP imbalances. DNA precursor levels were manipulated by disruption of the dCMP deaminase (*DCD1*) gene and by provision of excess dTMP or starvation for dTMP in a nucleotide-permeable strain. The primary dNTP disturbances produced by these different conditions in growing cells were increases in the dCTP or dTTP levels or a decrease in the dTTP pool, respectively. The mutation rate of the *SUP4*-o gene was enhanced 2-fold by the dCMP deaminase deficiency, 104-fold by provision of excess dTMP and 171-fold by starvation for dTMP. DNA sequencing was used to characterize 496 *SUP4*-o mutations that arose under these conditions and the resulting mutational spectra were compared to 334 spontaneous mutations recovered in an isogenic strain having balanced DNA precursor levels. Significantly more (>98%) of the changes resulting from nucleotide pool imbalance were single base-pair events, the majority of which could have been due to misinsertion of the nucleotides present in excess. Unexpectedly, expanding the dCTP pool did not increase the fraction of A·T to G·C transitions relative to the spontaneous value nor did enlarging the dTTP pool enhance the proportion of G·C to A·T transitions. Instead, the elevated levels of dCTP or dTTP were associated mainly with increases in the fraction of G·C to C·G or A·T to T·A transversions, respectively. Furthermore, T to C, and possibly A to C, events occurred preferentially in the *dcd1* strain at sites in *SUP4*-o where dCTP was to be inserted next. C to T and A to T events were induced most often by treatment with excess dTMP at sites where the next nucleotide to be inserted

was dTTP or dGTP[1] (dGTP levels were also elevated by this treatment).
Following dTMP starvation, a high dCTP:dTTP ratio and consequently an
increase primarily in A·T to G·C transitions were expected. However, the
largest change in the DNA precursor pools involved an enhanced dATP:dTTP
ratio and the substitutions recovered involved mainly replacements by an
A·T pair. In addition, no distinct preference for sites followed by a
particular 3′ base could be found, possibly because dATP as well as dGTP
and dCTP were elevated after dTMP starvation. Misinsertion of the
nucleotide in excess (dCTP, dTTP and dATP, respectively) did not exhibit
a strand bias for any of the three conditions (elevated dCTP or dTTP or
depleted dTTP). Collectively, the data suggest that increased levels of
dCTP, dTTP and dATP (in case of dTTP depletion) induce mutations via
nucleotide misinsertion and that inhibition of proofreading may play a
role in inducing mutations during dCTP imbalance or following treatment
with excess dTMP. However, other factors, possibly including the
regulation and specificity of proofreading and mismatch correction, must
also be involved.

The purpose of the second part of this study was to examine the
mutational specificity of selected monofunctional alkylating agents. A
total of 318 *SUP4*-o mutations induced by ethyl methanesulfonate (EMS) and
N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was analyzed. Only base-pair
substitutions were detected among the mutations examined and, for both

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[1]Even though nucleotides are present in DNA as monophosphates, it is
common in the literature to refer to '(mis-)incorporation' or '(mis-)in-
sertion' of dNTPs. This usage might not be completely accurate but it
stresses that it is dNTPs that are recognized by DNA polymerases and are
first involved in hydrogen-bonding with template deoxyribonucleotides. In
addition, it is less confusing to refer to dNTP misincorporation in the
context of this thesis where such replication errors are often attributed
to increased dNTP levels.

agents, the majority (>96%) were G·C to A·T transitions. This result was consistent with O^6-alkylguanine being the major premutational lesion for mutagenesis by monofunctional alkylating agents. There was considerable overlap among the SUP4-o sites that were mutated or not mutated by EMS and MNNG. However, EMS and MNNG mutagenesis differed with respect to the frequencies at which mutations were recovered at G·C pairs where the guanine was flanked (5') by a purine or pyrimidine. EMS exhibited no preference for either type of site, whereas a G·C site was 12- or 5-fold more likely to be mutated by MNNG if preceded by a 5' adenine or guanine, respectively, than if flanked by a 5' pyrimidine. Neither EMS nor MNNG mutagenesis showed a preference for G·C sites having the guanine on the transcribed or non-transcribed strand.

In the final portion of this project, the influence of elevated dCTP levels on EMS and MNNG mutagenesis was determined. A collection of 409 SUP4-o mutations induced by these alkylating agents in the dCMP deaminase deficient strain was characterized. SUP4-o mutation frequencies were reduced by 60% and 40%, respectively, after treatment with EMS or MNNG in the dcd1 strain compared to the corresponding values in the wild-type strain. In each case, the fraction of G·C to A·T transitions was reduced (90% and 70%, respectively) but by considerably more than the overall SUP4-o mutation frequency. The differences were due to 35- and 12-fold increases, for EMS and MNNG, respectively, in the frequencies of G·C to C··G transversions. Although G·C to C·G transversions were the major change (58%) arising spontaneously in the dcd1 strain, they occurred at least 7.5-fold more frequently after alkylation treatment. Misincorporation of dCTP during the repair of apurininc sites created by the removal of N-7-alkylguanine, the major DNA lesion produced by EMS and MNNG, was suggested

to account for the induction of G·C to C·G transitions. Again, MNNG- but not EMS-induced events showed a preference for G·C sites having a 5′ purine and neither EMS- nor MNNG-induced G·C to A·T transitions exhibited a strand bias indicating that the elevated dCTP levels had no effect on these features of alkylation mutagenesis.

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A	adenine
AC	anticodon
ADP	adenosine 5'-diphosphate
ala	alanine
AMP	adenosine 5'-monophosphate
AP	apurinic/apyrimidinic
2-APur	2-aminopurine
araCMP	9-ß-D-arabinofuranosylcytidine 5'monophosphate
AT	alkyltransferase
ATP	adenosine 5'-triphosphate
BDL	below detection level
BME	ß-mercaptoethanol
bp	base-pair
BrdUMP	5-bromo-2'-deoxyuridine 5'monophosphate
BrdUrd	5-bromo-2'-deoxyuridine
BrdUTP	5-bromo-2'-deoxyuridine 5'triphosphate
BSA	bovine serum albumin
C	cytosine
CDP	cytidine 5'-diphosphate
CHO	Chinese hamster ovary
Ci, mCi, μCi	curie, millicurie, microcurie
cm, mm, μm, nm	centimeter, millimeter, micrometer, nanometer
cpm	counts per minute
CTP	cytidine 5'-triphosphate
dADP	2'-deoxyadenosine 5'-diphosphate
dAMP	2'-deoxyadenosine 5'-monophosphate

dATP	2'-deoxyadenosine 5'-triphosphate
dCDP	2'-deoxycytidine 5'-diphosphate
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
ddNTP	2',3'-dideoxyribonucleoside 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
dGDP	2'-deoxyguanosine 5'-diphosphate
dGMP	2'-deoxyguanosine 5'-monophosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DHF	dihydrofolate
DMS	dimethylsulfate
DNA	deoxyribonucleic acid
dNMP(s)	2'-deoxyribonucleoside 5'-monophosphate(s)
dNTP(s)	2'-deoxyribonucleoside 5'-triphosphate(s)
dTDP	2'-deoxythymidine 5'-diphosphate
dTMP	2'-deoxythymidine 5'-monophosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUDP	2'-deoxyuridine 5'-diphosphate
dUMP	2'-deoxyuridine 5'-monophosphate
dUTP	2'-deoxyuridine 5'-triphosphate
EMS	ethyl methanesulfonate
FdUMP	5-fluoro-2'-deoxyuridine 5'-monophosphate
FdUrd	5-fluoro-2'-deoxyuridine
Freon	1,1,2-trichloro-1,2,2-trifluoroethane

G	guanine
g, mg, μg	gram, milligram, microgram
GAP	general amino acid permease
GDP	guanosine 5'-diphosphate
h	hour
I	intron
IdUMP	5-iodouridine 5'monophosphate
IMP	inosine 5'-monophosphate
kb	kilobase
l, ml, μl	liter, milliliter, microliter
M, mM, μM	molar, millimolar, micromolar
met	methionine
min	minute
MMS	methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MTHF	N^5, N^{10}-methylene tetrahydrofolate
N	normal
NADP	ß-nicotinamide adenine dinucleotide phosphate
NADPH	ß-nicotinamide adenine dinucleotide phosphate, reduced form
ND	not detected
Na$_2$EDTA	ethylenediaminetetraacetic acid
no.	number
NMR	nuclear magnetic resonance
NR	not reported
NTS	non-transcribed strand
OD	optical density

PEG	polyethylene glycol
phe	phenylalanine
pmol	picomol
PPi	pyrophosphate
Pur	purine
Pyr	pyrimidine
RNA	ribonucleic acid
rNDP(s)	ribonucleoside diphosphate(s)
s	second
S.D.	standard deviation
SDS	sodium dodecyl sulfate
T	thymine
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
THF	tetrahydrofolate
TNO	tri-n-octylamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TS	transcribed strand
TTS	transcription termination signal
U	unit
UDP	uridine 5'-diphosphate
UV	ultraviolet
val	valine
vs.	versus
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

2 INTRODUCTION

The remarkable stability of genes depends in large measure on the action of biochemical mechanisms that promote the fidelity of deoxyribonucleic acid (DNA) replication and the repair of damaged DNA. A balanced supply of the deoxyribonucleoside triphosphate (dNTP) precursors of DNA is a fundamental requirement for these processes. Perturbation of dNTP levels produces an impressive range of genetic effects associated with aberrant replication or failure to accurately repair DNA including mutation, recombination, enhanced cellular sensitivity to DNA-damaging agents, chromosome rearrangement, breakage and loss, and oncogenic transformation. Thus, it seems that normally the relative sizes of intracellular dNTP pools are carefully regulated so as to maximize genetic stability. Several informative review articles and volumes that are available collectively provide a far-ranging survey of the genetic consequences of dNTP pool imbalance (Kunz 1982; Meuth 1989; de Serres 1985; Haynes and Kunz 1986; MacPhee *et al.* 1988). In this literature review, the relations between dNTP pool imbalances and one manifestation of genetic instability, mutagenesis, will be the centre of interest. Relevant features of *de novo* dNTP synthesis and dNTP imbalance will be examined. The analysis of dNTP imbalance will focus on altered levels of deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP). In addition, phenomena that illustrate links between dNTP imbalance and the production of mutations, and potential mechanisms which may be responsible for these links will be considered. Finally, since use of the mono-functional alkylating agents ethyl methanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) constitutes a critical part of this study, alkylation mutagenesis will be reviewed.

2.1 *De Novo* dNTP Synthesis

2.1.1 Regulation of dNTP Synthesis
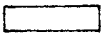
The *de novo* pathways for synthesis of dNTPs are connected, in part, by nucleotide interconversions (Figure 1). With the exception of dTTP, the DNA precursors appear initially at the diphosphate level via reduction of the corresponding ribonucleoside diphosphates (rNDPs) by ribonucleotide reductase (Thelander and Reichard 1979; Reichard 1985, 1988). Subsequently the deoxyribonucleoside diphosphates are phosphorylated by nucleoside diphosphate kinase for use in DNA synthesis. dTTP is formed by the sequential actions of thymidylate (dTMP) kinase and nucleoside diphosphate kinase on the monophosphate dTMP which is derived from deoxyuridine monophosphate (dUMP) via methylation by thymidylate synthase. N^5, N^{10}-methylene tetrahydrofolate serves as the methyl donor in the conversion of dUMP to dTMP and is oxidized to dihydrofolate so that the thymidylate synthase reaction constitutes a significant drain on intracellular tetrahydrofolate pools. The tetrahydrofolate pools are maintained during dTMP synthesis by the activities of dihydrofolate reductase and serine hydroxymethyltransferase. The major fraction of the dUMP for dTMP synthesis is derived either by: 1. hydrolysis of deoxyuridine triphosphate (dUTP, produced via ribonucleotide reductase) as in *Bacillus subtilis* and yeast (in this study, yeast always refers to *Saccharomyces cerevisiae*) (Møllgard and Neuhard 1978; McIntosh and Haynes 1984); 2. deamination of deoxycytidine monophosphate (dCMP) as observed for cultured mammalian cells and *Mycoplasma mycoides* (Neale *et al.* 1983; Reichard 1988); or 3. deamination of dCTP and hydrolysis of the resulting dUTP as for *Escherichia coli* and *Salmonella typhimurium* (Neuhard and Thomassen 1971; O'Donovan *et al.* 1971).

Figure 1. A Scheme for *De Novo* DNA Precursor Synthesis. MTHF: N^5, N^{10}-methylene tetrahydrofolate; DHF: dihydrofolate; THF: tetrahydrofolate. The following enzymes catalyze steps in dNTP synthesis: 1. ribonucleotide reductase; 2. dCMP deaminase; 3. 5′ nucleotidase/acid phosphatase; 4. nucleoside diphosphate kinase; 5. dUTPase; 6. thymidylate synthase; 7. dihydrofolate reductase; 8. serine hydroxymethyltransferase 9. thymidylate kinase; 10. dCTP deaminase (modified from Kunz and Kohalmi 1991).
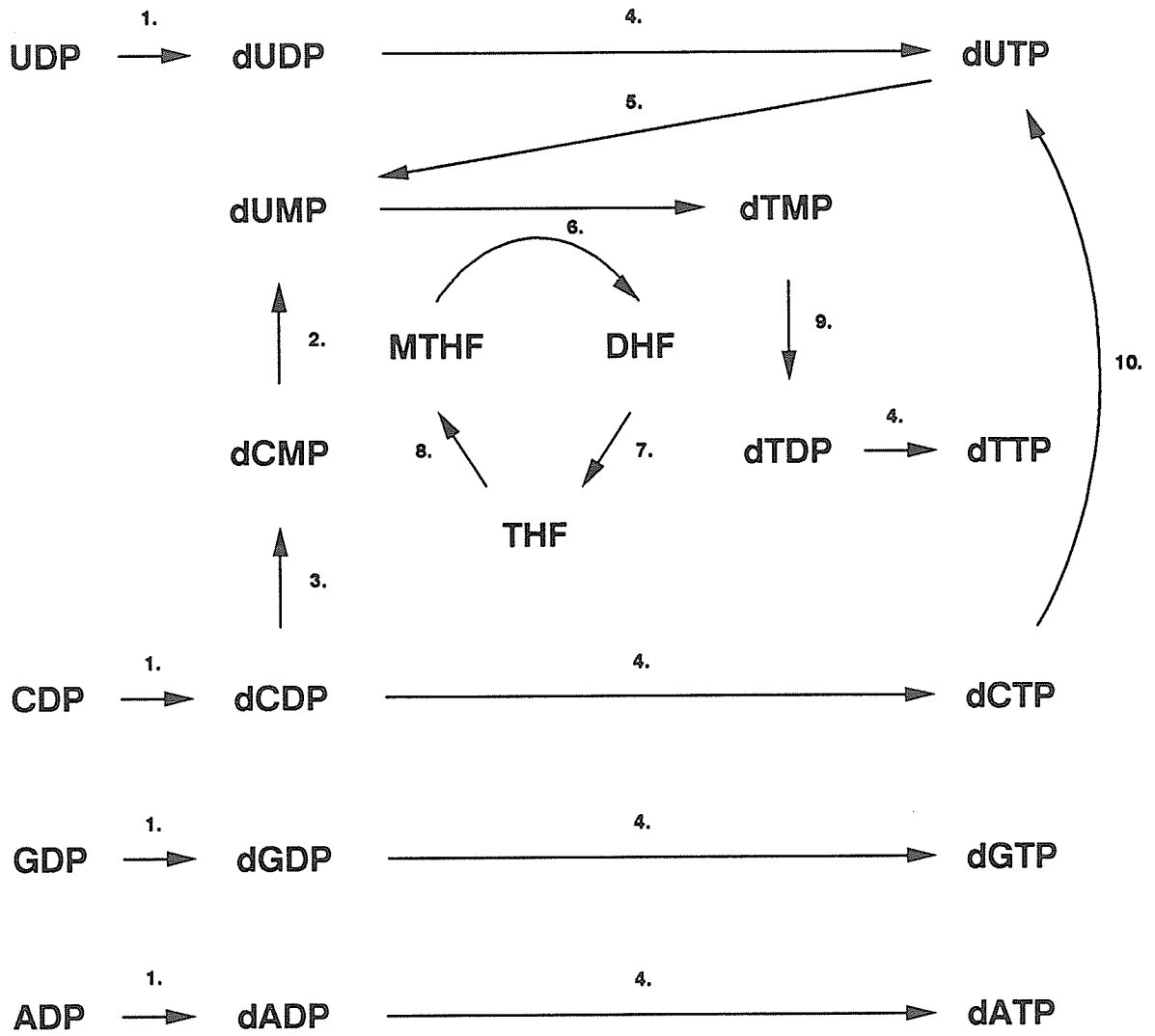
Allosteric regulatory mechanisms are of major importance for balancing *de novo* dNTP production and the enzymes ribonucleotide reductase and dCMP (or dCTP) deaminase play pivotal roles. Generally, deoxyadenosine triphosphate (dATP) inhibits reduction of the purine and pyrimidine rNDPs by ribonucleotide reductase, dTTP inhibits cytidine diphosphate (CDP) and uridine diphosphate (UDP) reduction but stimulates the reduction of guanosine diphosphate (GDP), and deoxyguanosine triphosphate (dGTP) inhibits CDP and UDP reduction while enhancing adenosine diphosphate (ADP) reduction (Figure 2) (Thelander and Reichard 1979; Lammers and Follmann 1984; Reichard 1985, 1988). Exceptions include the ribonucleotide reductases of bacteriophage T4 and herpes simplex virus which are relatively, or completely, insensitive to allosteric inhibition by dATP and dTTP (Huszar and Bacchetti 1983; Sargent *et al.* 1989). Interestingly, the regulation of ribonucleotide reductase does not distinguish between the two pyrimidine rNDPs. Instead, the relative dCTP and dTTP levels are controlled by dCMP or, depending on the organism, dCTP deaminase. The activity of these deaminases is stimulated by dCTP and inhibited by dTTP (Beck *et al.* 1975; Reichard 1988).

It is primarily through nucleotide interconversions and allosteric controls that fluctuations in particular dNTP pools often provoke alterations in others. Synthesis of dNTPs from deoxyribonucleosides (salvage pathways) and catabolism of deoxyribonucleotides also contribute to the overall balance of dNTP pools during cell growth. The enzymes involved, their regulation and their roles in substrate cycles have been discussed in detail (Bianchi *et al.* 1987; Reichard 1988). For the purposes of this study, it is important to note that although mammalian cells have the necessary kinases to salvage all common deoxyribonucleosides, only

Figure 2. **Allosteric Regulation of *De Novo* dNTP Synthesis.** The allosteric effects of effector nucleotides on the regulation of the ribonucleotide reductase are indicated by the following symbols: inhibition: ☐ ·····; activation: ----- (modified from Reichard 1978).
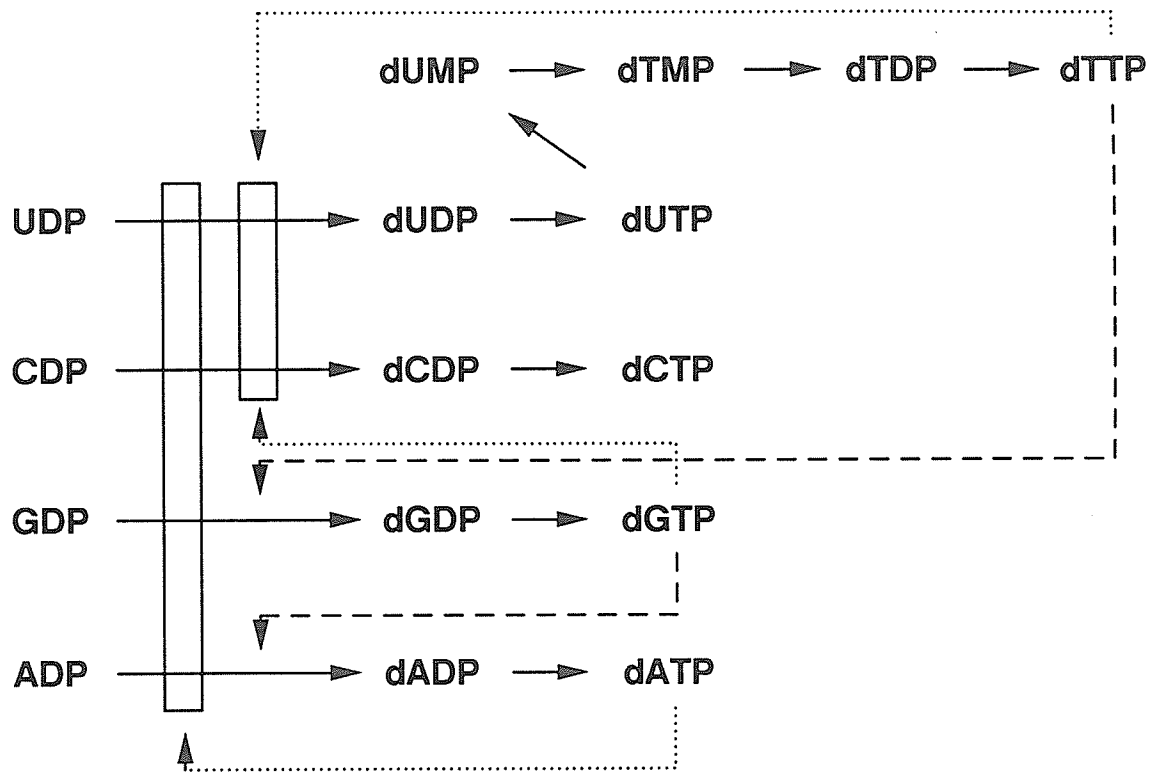
thymidine kinase is present in *E. coli* whereas several fungi including yeast, *Aspergillus nidulans* and *Neurospora crassa* lack this enzyme (Grivell and Jackson 1968) and probably the other deoxyribonucleoside kinases as well (McIntosh *et al.* 1986).

2.1.2 Sizes and Compartmentation of dNTP Pools

Generally, dNTP pools are small and correlate roughly with DNA content. The sizes of the dNTP pools in yeast (0.6 - 2.8 pmol/10^6 cells) are intermediate between those typically observed for bacterial (usually <0.2 pmol/10^6 cells) and mammalian cells (usually >5 pmol/10^6 cells with considerable variation among different cell lines) (Eckstein *et al.* 1974; Platz *et al.* 1985; Myers *et al.* 1987; Reichard 1988; Sargent *et al.* 1989; Kenne and Åkerblom 1990). Calculations indicate that the contents of the dNTP pools in eukaryotic cells would sustain chromosomal DNA synthesis for only a few minutes at best (Reichard 1988; see also Discussion). Even so, this is likely to be an overestimate. Most often dNTP pools have been measured using whole-cell extracts from asynchronous cultures. Yet, intranuclear dNTP pools can be considerably smaller than whole-cell pools (Mun and Mathews 1991). Furthermore, although dNTP levels vary during the eukaryotic cell cycle with the largest pool sizes found in S phase, measurable amounts of dNTPs are present in all phases (Albert and Gudas 1985; Andersson *et al.* 1988; Reichard 1988; Spyrou and Reichard 1988). Thus, some fraction of the dNTP pools measured in extracts from asynchronous cultures is not used for replicative chromosomal DNA synthesis. The dNTPs in question might be required for synthesis of repair patches, organellar DNA and/or deoxyliponucleotides (Bestwick *et al.* 1982; Friedberg 1985; Spyrou and Reichard 1987; Reichard 1988).

In vitro measurements of DNA precursor levels may not accurately represent the dNTP pools used for DNA replication for another reason. DNA synthesis requires that high local concentrations of dNTPs be maintained at replication forks. This functional compartmentation could be accomplished by organizing dNTP biosynthetic enzymes to couple them to the DNA replication apparatus and so coordinate dNTP synthesis with DNA replication. In prokaryotes, some enzymes of DNA precursor synthesis are aggregated into multienzyme complexes which may serve to channel dNTPs to sites of DNA replication (Mathews *et al.* 1988). Multienzyme complexes from *Streptococcus pneumoniae*, bacteriophage T4-infected *E. coli*, and *B. subtilis* contain ribonucleotide reductase and deoxyribonucleoside mono- and diphosphate kinase activities (Green and Firshein 1976; Mathews *et al.* 1988; Thylén and Mathews 1989; Laffan *et al.* 1990). Thymidine kinase activity is also present in the T4 and *B. subtilis* complexes and the former also possesses thymidylate synthase, dihydrofolate reductase, dCTPase-dUTPase, dCMP deaminase and dCMP hydroxymethylase activities (Mathews *et al.* 1988; Laffan *et al.* 1990). Consistent with a role for such complexes in dNTP biosynthesis, enzyme activities in the T4 complex (the one that is most well-characterized) are kinetically coupled (Mathews *et al.* 1988). This coupling depends on a functional complex and likely accounts for the finding that DNA replication in T4-infected permeabilized cells uses ribonucleoside diphosphates and deoxyribonucleoside monophosphates more efficiently than dNTPs (Mathews *et al.* 1988; Thylén and Mathews 1989). The apparent existence of dNTP concentration gradients in T4-infected *E. coli* (Mathews *et al.* 1988) points to a possible link between the T4 dNTP synthase complex and the DNA replication apparatus. That this may be a physical association is suggested by studies which

demonstrate: 1. interactions between T4 genes that encode enzymes for dNTP biosynthesis and those which specify DNA replication proteins (Chao *et al.* 1977; Macdonald and Hall 1984; Cook *et al.* 1988; Mathews *et al.* 1988); and 2. mutator phenotypes resulting from incubation of dCMP hydroxymethylase temperature-sensitive mutants at semi-permissive temperatures (Williams and Drake 1977) or forced dependency of T4 DNA replication on *E. coli* rather than T4 ribonucleotide reductase (Sargent *et al.* 1989). To date, the biochemical mechanisms that may coordinate synthesis of dNTPs by the T4 complex with phage DNA replication remain to be elucidated.

Among eukaryotes, multienzyme aggregates containing enzymes involved in dNTP biosynthesis have been reported for cultured carrot and mammalian cells (Mathews and Slabaugh 1986; Toth *et al.* 1987; Andersson *et al.* 1988; Mathews *et al.* 1988; Reichard 1988; Hammond *et al.* 1989). However, the kinetics of dNTP synthesis by these complexes have not been analyzed extensively and it is not clear that there is a physical relationship between DNA replication and enzymes that catalyze the synthesis of DNA precursors. A kinetic necessity for such channelling of DNA precursors is dubious given the relatively slow rates of chain growth and the numerous replicons characteristic of eukaryotic DNA replication. Indeed, current experimental evidence does not favour direct channelling of dNTPs to replication forks by a multienzyme complex in eukaryotic cells. Although several studies indicated the existence of dNTP pools that could be labelled with deoxyribonucleosides but were excluded from DNA replication, the results were obtained with asynchronous cells (Andersson *et al.* 1988; Reichard 1988). Recently, it has been determined that rodent cells more efficiently incorporate deoxycytidine and deoxyadenosine into dCTP and dATP, respectively, in G_0 and G_1 than in S phase (Leeds and Mathews 1987;

Duan and Sadée 1987). Thus, the replication-excluded dNTP pools may have accumulated in non-S phase cells so that compartmentation was temporal and intercellular rather than intracellular (Leeds and Mathews 1987). Channelling of dNTPs to sites of DNA replication would require that dNTP biosynthetic enzymes be situated in the nucleus during DNA replication. Yet ribonucleotide reductase and thymidylate synthase have been localized to the cytoplasm rather than the nuclei of rodent cells throughout the cell cycle suggesting that DNA precursors are synthesized in the cytoplasm of mammalian cells and then transported into the nucleus (Engström et al. 1984; Leeds et al. 1985, Kucera and Paulus 1986). Consistent with this idea, dCTP pools in extracts from synchronized whole Chinese hamster ovary (CHO) cells or their nuclei equilibrated to the same specific activities (Leeds and Mathews 1987). Also, microinjection experiments revealed that DNA was labelled to similar extents by radioactive dNTPs placed into the nuclei or cytoplasm of living mouse cells (Wawra 1988). These results argue against functional intracellular compartmentation of dNTPs used for chromosomal DNA replication in eukaryotic cells. On the other hand, mito-chondrial dNTPs do seem to occupy a separate intracellular compartment. Their production appears to be regulated somewhat independently from other cellular dNTPs and responds differently to inhibitors of DNA precursor synthesis (Bestwick and Mathews 1982; Bestwick et al. 1982).

2.1.3 Alteration of dNTP Levels by Mutagens

Exposure of bacterial, yeast, rodent or human cells to ultraviolet (UV) radiation, X-rays, EMS or MNNG has been shown to increase dNTP pool sizes (Eckstein et al. 1974; Das et al. 1983; Newman and Miller 1983; Suzuki et al. 1983). These alterations were detected within ten minutes to

four hours of mutagen treatment depending on the system used. The changes were dose-dependent where tested, and primarily dATP and dTTP pools were increased in bacterial and mammalian cells. A decrease in the dCTP and dATP pools was reported in one study involving CHO cells (Newman and Miller 1983) and a single X-ray or EMS dose elevated all four dNTP pools in yeast to similar extents (Eckstein *et al*. 1974). The UV-induced dNTP alterations in *E. coli* occurred in *recA⁻* or *umuC⁻* strains indicating that the modified dNTP levels were not a consequence of SOS induction or UV mutagenesis. Despite these intriguing findings, the generality of induction of substantial dNTP imbalances in mammalian cells by mutagenic agents has been questioned. Several groups using rodent or human cells reported that treatment with radiation or alkylating agents produced at best only small variations in dNTP levels, although results qualitatively similar to some of those described above were observed (Slaby *et al*. 1971; Hyodo *et al*. 1984; Collins and Oates 1987; Arecco *et al*. 1988; Snyder and Davis 1988).

How, when observed, might DNA precursor imbalances following mutagen exposure be explained? It was suggested that they might reflect DNA degradation, excision of nucleotides during repair or decreased dNTP incorporation into DNA due to inhibition of DNA synthesis (Slaby *et al*. 1971; Eckstein *et al*. 1974; Meuth 1989). If so, one might expect all four dNTP pools to expand in a more or less balanced fashion. However, this was seen to occur only in yeast (Eckstein *et al*. 1974). Furthermore, MNNG treatment that enhanced the dATP and dTTP pools in Chinese hamster cells did not significantly increase the fraction of cells in S phase (Das *et al*. 1983). In addition, drug mediated inhibition of DNA polymerization in mouse fibroblasts resulted in only a moderate elevation at best in dNTP

levels (Reichard 1988). To account for a very rapid (within ten minutes) dTTP pool increase and concurrent dCTP pool decrease in one CHO cell line following UV irradiation, it was proposed that UV promoted degradation of dCTP to dCMP and conversion of the latter nucleotide to dUMP, the substrate for thymidylate synthase (Newman and Miller 1985). This suggestion was based in part on an immediate, UV-induced, dose-dependent stimulation of dCMP deaminase activity which quickly diminished. Unfortunately, the question of how UV irradiation was able to enhance enzyme activity so rapidly and transiently was not addressed.

An interesting alternative explanation is that exposure to mutagens stimulates the synthesis of enzymes involved in dNTP production as a means of facilitating DNA repair (Elledge and Davis 1990; Yagle and McEntee 1990). Expression of the yeast genes encoding either dTMP kinase (*CDC8*) or the large (*RNR1, RNR3*) or small (*RNR2*) subunits of ribonucleotide reductase is increased following treatment with several DNA damaging agents (Elledge and Davis 1987, 1990). Two different consensus sequences have been associated with damage inducibility of the *RNR2* and *RNR3* genes (Sebastian *et al.* 1990; Yagle and McEntee 1990). Near matches to these sequences are also present in the regions 5′ to the yeast *CDC8, DCD1* (dCMP deaminase), *DFR1* (dihydrofolate reductase), *DUT1* (dUTPase) and *TMP1* (thymidylate synthase) genes (Birkenmeyer *et al.* 1984; McIntosh and Haynes 1986; Lagosky *et al.* 1987; Taylor *et al.* 1987; M. H. Gadsden: personal communication), although whether the latter four genes are damage inducible is not known. Expression of *RNR1, RNR2* and *CDC8* also increases during S phase in the absence of mutagen treatment (Elledge and Davis 1987, 1990), presumably because their products are required for DNA replication. Thus, induction of these damage-responsive genes by mutagen

treatment might actually reflect blocking cell cycling at a stage (DNA synthesis) when expression of these genes is normally enhanced (Elledge and Davis 1987). This appears to contribute in part to induction of *RNR2* following mutagen treatment (Elledge and Davis 1989) but both *RNR1* and *RNR2* can be induced by DNA damage in G_1 (Elledge and Davis 1989, 1990) indicating that the S phase response and damage inducibility of these genes can be uncoupled. There is no evidence yet that expression of genes controlling dNTP biosynthesis is damage inducible in other organisms.

2.2 dNTP Pool Imbalances

Alterations in dNTP levels can be produced in cultured cells in at least three ways (Kunz 1982): 1. mutational inactivation of genes controlling steps in DNA precursor synthesis; 2. drug treatment to inhibit specific enzymes involved in the biosynthesis or catabolism of dNTPs; and 3. supplementation of growth medium with DNA bases or nucleosides (for organisms having the appropriate salvage pathways) or deoxyribonucleotides (for nucleotide-permeable cells). These approaches generally result in the expected perturbation of a particular DNA precursor pool although the levels of other dNTPs can also be modified as a consequence of the nucleotide interconversions and allosteric regulatory mechanisms discussed above. dNTP imbalances involving dTTP excess and depletion and elevated dCTP levels are of particular interest for this study and are examined in detail below.

2.2.1 dTTP Depletion

Mutants defective in thymidylate synthase die if thymidine or dTMP (for permeable yeast cells) is not provided during cell growth (Kunz 1982). This 'thymineless death' was first described for *E. coli* by Barner

and Cohen (1954). Starvation for thymine nucleotides can also be induced by drug treatment (Neuhard 1966; Smith *et al*. 1973; Genther *et al*. 1977; Wurtz *et al*. 1979; Jackson 1980; Kunz *et al*. 1980; Ritter *et al*. 1980; Peterson *et al*. 1983; Hoar and Dimnik 1985; Clements *et al*. 1990). Cells can be treated with drugs that block thymidylate synthase such as fluoro-deoxyuridine (FdUrd), which is converted by thymidine kinase to the potent inhibitor fluorodeoxyuridine monophosphate (FdUMP), and FdUMP itself (in nucleotide-permeable strains) (Figure 3). Alternatively, replenishment of the tetrahydrofolate consumed during dTMP synthesis can be prevented by using antifolate drugs such as methotrexate and sulfanilamide (Figure 3).

It is now well-established that starvation for thymine nucleotides can elicit a wide range of genetic and biochemical effects. In bacteria it was shown to cause recombination (Gallant and Spottswood 1964; Norin and Goldschmidt 1979), prophage induction (Korn and Weissbach 1962), sensitization to UV light (Gallant and Suskind 1961; Little and Hanawalt 1973), induction of *recA*-dependent SOS functions (Bresler *et al*. 1970; Witkin 1976; Witkin and Wermundsen 1978), non-conservative DNA replication (Pauling and Hanawalt 1965), and degradation of single- and double-stranded DNA (Freifelder 1969; Ramareddy and Reiter 1970; Reiter and Ramareddy 1970; Walker 1970; Reichenbach *et al*. 1971; Breitman *et al*. 1972; Hill and Fangman 1973; Buick and Harris 1975; Nakayama and Hanawalt 1975; Bousque and Sicard 1976). In yeast, thymineless conditions lead to the loss of mitochondrial DNA (Barclay and Little 1978; Zelikson and Luzzati 1982), DNA strand breaks (Barclay *et al*. 1982), mitotic inter- and intrachromosomal recombination (gene conversion and crossing-over), unequal sister chromatid crossing-over (Kunz *et al*. 1980; Barclay *et al*. 1982; Kunz and Haynes 1982; Eckardt *et al*. 1983; Kunz *et al*. 1984, 1985),

Figure 3. Inhibition of dTMP Biosynthesis by Fluorodeoxyuridine Monophos-
phate (FdUMP), Methotrexate and Sulfanilamide. Enzymes involved:
1: dihydrohydroxymethylpterin pyrophosphokinase; 2: dihydro-
pteroate synthase; 3: dihydrofolate synthetase; 4: dihydrofolate
reductase; 5: serine hydroxymethyltransferase; 6: thymidylate
synthase. Sulfanilamide, an analogue of p-aminobenzoate, poisons
an initial step of *de novo* folate synthesis (2). Methotrexate,
a dihydrofolate analogue, inhibits dihydrofolate reductase (4).
FdUMP blocks thymidylate synthase (6) activity (modified from
Kisliuk 1984; Kunz *et al.* 1984).

dUMP $\xrightarrow{\quad 6 \quad}$ dTMP

$N_5 N_{10}$-methylene
tetrahydrofolate

dihydrofolate

glycine

NADPH + H

NADP

serine

5

4

tetrahydrofolate

3

glutamate

2-amino-
4-hydroxy-6-
methylpterin $\xrightarrow{\quad 1 \quad}$ 2-amino-4-hydroxy-6-
methylpterin
pyrophosphate $\xrightarrow{\quad 2 \quad}$ dihydro-
pteroate

ATP

AMP

p-aminobenzoate
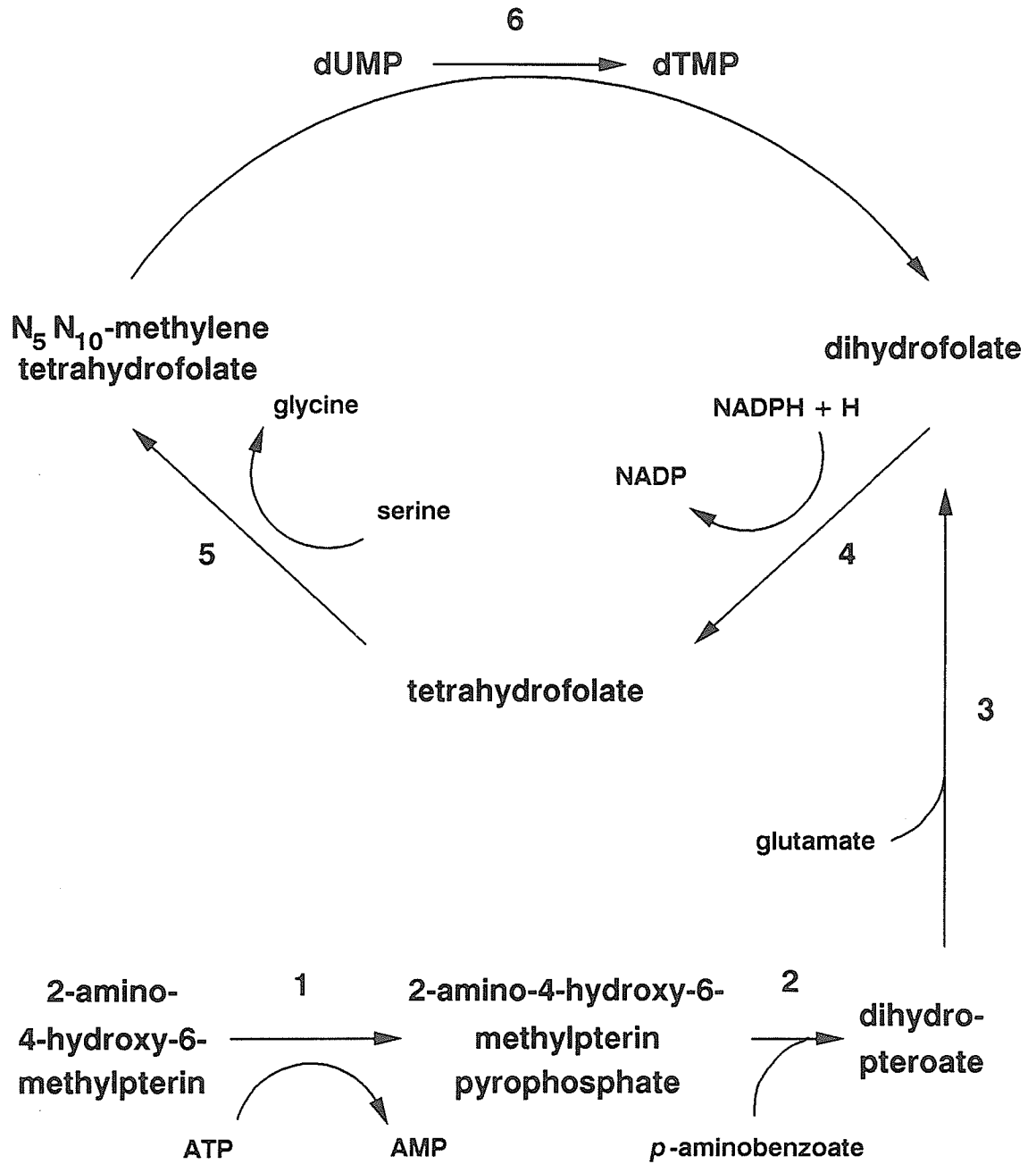
formation of the 'nuclear dense body', a cellular organelle which usually develops only during meiosis (Moens *et al.* 1981), and induction of mating-type switching (Kunz *et al.* 1985). Additionally, there are a number of reports showing that thymine starvation in mammalian cells can cause DNA strand breaks (Sedwick *et al.* 1981; Ayusawa *et al.* 1983; Greer and Kaplan 1983), chromosome and chromatid breaks (Ockney *et al.* 1968; Melnyk *et al.* 1971; Hansmann 1974; Benedict *et al.* 1977; Jensen and Nyfors 1979), and chromosome rearrangements (Seno *et al.* 1985). Furthermore, it was demonstrated that thymine deprivation could induce fragile sites in human cells, including the fragile X site responsible for a rare genetically inherited disorder (Sutherland 1988). Of particular interest for this study is that starvation for thymine nucleotides has been associated with increased mutagenesis in *E. coli* (Coughlin and Adelberg 1956, Weinberg and Latham 1956), *S. typhimurium* (Holmes and Eisenstark 1968), *B. subtilis* (Bresler *et al.* 1970), bacteriophage T4 (Smith *et al.* 1973), yeast (Eckardt *et al.* 1983) and mammalian cells (Koyama *et al.* 1982). Where measured, thymineless conditions have been found to result in dTTP and possibly dGTP depletion and expansion of the dCTP, dATP and dUTP pools (Goulian and Beck 1966; Neuhard 1966; Meuth 1981b; Kunz 1982).

2.2.2 dTTP Excess

Addition of thymine or thymidine, or for permeable cells, dTMP, to actively growing cultures has been found to induce mutations in bacterio-phage T4 (Bernstein *et al.* 1972; DeVries and Wallace 1982), yeast (Barclay and Little 1981, Eckardt *et al.* 1983), *Drosophila melanogaster* (Parkash 1967) and mammalian cells (Rossman and Stone-Wolff 1982; Arecco *et al.* 1988; Wilkinson and McKenna 1989). The degree of mutagenicity provoked by

excess thymidine or dTMP appeared to reflect the magnitude of the dTTP pool expansion (Meuth 1981b; Weinberg et al. 1985; Wilkinson and McKenna 1989; Mun and Mathews 1991) and simultaneous provision of deoxycytidine substantially reduced the mutagenic effects of excess thymidine in Chinese hamster cells, presumably by restoring the dTTP/dCTP balance (Rossman and Stone-Wolff 1982; Goncalves et al. 1984).

In addition to the induction of mutations, provision of excess thymidine or dTMP has a number of other effects. One of the earliest observations was that elevated thymine nucleotide levels were associated with cytotoxicity in yeast and mammalian cells (Kim et al. 1965; Morse and Potter 1965; Langjahr et al. 1975). Evidence that this effect was more pronounced in malignant than in 'normal' cells led to the use of thymidine in anticancer chemotherapy (Blumenreich et al. 1980; Chiuten et al. 1980; Kufe et al. 1980, 1981). Conditions expected to enhance dTTP pools were also found to inhibit DNA and ribonucleic acid (RNA) synthesis and induce recombination in yeast (Langjahr et al. 1975; Eckardt et al. 1983), cause sister chromatid exchange, chromosome and chromatid aberrations in mammalian cells (Yang et al. 1966; Luchnik et al. 1976; Anderson et al. 1981; Perry 1983) and lead to teratogenesis in D. melanogaster (Parkash 1967, 1971).

2.2.3 Excess dCTP

Lesions in several genes involved in de novo dCTP synthesis, including cytidine triphosphate (CTP) synthase, dCMP deaminase and dCMP hydroxy-methylase, confer mutator phenotypes in phage T4, E. coli, or mammalian cells but dNTP imbalances (predominantly increases in the dCTP levels) have so far been confirmed only for defects in the first two genes

(Williams and Drake 1977; Weinberg *et al.* 1985; Aronow *et al.* 1984; Sargent and Mathews 1987). The mutator phenotypes associated with elevated dCTP pools resulting from CTP synthase and dCMP deaminase deficiencies in mammalian cells were reduced by offsetting the dCTP:dTTP imbalance through provision of thymidine (Meuth 1981b; Weinberg *et al.* 1985; Mun and Mathews 1991). In contrast to the multitude of studies on perturbation of thymidine nucleotide levels, there is little information about effects other than induction of mutations by increased dCTP pools. However, Anderson *et al.* (1981) reported that treatment with deoxycytidine can induce chromosome aberrations in mammalian cells.

2.2.4 Other Types of dNTP Pool Imbalances

Lesions in at least two other genes involved in dNTP synthesis, ribonucleotide reductase and dUTPase, confer mutator phenotypes in *E. coli* or mammalian cells but dNTP imbalances have been confirmed only for ribonucleotide reductase (Hochhauser and Weiss 1978; Mun and Mathews 1991). Treatment with the ribonucleotide reductase inhibitor hydroxyurea imbalances dNTP pools and also is mutagenic (Moore and Hurlbert 1985; Snyder 1988). Increases in the levels of purine deoxyribonucleotide pools can be caused either by addition of excess purines or by deficiency or inhibition of enzymes involved in nucleoside catabolism such as purine nucleoside phosphorylase or adenosine deaminase (Ullman *et al.* 1976; Carson *et al.* 1977; Cohen *et al.* 1978a,b; Gudas *et al.* 1978; Ullman *et al.* 1978, 1979; Tu and Patterson 1978; Ross *et al.* 1981; Simmonds *et al.* 1982a,b). Elevated dGTP levels have been found to increase dATP levels and decrease dCTP and dTTP levels (Gudas *et al.* 1978; Ross *et al.* 1981) while increased dATP concentrations decrease the levels of all three remaining

DNA precursors (Meuth *et al*. 1976; Ullman *et al*. 1978). It is believed that the effects of elevated purine dNTP levels on other dNTP concentrations are a consequence of dATP and dGTP inhibiting the activity of ribonucleotide reductase (Meuth *et al*. 1976; Gudas *et al*. 1978; Ullman *et al*. 1978, 1979; Ross *et al*. 1981; Waddell and Ullman 1983; Reichard 1988). Accumulation of purine dNTPs has been associated with loss of cell viability, induction of mutations, formation of DNA single- and double-strand breaks, inhibition of repair of single strand breaks after mutagen treatment and the occurrence of chromosome breaks and aberrations (Kihlman 1963; Ullman *et al*. 1978; Anderson *et al*. 1981; Kefford and Fox 1982; Brox *et al*. 1984; Cohen and Thompson 1986; Seto *et al*. 1986; Yoshioka *et al*. 1987a; Mattano *et al*. 1990). In addition, imbalances in the levels of purine dNTPs have been implicated in immuno-deficiency diseases (Cohen *et al*. 1978a,b; Gudas *et al*. 1978; Ullman *et al*. 1978, 1979; Simmonds *et al*. 1982a,b; Carrera and Carson 1987).

Despite the foregoing, dNTP imbalances do not invariably enhance spontaneous mutagenesis. Changes in dNTP levels in methyl methanesulfonate (MMS) sensitive mutants of *N. crassa* did not correlate with the magnitudes of spontaneous mutation frequencies in these mutants (Srivastava and Schroeder 1989). A thymidine kinase-deficient mouse cell subclone, with a 6.5-fold greater dCTP:dTTP ratio than the line from which it was derived, exhibited the same spontaneous mutation frequency as that line (Wilkinson and McKenna 1989). In addition, the increased spontaneous mutagenesis due to an alteration in the ribonucleotide reductase from mouse cells could be uncoupled from expansion of the dATP and dGTP pools caused by that alteration (Caras and Martin 1988). A different reductase defect associated with much higher spontaneous mutation rates in the same mouse

cell line decreased all four dNTP pools but did not change the relative balance of the dNTPs compared to wild type cells (Roguska and Gudas 1984). Thus, there may also be very subtle links between dNTP imbalance and spontaneous mutation.

2.2.5 Characterization of Mutations Resulting from dNTP Imbalance

Recently, DNA sequencing techniques have been applied to characterize mutations arising under conditions of dNTP stress (for details concerning earlier specificity studies, see Williams and Drake 1977; DeVries and Wallace 1982; Kunz 1985). So far, mainly pyrimidine dNTP imbalances have been examined. Briefly, the systems used and principal results were as follows. First, a phage T4 amber mutation (*rIIamHB74*) was reverted by a mutator due to T4 dCMP deaminase deficiency (Sargent and Mathews 1987). The hydroxymethyl dCTP pool was shown to be elevated in *E. coli* infected with this T4 mutator strain and ten revertants were analyzed. All ten arose via A·T to G·C transitions though any change that resulted in a non-terminating codon should have reverted the amber codon. Second, forward mutations were induced in the *E. coli* *lacI* gene by thymidine starvation using either a thymidine auxotroph (71 mutants analyzed) (Glickman *et al.* 1988) or treatment with the antifolate trimethoprim (43 mutants analyzed) (Veigl *et al.* 1991). The dNTP pools were not measured in either study but thymidine starvation of *E. coli* depletes dTTP levels while enhancing the dATP, dCTP and dUTP pools (Goulian and Beck 1966; Neuhard 1966) and trimethoprim is expected to affect dNTP levels similarly. The majority (>75%) of the analyzed *lacI⁻* mutations were base-pair substitutions but A·T to G·C transitions did not predominate as predicted on the bases of an elevated dCTP:dTTP ratio and earlier specificity studies. Furthermore, G·C

to C·G transversions and frameshifts, but not deletions or A·T to C·G transversions, were detected after starvation of the auxotroph for two hours. Conversely, deletions and A·T to C·G transversions, but not G·C to C·G transversions or frameshifts, were recovered after starvation of the auxotroph for six hours. Third, *aprt⁻* mutations were induced in Chinese hamster cells by thymidine treatment or by the mutator effect attributed to a putative CTP synthase deficiency (Phear and Meuth 1989a). These two conditions were known to elevate the dTTP or dCTP pools, respectively (Meuth 1981b), but the degrees of the dNTP imbalances assumed to have been induced in this study were not determined. Most (38/44) *aprt⁻* mutations induced by thymidine were G·C to A·T transitions, a result consistent with the expected increase in the dTTP:dCTP ratio. Although the mutator strain was believed to have an enhanced dCTP:dTTP ratio, only 16/40 mutations were A·T to G·C transitions. The remainder were almost exclusively trans- versions at G·C pairs. Fourth, *hprt⁻* mutations were induced in human cells by treatment with deoxyguanosine or deoxyadenosine (Mattano *et al.* 1990). The dGTP or dATP levels were increased under these conditions but only these two dNTP pools were measured. Analysis of 6 or 14 *hprt⁻* mutations induced by deoxyguanosine or deoxyadenosine, respectively, revealed that primarily single base-pair substitutions, deletions and insertions occurred.

These studies demonstrated that for the same dNTP perturbation, the precise changes recovered seemed to depend on the assay system used and even on the duration of treatment. Nevertheless, the majority of mutations characterized were single base-pair events that could have been due to misinsertion of the dNTP(s) expected or shown to be present in excess (dNTP misincorporation during DNA synthesis can initiate frameshifting;

Bebenek and Kunkel 1990). In the one investigation where the results did not support dNTP misinsertion as the cause of mutation (Mattano *et al.* 1990), very few mutations were analyzed clouding the significance of this difference. Despite the apparent bias for misincorporation of the dNTPs in excess, the transitions expected on the basis of pyrimidine dNTP imbalances were seldom the most frequent substitutions (Glickman *et al.* 1988; Phear and Meuth 1989a; Veigl *et al.* 1991). This argues against a 'mass action' hypothesis as the only explanation for the induction of substitutions.

2.2.6 Next-Nucleotide Effects

The accuracy of *in vitro* DNA synthesis is reduced by a bias in the relative dNTP concentrations present in the reaction mixture and the decreased replicational fidelity can reflect misinsertion of the dNTP in excess (Kunz 1982). The sequencing data detailed above support the possibility that dNTP imbalance induces mutations *in vivo* by promoting nucleotide misincorporation during DNA replication. However, the mutations were unlikely to occur solely in this fashion. In most cases, there seemed to be a preference for substitutions at positions where the next dNTP to be incorporated was one present in excess. This is consistent with two mechanisms whereby increased levels of the next correct nucleotide can reduce the fidelity of replication *in vitro*: 1. an increased probability of extending mismatched primer termini (Mendelman *et al.* 1990); and 2. a decreased probability of 3' to 5' editing (proofreading) due to an enhanced rate of polymerization, a phenomenon termed the 'next-nucleotide effect' (Kunkel 1988). The influence of dNTP concentration on extension of mismatched primer termini is thought to apply only to situations where

extension occurs in the absence of proofreading (Mendelman *et al.* 1990). Since replicative DNA polymerases from *E. coli*, yeast and mammalian cells are known to have proofreading ability, or 3′ to 5′ exonuclease activities that probably function in proofreading (Kunkel *et al.* 1987; Kunkel 1988; Bialek *et al.* 1989; Morrison *et al.* 1990), it seems reasonable to think that the next-nucleotide-like effects observed *in vivo* were a consequence of inhibiting proofreading rather than enhancing extension of mismatched primer termini.

2.3 dNTP Imbalances and Induced Mutagenesis

2.3.1 dNTP Imbalances Influence Induced Mutagenesis

Alterations in dNTP pools, or conditions expected to modify DNA precursor levels, modulate induced mutagenesis in bacterial, yeast and mammalian cells (Kunz 1988). The approaches taken to produce DNA precursor imbalances in such studies include using strains with defects in adenine phosphoribosyltransferase, CTP synthase, deoxycytidine kinase, dCMP deaminase, dTMP kinase, thymidylate synthase, glycinamide ribonucleotide synthase, ribonucleotide reductase or thymidine kinase (Collins *et al.* 1988; Kunz 1988; Amara *et al.* 1991), inhibition of dTMP biosynthesis by treatment with FdUrd, and supplementation of growth medium with deoxy-nucleosides or nucleotides (Öhnfeld and Jenssen 1982; Arecco *et al.* 1988; Kunz 1988). However, the magnitudes of the dNTP pool disturbances were measured only in a minority of studies (Meuth 1981a, 1983; Roguska and Gudas 1984; Arecco *et al.* 1988; Amara *et al.* 1991). Not knowing how and to what extent the dNTP pools are affected can present interpretational difficulties because unanticipated dNTP pool imbalances may occur. For example, the effects of excess deoxycytidine might be attributable to high

levels of dTTP, rather than dCTP, resulting from deamination of the nucleoside or dCMP to deoxyuridine or dUMP, respectively, and its subsequent conversion to dTMP via dUMP. The mutagens tested to date include UV radiation, DNA base, nucleoside or nucleotide analogs, aflatoxin B1, benzo(a)pyrene and mono- or bifunctional alkylating agents (Öhnfeld and Jenssen 1982; Fox 1985; Peterson *et al.* 1985a; Arecco *et al.* 1988; Kunz 1988).

The following conclusions can be derived from these studies. First, whether induced mutagenesis is affected by DNA precursor disturbances and, if so, the manner and extent to which it is affected appears to vary with treatment conditions or mutational systems. For example, UV mutagenesis was enhanced by thymidine starvation of *E. coli* strains auxotrophic for the nucleoside prior to irradiation (Witkin and Wermundsen 1978; B. A. Kunz, personal communication) but treatment of wild-type bacteria with FdUrd after UV irradiation was antimutagenic (Ohta *et al.* 1986) as was decreased dTMP kinase (*CDC8*) activity in yeast (Prakash *et al.* 1979) even though dTTP levels should be reduced in each case. (In the latter study, temperature-sensitive *cdc8* alleles were used. Mutants bearing such alleles have reduced dTMP kinase activity even at permissive temperatures, Sclafani and Fangman 1984.) A deoxycytidine kinase deficiency, which diminishes dCTP pools, increased the mutagenicity of EMS in Chinese hamster but not murine leukemia cells (Meuth 1983; McKenna and Boullier 1988). Post-treatment provision of deoxythymidine was found to increase, reduce or have no effect at all on EMS-, ethylnitrosourea-, MMS- or 6-amino-2-hydroxypurine-induced *hprt⁻* reversion in Chinese hamster cells, depending on the mutant allele used (Randazzo *et al.* 1987). Furthermore, the magnitude of potentiation or reduction of mutagenesis in this study

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ranged from less than 2-fold to more than 300-fold. Second, there appears to be a direct relationship between dNTP pool imbalance and mutation induction by certain agents. Thymidine-mediated enhancement of methyl-nitrosourea-induced mutagenesis could be reversed by simultaneous provision of deoxycytidine (Fox 1985). More to the point, the mutagenicity of 2-aminopurine (2-APur), bromodeoxyuridine (BrdUrd) and EMS could be modulated by manipulation of dNTP levels (Meuth 1981a; Caras *et al.* 1982; Ross *et al.* 1987; Kaufman 1988). Third, for alkylating agents the dNTP pool imbalances were not simply altering mutagen transport or the alkylation reactions. EMS and MNNG mutagenesis was potentiated by treatment with nucleosides following exposure to the alkylating agents (Fox 1985; Meuth 1981a; Peterson *et al.* 1985a). Fourth, the different responses to dNTP imbalance argue against a single mechanism being responsible for the effects of DNA precursor levels on mutation induction. In fact, there appears to be at least one mechanism related to dNTP pools which does not involve imbalance. A defect in ribonucleotide reductase of mouse lymphoma cells was associated with a ten-fold increase in the frequency of MNNG-induced *hprt⁻* mutations (Roguska and Gudas 1984). Yet the reductase mutation did not cause an imbalance in the dNTP pools relative to a comparable wild-type strain. Instead the levels of all four dNTPs were uniformly reduced by 50%.

2.3.2 Links Between dNTP Pool Imbalances and Induced Mutation

Ostensibly, a number of mechanisms might link alterations in DNA precursor levels with cellular responses to mutagens.

Enhanced mutagenesis might result from misinsertion of the dNTP(s) in excess opposite template damage (Meuth 1981a). This seems to be a

reasonable explanation for the effects of post-treatment dNTP imbalances on the mutagenicity of monofunctional alkylating agents. O^6-alkylguanine is considered to be the major premutational lesion produced by chemicals such as EMS and MNNG (Horsfall *et al*. 1990). This lesion can mispair with thymine during DNA synthesis *in vitro* and *in vivo* and this mispairing specificity can be increased *in vitro* by a reduction in the level of dCTP present in the reaction (Snow and Mitra 1988; Ellison *et al*. 1989). Consistent with these findings, the mutagenic efficiencies of EMS and MNNG are increased by high dTTP:dCTP ratios in Chinese hamster cells (Meuth 1981a, 1983; Peterson *et al*. 1985a) [it should be noted, however, that contradictory findings have been reported for mouse cells (McKenna and Boullier 1988)]. This dNTP imbalance would be expected to lead, respectively, to an increase or decrease in the frequency of G·C to A·T transition, the predominant mutational event induced by EMS and MNNG *in vivo* (Horsfall *et al*. 1990).

In cases where the dNTP levels are perturbed prior to or during mutagen treatment, it is also possible that DNA precursor pools may be targets for alkylating agents (Topal and Baker 1982). This suggestion was based on the finding that dNTPs in the DNA precursor pool are more susceptible to methylation by methylnitrosourea than those in duplex DNA and is supported by demonstrations that alkylated dNTPs can be incorporated into DNA during replication *in vitro* (Snow and Mitra 1988). Thus, the potentiating effect of elevated dTTP levels on alkylation mutagenesis might be explained as an allosteric interaction of dTTP with ribonucleotide reductase leading to a larger dGTP pool as a target for alkylation. The O^6-alkylguanine DNA precursors formed in this way could then be incorporated opposite template thymines during DNA replication and

following demethylation would produce A·T to G·C transitions if the resulting T-G mismatch was not corrected prior to the next round of DNA replication. Such a mechanism predicts that: 1. modified DNA precursors would be mutagenic in cells; 2. increasing the target size prior to treatment with the alkylating agent should potentiate mutagenesis; and 3. A·T to G·C transitions would be enhanced in the case of elevated dGTP levels. Whereas certain modified DNA bases introduced into cells apparently can be converted to dNTPs and incorporated into DNA, O^6-methyl-guanine, O^6-methyldeoxyguanosine and O^6-methyldGTP were generally not mutagenic in bacterial or mammalian systems (Foote *et al.* 1988; Snow and Mitra 1988). This was attributed to failure of the base or nucleoside to be phosphorylated due to the modification and to degradation of the methylated dGTP to guanine. Although O^6-methylguanine was reported to induce *hprt⁻* mutations in Chinese hamster V79 cells (Kaina *et al.* 1983), incorporation of the modified base into DNA was not confirmed. Elevation of dATP, dGTP or dTTP pools prior to mutagen treatment enhanced methyl-nitrosourea mutagenesis in Chinese hamster cells by 2- to 5-fold but the mutations were not characterized (Arecco *et al.* 1988). Thus, these results must be viewed as indirect support for the possibility that elevated DNA precursor pools may potentiate alkylation mutagenesis *in vivo* by acting as targets for alkylating agents.

Altered dNTP pools may modulate base analog mutagenesis by facili-tating incorporation of a nucleotide in excess opposite the analog or promoting misincorporation of an analog (Hopkins and Goodman 1980). During *in vitro* DNA synthesis, 2-APur can be misincorporated opposite template cytosine or, when in the template, can mispair with cytosine (Mhaskar and Goodman 1984). Genetic studies demonstrated that 2-APur induces primarily

A·T to G·C transitions suggesting that most often cytosine mispairs with template 2-APur (Coulondre and Miller 1977; Ripley 1981). On this basis, conditions that raise or lower dCTP levels should increase or decrease 2-APur mutagenesis, respectively. This has been found to occur in viral, bacterial and mammalian systems and a similar result was obtained for the related analog 6-amino-2-hydroxypurine in mammalian cells (Kunz 1988). Although it was suggested that perturbation of dNTP pools by 2-APur might contribute to its mutagenicity (Hopkins and Goodman 1980), it was subsequently demonstrated that the analog had no effect on dNTP levels in bacteriophage T4-infected *E. coli* or mouse lymphosarcoma cells (Caras *et al*. 1982; Hopkins and Goodman 1985). While BrdUrd in a synthetic template directed mispairing with guanine, BrdUrd was misincorporated opposite template guanine at a rate 200-fold greater and this misincorporation was reduced by high levels of dCTP (Lasken and Goodman 1984). *In vivo*, excess dCTP, which should compete with bromodeoxyuridine triphosphate (BrdUTP) for insertion opposite template guanine, suppressed BrdUrd mutagenesis in yeast and mammalian cells whereas elevated dTTP levels, which reduce the dCTP pool and so should favour BrdUTP incorporation, enhanced BrdUrd mutagenesis (Ross *et al*. 1987; Kaufman 1988). These results plus the fact that allosteric interaction of BrdUTP with ribonucleotide reductase decreases the dCTP level in mammalian cells led to the conclusion that the BrdUTP:dCTP ratio is an important determinant of mutagenesis by BrdUTP incorporation (Kaufman 1988). DNA sequencing revealed that the majority of mutations induced by misincorporation of BrdUTP into a shuttle vector-borne *E. coli* *gpt* gene in mouse cells were G·C to A·T transitions as anticipated (Davidson *et al*. 1988). Enhancement of the dGTP:dATP ratio during DNA replication to promote incorporation of dGTP opposite template

BrdUrd caused a large increase in mutagenesis (Kaufman 1988). As expected, mutations induced in bacteriophage λ or mouse cells by replication of BrdUrd-substituted DNA were primarily A·T to G·C transitions (Skopek and Hutchinson 1982; Xu *et al.* 1990). However, a recent study of T4-infected *E. coli* demonstrated that the relative frequencies of BrdUrd-induced A·T to G·C and G·C to A·T transitions depended on whether the replication of phage DNA relied on the bacterial or T4 ribonucleotide reductase (Sargent *et al.* 1989). This suggests that the composition of the T4 multienzyme complex for synthesis of DNA precursors might influence BrdUrd mutagenesis. Furthermore, BrdUrd had little effect on the dCTP levels indicating potential insensitivity of the T4 reductase to allosteric inhibition by BrdUTP. Consequently, there may be unexpected complexities to the relationship between BrdUrd mutagenesis and dNTP metabolism, at least for T4-infected *E. coli*.

Finally, altered dNTP levels might influence the repair of damaged DNA (Francis *et al.* 1979). Consistent with this possibility, fluctuations in DNA-precursor pools are often associated with increased or decreased cyto-toxicity in response to mutagen treatment (Kunz 1988). DNA repair could be affected in several ways. Hydroxyurea, an inhibitor of ribonucleotide reductase and methotrexate, which interferes with *de novo* purine nucleotide and dTMP synthesis, inhibit excision repair of UV-, EMS- or MMS-induced DNA damage in rodent or human cells (Snyder 1988; Borchers *et al.* 1990). These results argue that inhibition of *de novo* DNA precursor synthesis blocks repair replication as a consequence of dNTP depletion. Even so, the situation is not clear. Evidence as to whether dNTP depletion can inhibit the incision step of excision repair is contradictory (Collins *et al.* 1988; Snyder 1988; Borchers *et al.* 1990). In addition, elevated

levels of dATP, but not other dNTPs, may also inhibit repair of UV damage (Snyder 1988). To complicate the picture further, depletion of the dGTP pool to undetectable levels in CHO cells prevented removal of UV-induced pyrimidine dimers but did not block repair replication and it was suggested that repair might be 'aberrant' (e.g., terminating prematurely) under these conditions (Collins *et al.* 1988). Similarly, thymidine kinase deficiency appeared to inhibit excision repair in mouse and human cells (McKenna *et al.* 1985; Intine and Rainbow 1990) but repair replication occurred in the mouse cells (McKenna and McKelvey 1986) where the dTTP pool was reduced by about 50% (Wilkinson and McKenna 1989). In view of the latter findings, it was suggested that dNTP misincorporation during repair synthesis under conditions of DNA precursor imbalance might contribute to the potentiation of mutation induction by physical and chemical agents in thymidine kinase deficient mouse cells (McKenna and McKelvey 1986; Wilkinson and McKenna 1989). There might be at least three other mechanisms linking dNTP pools and repair. First, dNTP imbalances might lead to the accumulation of DNA damage that could saturate repair systems leaving an increased number of mutagen-induced DNA lesions unrepaired. It is known, for example, that conditions causing dTTP depletion result in DNA breakage that might arise from misincorporation of uracil into DNA (Haynes and Kunz 1986). Second, it was suggested that a slight sensitization of mouse cells to the lethal and mutagenic effects of EMS and MMS by adenine phosphoribosyltransferase deficiency might be due to a reduction in adenosine triphosphate (ATP) for ATP-dependent repair enzymes rather than to dNTP imbalance (Amara *et al.* 1991). Third, repair enzyme activity might somehow be indirectly regulated by DNA precursor levels. This admittedly speculative suggestion is based on the finding that the

activity of *E. coli* photolyase, which monomerizes UV-induced cyclobutane dimers, is increased post-translationally by adenine deprivation (Alcorn and Rupert 1990).

2.4 Consequences of dNTP Pool Imbalances in Human Cells *In Vivo*

Imbalances in the DNA precursor pools of growing cells in culture can enhance spontaneous mutation rates and modulate cellular responses to physical and chemical mutagens. The means by which these effects may be accomplished are beginning to be understood but puzzling observations remain. For example, dNTP excess seems to promote nucleotide misincorporation during DNA replication and to inhibit proofreading of the mis-inserted nucleotides. Yet the mutational specificities observed are seldom those predicted on the basis of this and the expected dNTP imbalances and it seems that additional factors must influence the types and locations of substitutions that occur. Similarly, mechanisms proposed to account for the potentiation or reduction of induced mutagenesis fit well in some cases but not in others. Certainly, a goal for the future will be to improve our understanding of the mechanisms linking dNTP imbalances with spontaneous and induced mutagenesis. Such knowledge may have broad implications since dNTP imbalances can occur in human cells *in vivo*. Evidence that expression of human fragile sites (detected as constrictions, gaps or breaks in human metaphase chromosomes) and folate deficiency (the most common vitamin deficiency world-wide) are associated with perturbations in dNTP pools has been reviewed previously (Kunz 1988; MacPhee *et al.* 1988). Recently, dNTP pools in cells from patients with *ataxia telangiectasia* (a genetic chromosome instability disease which predisposes to cancer) were found to be approximately two to three-fold

larger than those for normal cells when the proportions of cells in S phase were taken into account (Kenne and Åkerblom 1990). The greatest differences were observed for the pyrimidine dNTPs and treatment of *ataxia telangiectasia* cells with thymidine caused a more pronounced elevation of the dTTP pool than for the normal strain implying altered regulation of dNTP levels. It is an intriguing possibility that these irregularities may contribute to the *ataxia telangiectasia* phenotype. Unequivocal evidence that dNTP imbalances can occur *in vivo* comes from studies of certain human immunodeficiency syndromes caused by the absence of adenosine deaminase and purine nucleoside phosphorylase (Boss and Seegmiller 1982). These enzymes participate in the catabolism of adenine or guanine dNTPs, respectively, and affected individuals have elevated levels of dATP or dGTP which are toxic to T cells (Simmonds *et al.* 1982b; Barankiewicz and Cohen 1984). Under more normal conditions, dNTP imbalances might be caused by the breakdown of DNA from dead or dying cells and it has been suggested that blood platelets may play a role in buffering DNA precursor imbalances in the peripheral circulation (Shaw 1988). These observations plus the ability of engineered disturbances in DNA precursor levels to modulate spontaneous and induced mutagenesis argue that dNTP pool balance may be an important contributory factor to the maintenance of genetic stability in human cells *in vivo*.

2.5 Alkylation Mutagenesis

EMS and MNNG (Figure 4), the two chemical mutagens used in this project, are well-known monofunctional alkylating agents that are known to be mutagenic and carcinogenic (for reviews, see Gichner and Veleminský 1982; Singer and Grunberger 1983; Sega 1984; Saffhill *et al.* 1985).

Figure 4. Chemical Structures of EMS and MNNG. The reactive groups are shown in boxes. A: ethyl methanesulfonate (EMS); B: N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

A

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-O-\boxed{CH_2-CH_3}$$

B

$$O=N-\overset{\boxed{CH_3}}{N}-\overset{\underset{\displaystyle \|}{C}}{\underset{NH}{\|}}-\overset{H}{\underset{NO_2}{N}}$$
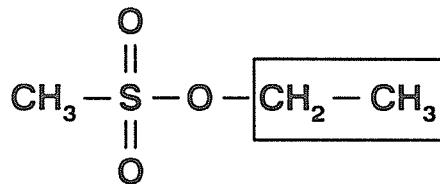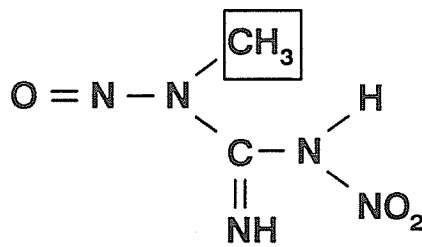
Neither compound occurs naturally in the environment. EMS was synthesized for the first time in 1905 by reaction of methanesulfonite anhydride with ethyl alcohol (Billeter 1905) and MNNG in 1947 by nitrosation of N-methyl-N'-nitroguanidine (McKay and Wright 1947). Nevertheless, both alkylating agents have been widely used for scientific investigations, and studying these compounds has helped to understand the interaction between mutagens and DNA, mechanisms of mutation induction and the repair of alkylation damage in DNA.

Alkylation of DNA is believed to be the primary reason for mutation induction by chemical agents such as EMS and MNNG. However, both agents are able to alkylate proteins and some of their cytotoxicity could be due to this effect. Interestingly, in addition to the transfer of an alkyl group, MNNG has a second way to interact with proteins (at lysine or cysteine residues) due to its guanidino group which can cause nitro-amidation of proteins (McCalla and Reuvers 1968; Nagao *et al.* 1969; Lawley and Thatcher 1970). In particular, this nitroamidation reaction is thought to be the reason that MNNG, more than other mutagens and carcinogens, can inhibit enzymes including the DNA polymerase in hamster fibroblasts and DNA polymerases I and III of *E. coli* (MNNG can inhibit the polymerase and endonuclease functions of polymerase III) (Anderson and Burdon 1970; Jiménez-Sánchez 1976; Hellermann and Billen 1978).

Alkylating agents can transfer their reactive alkyl group to a variety of cellular, nucleophilic centres such as the ring N atoms and exocyclic O atoms of purines and pyrimidines or O atoms of the sugar-phosphate backbone (Singer and Kusmierek 1982). Possible alkylation targets in the DNA are depicted in Figure 5. Nucleophilic substitutions can either follow an S_N1- or S_N2-type reaction. S_N1-reactions are two-step reactions, where

Figure 5. Possible Targets in DNA for Alkylating Agents. One A·T (top) and
one G·C (bottom) pair from a DNA molecule are shown along with
the sugar-phosphate backbone on the purine side of the base-
pairs. Potential alkylation sites are shown in circles
(information given in this figure is reviewed in Beranek 1990).
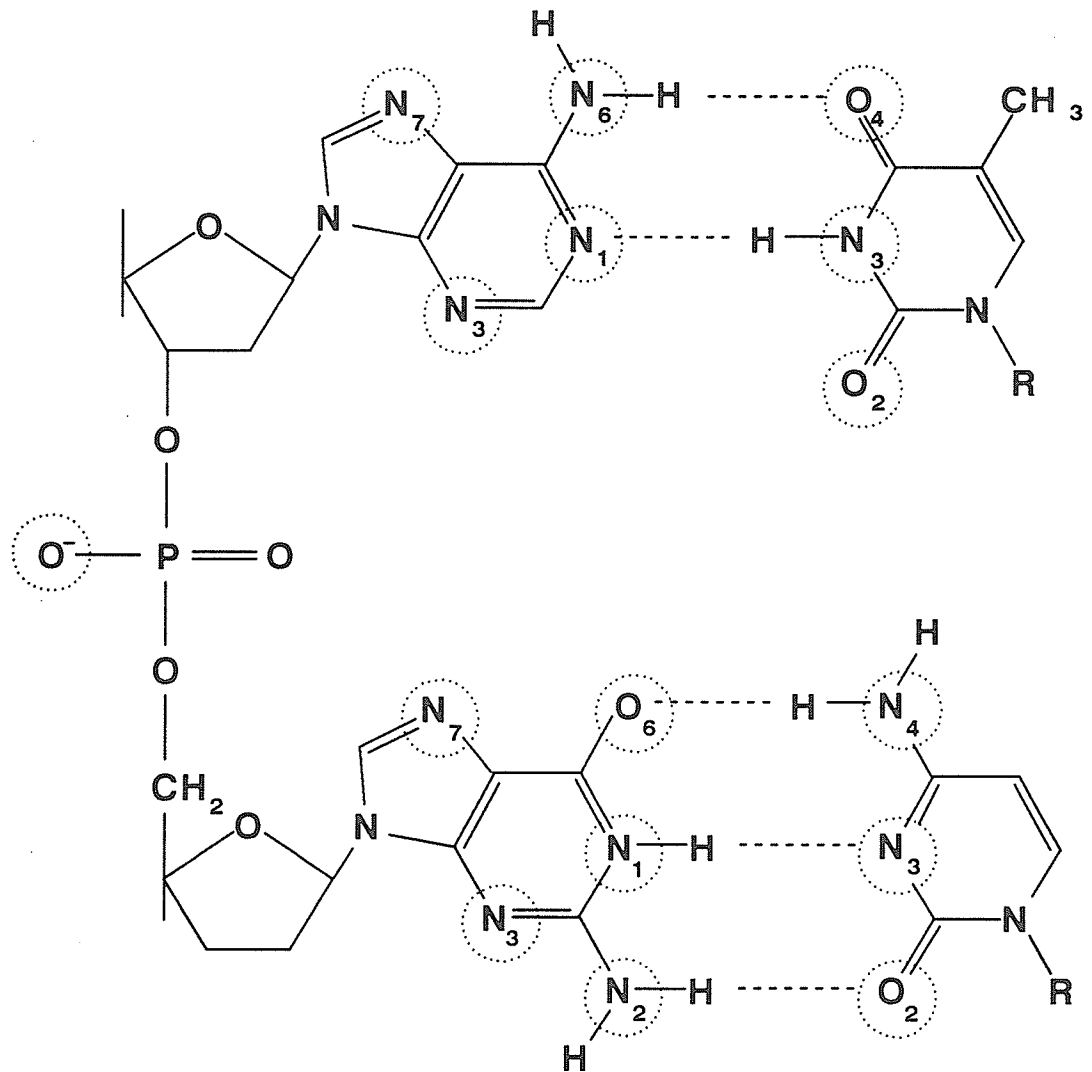
the generated electrophilic carbonation intermediate (the first rate-limiting step) is rapidly trapped by a nucleophilic centre on the macro-molecule (DNA) forming a covalently bound adduct (second step). In an S_N2-reaction, the agent carrying the alkyl group forms a transition complex with the macromolecule (DNA). This complex then gives rise to an alkylated product with the release of the leaving group (one-step reaction). S_N2-reactions are therefore strictly dependent on steric accessibility. The transfer of an ethyl group from EMS can occur either in an S_N1 or S_N2 fashion, while MNNG-induced methylations follow only the S_N1-reaction pathway (Lawley 1974; O'Connor 1981; Swenson 1983). The difference in the type of reaction is reflected in the individual *in vitro* alkylation patterns identified for EMS and MNNG (Table 1). A further point which is relevant to the mutagenicity of each agent is that with the exception of O^6-alkylguanine and *N*-3-alkyladenine, ethylated adducts tend to have a longer halflife *in vivo* than their corresponding methylated species (Den Engelse *et al.* 1987; Singer 1985).

2.5.1 Mechanisms Involved in Alkylation Mutagenesis

The mutagenicity and carcinogenicity of an alkylating agent was linked early on to its ability to alkylate the O^6 position of guanine (Loveless 1969). For both alkylating agents used in this study, the major premutational lesion is believed to be O^6-alkylguanine (Loveless 1969; Lawley and Shah 1972) which can mispair with thymine *in vitro* (Abbott and Saffhill 1979; Dodson *et al.* 1982; Eadie *et al.* 1984; Loechler *et al.* 1984; Snow *et al.* 1984a; Saffhill and Hall 1985; Bhanot and Ray 1986; Topal *et al.* 1986; Williams and Shaw 1987). *In vivo*, this should lead to G·C to A·T transitions and, indeed, analysis of EMS- and MNNG-induced mutations in a

Table 1. *In Vitro* Alkylation Patterns of DNA[a]

Site of alkylation	EMS	MNNG
Adenine		
N-1	1.7[b]	1.0[b]
N-3	4.2-4.9	12.0
N-6	ND[c]	NR[d]
N-7	1.1-1.9	NR
Cytosine		
O^2	0.3	NR
N-3	0.4-0.6	2.0
Guanine		
N-1	ND	NR
N-2	ND	NR
N-3	0.3-0.9	NR
O^6	2.0	7.0
N-7	58.0-65.0	72.0
Thymidine		
O^2	ND	NR
N-3	ND	NR
O^4	ND	NR
Phosphodiesters	12.0-13.0	NR

[a] Data as reviewed in Beranek (1990).

[b] Percent of total DNA alkylation.

[c] Not detected / below limits of detection.

[d] Not reported.

variety of systems has revealed that G·C to A·T substitutions predominate
(Prakash and Sherman 1973; Coulondre and Miller 1977; Dodson *et al.* 1982;
Loechler *et al.* 1984; Burns *et al.* 1986, 1987; Lebkowski *et al.* 1986;
Lucchesi *et al.* 1986; Ashman and Davidson 1987a; Reed and Hutchinson 1987;
Richardson *et al.* 1987a; Gordon *et al.* 1988; Ingle and Drinkwater 1989).
There is also evidence that alkylation at the O^4 position of thymine
(Lawley *et al.* 1973), which could lead to A·T to G·C transitions, might
play a minor role in EMS and MNNG mutagenesis (Prakash and Sherman 1973;
Ripley 1975; Coulondre and Miller 1977; Saffhill and Hall 1985; Burns *et
al.* 1986, 1987; Preston *et al.* 1986, 1987; Singer 1986). It was assumed
that the mispairing of O^6-alkylguanine with T and of O^4-alkylthymine with
G were directed by the formation of stable hydrogen bonds. This notion
arose from the belief that base selection during DNA synthesis is directed
by the formation of a suitable Watson-Crick base-pair with the newly
inserted dNTP (McHenry 1988). In order for the alkylated base and T or G
to hydrogen bond stably, it was thought that the alkyl group had to be
positioned *anti* to the *N*-1 of guanine or *N*-3 of thymine, respectively.
This led to the predicted mispairing structures shown in Figure 6 (Singer
1980; Brennan *et al.* 1986; Williams and Shaw 1987). More recently, the
results from nuclear magnetic resonance (NMR) and X-ray crystallography
studies indicated that the alkyl group is actually positioned *syn* to the
N-1 of guanine or *N*-3 of thymine, respectively (Brennan *et al.* 1986;
Parthasarathy and Fridey 1986; Yamagata *et al.* 1988; Kalnik *et al.* 1988a,
1989a). Positioning the alkyl group in *syn* to the *N*-3 of guanine or *N*-1 of
thymine, limits the formation of hydrogen bonds and the mispairing
structures shown in Figure 7 were predicted (Kalnik *et al.* 1988a, 1989a).
The lack of stable hydrogen bonds would make mispairing with an alkylated

Figure 6. Possible Structures for O^6-MethylG·T and O^4-MethylT·G Mispairs Assuming the Formation of Stable Hydrogen Bonds (modified from Swann 1990). Note that all alkyl groups (shown in circles) are positioned *anti* to the *N*-1 of guanine and *N*-3 of thymine, respectively. The configuration of O^4-methylthymine allows the prediction of two hypothetical hydrogen bond formations. A: O^6-methylG·T; B and C: O^4-methylT·G.
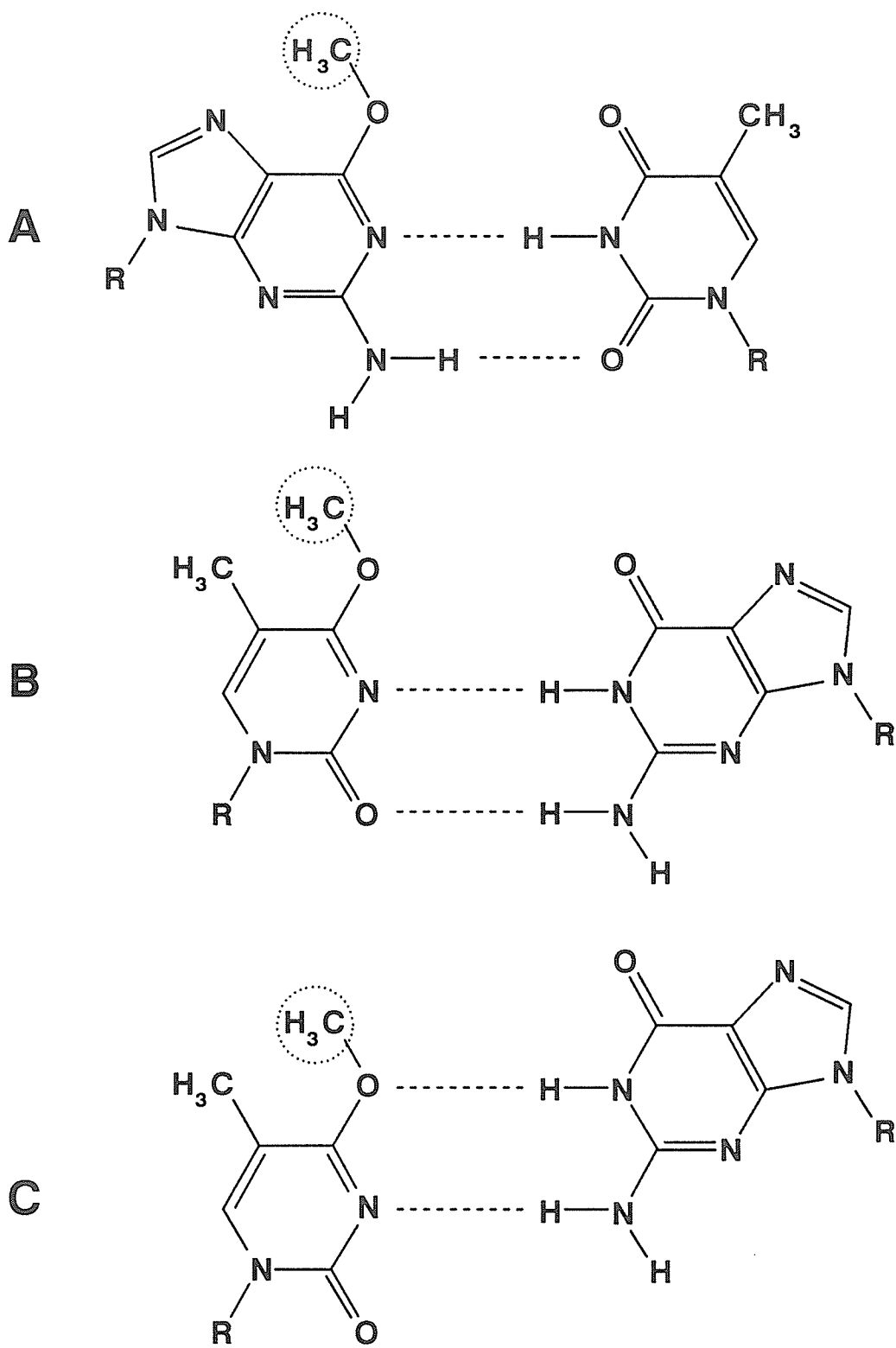
Figure 7. Hypothetical Structures for the Formation of O^6-MethylG·T and O^4-MethylT·G Mispairs Suggested from the Results of NMR and X-Ray Crystallography Studies (modifed from Swann 1990). Note that all alkyl groups (shown in circles) are postioned *syn* to the *N*-1 of guanine and *N*-3 of thymine, respectively. A: O^6-methylG·T; B: O^4-methylT·G.
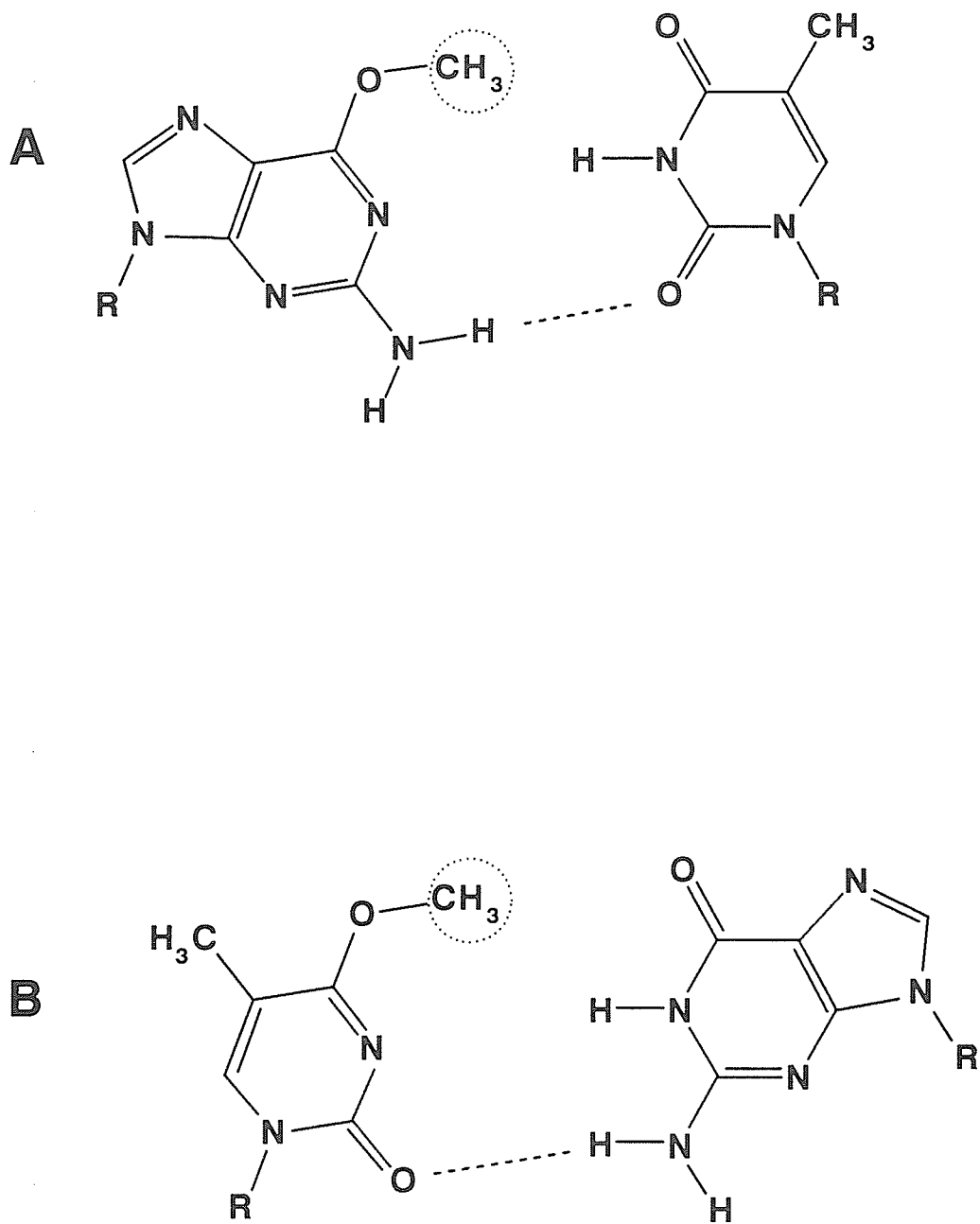
base a rather weak link in the DNA structure. Nevertheless, two different observations support the assumption that this could be the structural basis for the mispairing of alkylated bases in DNA. First, poly O^6-methylguanine is not able to form a stable complex with a poly(U) oligonucleotide (uridine is preferentially incorporated opposite O^6-alkylguanine by RNA polymerase; Gerchman and Ludlum 1973) as would be expected if the formation of stable hydrogen bonds was involved in the mispairing (Mehta and Ludlum 1976). Second, melting point studies of DNA duplexes containing O^6-methylguanine (or O^4-methylthymine) showed that oligomers which had the alkylated base paired with a C (or A) had higher melting points than oligomers containing O^6-methylG·T (or O^4-methylT·G) pairs (Gaffney *et al.* 1984; Li *et al.* 1987; Gaffney and Jones 1989). Both observations agree with the presence of destabilized mispairs and the lack of stable hydrogen bonding. Several possibilities have been discussed to explain why O^6-alkylguanine and O^4-alkylthymine should mispair without the involvement of stable hydrogen bonds (Leonard *et al.* 1990; Swann 1990). O^6-alkylguanine and O^4-alkylthymine share physical similarities with adenine and cytosine, respectively. If DNA polymerase actively recognizes the template nucleotide and selects the nucleotide to be incorporated, as some data suggest (Fersht *et al.* 1983; Boosalis *et al.* 1987; El-Deiry *et al.* 1988), the alkylated bases might be mistaken for an A or C. Consequently, a T or G, respectively, could be selected for incorporation opposite the alkylated bases. In addition, O^6-methylG·T and O^4-methylT·G pairs have the alignment of normal Watson-Crick base-pairs with N-1 of the purine positioned across from the N-3 of the pyrimidine. O^6-methylG·C and O^4-methylT·A base-pairs have a wobble alignment where the alkylated base is moved into the major groove of the DNA structure causing a distortion in the phospho-

diester-sugar backbone (Kalnik *et al.* 1988a,b, 1989a,b; Leonard *et al.*
1990). Such distortions could interfere with polymerase action and slow
down the rate of insertion of the next nucleotide thereby increasing the
probability that the inserted base is removed by proofreading. O^6-methylG·T
and O^4-methylT·G pairs are sterically much more acceptable and could confer
a greater stacking stability. Therefore, mispairing involving alkylated
bases could be the result of a 'better fit' within the larger structure of
the DNA molecule rather than being the consequence of stable hydrogen bond
formation.

Next to mispairing of alkylated bases during DNA replication, two
other possible mechanisms for the mutagenicity of alkylating agents have
been discussed. First, cytosines which are situated across from O^6-alkyl-
guanine could be more prone to deaminate as a result of 'cross strand
protonation' (Williams and Shaw 1987). Deamination of cytosine generates
uracil, which, if left in place pairs with adenine during DNA replication.
Therefore, it was suggested that an increased probability of deamination
due to alkylation at the O^6 position of guanine could lead to a rise in the
formation of G·C to A·T transitions (Richards *et al.* 1984; Sowers *et al.*
1987). Recently, a comparison of EMS mutagenesis in wild-type and uracil-
DNA glycosylase deficient strains of *E. coli* indicated that cytosine
deamination could be a potential source of alkylation mutagenesis (Fix *et
al.* 1990). Second, apurinic/apyrimidinic (AP) sites have been implicated
as a mutagenic lesion produced by monofunctional alkylating agents
(Drinkwater *et al.* 1980; Foster and Eisenstadt 1985; Loeb 1985). AP sites
can occur due to spontaneous loss of alkylated bases (i.e. *N*-7-alkyl-
guanine) or as a consequence of enzymatic removal of alkylbases including
N-3-alkylpurines and O^2-alkylpyrimidines (Evensen and Seeberg 1982; Karran

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et al. 1982; Lindahl 1982; McCarthy *et al*. 1984). Subsequently, mutations might arise as a result of DNA polymerase preferentially misinserting purines across from AP sites during replicative by-pass of these DNA lesions (Sagher and Strauss 1983). That this mechanism might contribute to the generation of mutations was supported by the observation that *E. coli* mutants deficient for AP endonuclease (an enzyme required for the repair of AP-sites; see below for details) showed increased lethality and muta-genicity following MNNG treatment compared to wild-type cells (Foster and Eisenstadt 1985). These effects were counteracted by the presence of a second mutation disabling the *N*-3-methyladenine-DNA glycosylase, an enzymatic activity that creates AP-sites at methylated nucleotides (Foster and Eisenstadt 1985).

2.5.2 Site and Strand Specificity of Alkylation Mutagenesis

Recently, MNNG-induced mutations in the *lacI* and *gpt* genes of *E. coli* and the *cI* gene of bacteriophage λ have been found to occur preferentially at G·C pairs where the guanine is preceded (5′) by a purine, most often guanine (Burns *et al*. 1987; Reed and Hutchinson 1987; Richardson *et al*. 1987a; Glickman *et al*. 1987; Gordon *et al*. 1988). It was suggested (Horsfall *et al*. 1990) that this preference for sites which are preceded by 5′ purines is correlated to the reaction mechanism of the alkylation agents. This was based on the observations that: 1. the same site preference was found in bacterial and mammalian systems for a number of other S_N1 type agents including *N*-methyl-*N*-nitrosourea, *N*-ethyl-*N*-nitroso-urea, *N*-propyl-*N*′-nitro-*N*-nitrosoguanidine and dialkylnitrosamines (DuBridge *et al*. 1987; Richardson *et al*. 1987b; Burns *et al*. 1988a,b; Horsfall and Glickman 1988, 1989; Horsfall *et al*. 1989; Van der Vliet *et*

al. 1989) and 2. the site preference was missing for agents which reacted more by a S_N2 type pathway such as dimethylsulfate and EMS- (Burns *et al*. 1986; Ashman and Davidson 1987a; Ingle and Drinkwater 1989; Zielenska *et al*. 1989). Conceivably, several factors could determine where mutations occur during alkylation mutagenesis. First, DNA sequences surrounding a potential target site might influence the reactivity of this site with the alkylating agent and determine its accessability to the agent (Horsfall *et al*. 1990). Second, neighbouring bases could affect nucleotide insertion across from alkylated bases (Singer *et al*. 1989). Third, the efficiency of DNA repair of alkylation damage might be sequence dependent (Burns *et al*. 1986; Dolan *et al*. 1988). However, involvement of DNA repair in site specificity is controversial. Analysis of MNNG-induced sequence alterations in *E. coli* having defects in various repair genes showed no indication that the defects altered the 5' specificity observed in wild-type strains (Burns *et al*. 1987; Richardson *et al*. 1987a; Gordon *et al*. 1988). It was therefore concluded that the preference for a 5' purine reflected the distribution of the alkylation damage rather than the specificity of its repair. On the other hand, a number of studies showed that the repair efficiencies of prokaryotic and eukaryotic enzymes are sequence dependent and that alkylated bases having a 5' G are repaired at the slowest rate compared to bases positioned 5' to pyrimidines (Topal *et al*. 1986; Dolan *et al*. 1988; Topal 1988; Pegg and Dolan 1989). Even though EMS mutagenesis showed no preference for occurring at sites having a 5'-purine, excision repair might affect the site specificity of EMS-induced damage. It was demonstrated that only in excision repair pro-ficient strains of *E. coli* were EMS-induced mutations more likely to occur at sites which were flanked 3' and 5' by a G·C pair (Burns *et al*. 1986).

A bias for MNNG-induced mutations at sites where the guanine is on the non-transcribed strand has been reported for the λ *cI* and *E. coli gpt* genes (Reed and Hutchinson 1987; Richardson *et al.* 1987a). Such a bias might be predicted on the basis of strand-specific replication or repair (Mellon *et al.* 1987; Wu and Maeda 1987). However, the removal of *N*-methyl-purines from the dihydrofolate reductase gene of CHO cells has been reported to occur at similar rates on the transcribed and non-transcribed strands (Scicchitano and Hanawalt 1989, 1990). Since the strand preference was limited to a few mutational studies analyzing MNNG-induced mutations, it was suggested that the bias could have been due to a non-random distribution of sequences particularly prone to target MNNG mutagenesis rather than to preferential repair (Gordon and Glickman 1988). Strand preferences have not been detected for EMS mutagenesis in *E. coli* (Burns *et al.* 1986).

2.5.3 Repair of Alkylation Damage in the DNA

As indicated above, repair of alkylation damage might play a role in the specificity of alkylation mutagenesis. Repair systems for alkylation damage in prokaryotes, namely *E. coli*, have been much better described so far than those in eukaryotes, including yeast. However, initial studies have indicated that there could be differences in the repair systems and particularly in the way they are regulated in prokaryotic and eukaryotic cells.

Generally, three different repair pathways have been associated with the removal of alkylation damage: 1. elimination of the alkylated nucleotide by enzymes of the excision repair complex; 2. detachment of the alkyl group by an alkyl transferase; and 3. excision of the alkylated base

by an alkyl-DNA glycosylase. Even though ethylation damage is recognized by enzymes of the excision repair complex, and nucleotides ethylated at the O^6 or O^4 position of guanine or thymine, respectively, are excised (Warren and Lawley 1980; Todd and Schendel 1983; Samson *et al.* 1988; Voigt *et al.* 1989), excision repair seems to be required primarily for the removal of nucleotides carrying more complex alkylation groups (Todd and Schendel 1983; Samson *et al.* 1988). The repair of short-chained alkylation groups, such as those transferred by the agents used in this study, apparently rely mainly on the activity of alkyl transferases and alkyl-DNA glycosylases.

Repair of the major premutational lesion O^6-alkylguanine is carried out by the O^6-alkylguanine-DNA alkyltransferase (AT). This protein catalyzes the transfer of the alkyl group and also acts as the alkyl group acceptor protein. The alkyl group is transferred to an internal cysteine and the AT activity is inactivated by this process. Therefore, the repair of O^6-alkyl-guanines in this fashion is limited by the number of AT molecules present in a cell (Demple and Karran 1983; Yarosh 1985; Lindahl *et al.* 1988). Since the AT acts only once as an alkyl acceptor, it is not an enzyme in the strict sense of the definition. However, even though there is no evidence for the restoration of the protein activity under various conditions *in vitro*, the complete absence of regeneration of the acceptor site *in vivo* has not been proven (Pegg 1990).

Two distinct AT activities are encoded by the *E. coli ada* and *ogt* genes. The *ada* gene product is the larger protein and provides a dual function as an AT and transcriptional activator of the 'adaptive response' (for details, see below) (Teo *et al.* 1984; Sedgwick *et al.* 1988). The *ogt* gene product is a non-inducible enzyme which only has AT activity and is

the major AT protein present in uninduced cells (Rebeck *et al*. 1989; Wilkinson *et al*. 1989). Both proteins remove alkyl groups not only from O^6-alkylguanines but also from O^4-alkylthymines and methylphosphodiesters (Foote *et al*. 1980; Olsson and Lindahl 1980; Demple *et al*. 1982; McCarthy *et al*. 1983; McCarthy and Lindahl 1985; Potter *et al*. 1987; Wilkinson *et al*. 1989). Generally, methyl groups are the preferred substrate but ethyl groups can be removed as well although at slower rates (Sedgwick and Lindahl 1982; Pegg *et al*. 1984). ATs have been identified in a number of different eukaryotic organisms, including yeast, rodents and humans (Yarosh 1985; Koike *et al*. 1990; Pegg 1990; Sassanfar and Samson 1990; von Wronski *et al*. 1991). They are able to remove ethyl and methyl groups but their substrate specificity seems to be limited to O^6-alkylguanines. Their capability to deal with O^4-methylthymine is still under debate (Pegg *et al*. 1984; Yarosh 1985; Brent *et al*. 1988; Sassanfar *et al*. 1991; Xiao *et al*. 1991).

Removal of an alkylated base from DNA by hydrolysis of the *N*-glyco-sylic bond between the deoxyribose and the alkylated base requires the activity of an alkyl-DNA glycosylase. In *E. coli*, two different alkyl-DNA glycosylases have been identified, *N*-3-methyladenine-DNA glycosylases I and II (encoded by the *tag* and *alkA* genes, respectively). Similar to the regulation of the two alkyltransferases found in *E. coli*, one of the alkyl-DNA glycosylases is expressed constitutively (*tag* gene product) while the other is part of the adaptive response (see below) to alkylating agents (*alkA* gene product) (Riazuddin and Lindahl 1978; Evensen and Seeberg 1982; Karran *et al*. 1982; Nakabeppu *et al*. 1984). These alkyl-DNA glycosylases seem to repair primarily alkylation damage that is cytotoxic. Both are able to remove *N*-3-methyladenine and *N*-3-ethyladenine. In

addition, N-3-alkyladenine-DNA glycosylase II can excise N-3- and N-7-methyladenine, N-3- and N-7-methylguanine, O^2-methylcytosine, O^2-methylthymine and their ethylated analogs (Friedberg 1985; Walker 1985; Claverys and Lacks 1986; Lindahl *et al.* 1988). Mutants deficient in alkyl-DNA glycosylase activity are extremely sensitive to the lethal effects of alkylating agents (Evensen and Seeberg 1982; Clarke *et al.* 1984). N-3-methyladenine-DNA glycosylases have also been identified in mammalian cells (Cathcart and Goldwaith 1981; Gallagher and Brent 1982; Male *et al.* 1985) and yeast (Chen *et al.* 1989, 1990; Berdal *et al.* 1990). The yeast enzyme is capable of removing N-3-methyladenine as well as N-7-methylguanine, its gene is alkylation inducible and its amino acid sequence shows homology to the *E. coli alkA* gene product (Berdal *et al.* 1990; Chen *et al.* 1990).

The action of an alkyl-DNA glycosylase creates an AP site which needs further processing by an AP endonuclease to continue the repair process. AP endonucleases hydrolyze the phosphodiester bond next to the AP site. The AP endonucleases associated with alkylation repair are either characterized as class I or class II enzymes (Sancar and Sancar 1988). Class I enzymes create 3'-OH and 5'-P termini 3' to the DNA lesion whereas class II enzymes create 3'-OH and 5'-P termini 5' to the lesion. Although AP endonucleases are usually very specific in the way they catalyze the incision reaction, one enzyme has been described which can be classified in more than one category. This AP endonuclease (isolated from human placenta) can either act as a class I or a class II enzyme (Grafstrom *et al.* 1982). In *E. coli*, endonucleases III and IV (the *xth* and *nfo* gene products, respectively), have been implicated in the repair of AP sites following alkylation mutagenesis (Foster and Davis 1987; Sancar and Sancar

1988). Several class I and II AP endonucleases have been identified in yeast (Armel and Wallace 1984; Johnson and Demple 1988a;b) and one of these (encoded by the *APN1* gene) shows sequence homology to the *nfo* gene in *E. coli*. Therefore, *APN1* could possibly be involved in the repair of AP sites arising after alkylation damage in yeast (Popoff *et al.* 1990).

Subsequent to phosphodiester hydrolysis by the AP endonuclease, the abasic sugar is removed by an exonuclease and the gap is then filled with the correct nucleotide by DNA polymerase and the remaining nick sealed by DNA ligase.

Samson and Cairns (1977) discovered that *E. coli* cells which were exposed to low doses of MNNG displayed a markedly increased resistance to the mutagenic and killing effects of MNNG and other alkylating agents. This inducible defense reaction to alkylation damage was termed the 'adaptive response'. Since then, it has been established that the adaptive response involves the induction of genes encoding enzymes specific for the repair of alkylation damage: O^6-alkylguanine-DNA alkyltransferase (*ada*) and *N*-3-methyladenine DNA glycosylase (*alkA*) (Evensen and Seeberg 1982; Lindahl 1982; Karran *et al.* 1982; McCarthy *et al.* 1984; Demple *et al.* 1985). In addition, two genes with unknown functions (*alkB*, *aidB*) are also part of the adaptive response (Kondo *et al.* 1986; Volkert *et al.* 1986). The expression of these genes is regulated by the *ada* gene product, a protein that functions as an alkyltransferase and a transcriptional activator (Teo *et al.* 1984; Nakabeppu and Sekiguchi 1986). The Ada protein contains several cysteines, one of which (position 69) accepts methyl groups removed from methylphosphotriesters while another (position 321) acts as an acceptor for alkyl groups from O^6-alkylguanines and O^4-alkyl-thymines (Demple *et al.* 1985; Teo *et al.* 1986; Sedgwick 1987). Methylation

of the phosphotriester methyl acceptor site, but not the methyl acceptor site, is required for the activation of the Ada protein as a transcriptional activator. The activated form then stimulates transcription from promoters of genes involved in the adaptive response (Teo *et al.* 1986).

As for mammalian cells (reviewed in Frosina and Abbondandolo 1985; Wani *et al.* 1989), the evidence for the existence of an adaptive response in *S. cerevisiae* is still controversial. There are several reports that yeast lacks an adaptive response to alkylation treatment (Maga and McEntee 1985; McBlane and Kilbey 1985; Polakowska *et al.* 1986) but a number of genes can be induced by treatment with alkylating agents including the genes for DNA ligase, DNA polymerase I, ribonucleotide reductase, ubiquitin and *N*-3-methyladenine DNA glycosylase and several damage inducible genes whose functions are still unidentified (Johnston and Nasmyth 1978; Peterson *et al.* 1985b; Ruby and Szostak 1985; Johnson *et al.* 1986; Elledge and Davis 1987, 1989; Johnston *et al.* 1987; Hurd *et al.* 1987; Treger *et al.* 1988, Chen *et al.* 1990). The presence of these damage inducible genes suggests that yeast might be capable of a coordinated response to DNA damaging agents. However, although the AT protein of eukaryotes shows homology to the *E. coli ada* gene product, it lacks the portion corresponding to the *ada* transcriptional activator region (Tano *et al.* 1990). Therefore, it still remains to be determined if regulation of enzymes involved in the repair of alkylation damage in eukaryotes is comparable to that for *E. coli*.

2.6 Purpose of This Study

The aim of this project was to investigate the relationships between dNTP imbalances and alkylation mutagenesis in the yeast *S. cerevisiae*.

Three related parts were involved. First, the mutational specificities of three different types of dNTP imbalance were determined: dTTP depletion, dTTP excess and dCTP excess. Second, EMS- and MNNG-induced mutations were characterized under conditions of balanced dNTP pools. Third, the effects of elevated dCTP levels on the lethality and mutagenicity of EMS and MNNG were measured. In each of these three parts, DNA sequencing was used to characterize forward mutations in the yeast suppressor transfer RNA (tRNA) gene *SUP4*-o. Knowledge of the types of sequence alteration and their frequency and location within a single gene can provide valuable clues about the lesions and mechanisms responsible for the changes. This information, plus comparisons of mutational spectra, facilitated an examination of the following questions. 1. Are mutations in strains with imbalanced dNTP levels caused by misinsertion of the dNTP(s) in excess? 2. Are such misinsertion events influenced by DNA sequence context and is there evidence for a next-nucleotide effect in yeast *in vivo*? 3. Do mutations induced by dNTP imbalances show a strand bias? 4. Are O^6-alkyl-guanines the major premutational lesion for EMS and MNNG mutagenesis in yeast? 5. Is there evidence for influence of DNA sequence context on EMS- and MNNG-induced mutations and do alkylation-induced mutations occur more frequently on the transcribed or non-transcribed strand? 7. Do elevated dCTP levels decrease EMS- and MNNG-induced mutations in yeast and, if so, is this decrease associated with a reduction in G·C to A·T transitions?

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Strains and Plasmids

All haploid strains of the yeast *S. cerevisiae* used throughout this study are isogenic derivatives of the wild-type strain MKP-o. MGK-d is deficient for dCMP deaminase (*dcd1*), SBT is dTMP-permeable (*tup*), SBT-T is dTMP-permeable and auxotrophic for dTMP (*tmp1*), while SBT-TL is a variant of SBT-T that requires less dTMP for normal growth. Complete genotypes of these strains are given in Table 2. Construction of MKP-o and MGK-d is described elsewhere (Pierce *et al.* 1987; Kohalmi *et al.* 1991) whereas the construction of the various SBT strains is detailed in the Results section. Strains carrying the plasmid YCpMP2 (see below) are designated MKP-op, MGK-dp, SBT-p, SBT-Tp and SBT-TLp. The diploid yeast strain gt12T (Taylor *et al.* 1987) was used as a source of DNA for hybridization controls. The *E. coli* strain JF1754 (Table 2) was used to recover plasmid DNA from yeast cells.

YCpMP2 (Pierce *et al.* 1987) is a yeast-bacterial shuttle vector which can replicate autonomously in yeast and in *E. coli* (Figure 8). The vector contains a replication origin (*ARS1*), a centromere sequence (*CEN4*) and a selectable marker (*URA3*) from yeast and the replication origin (*REP*) and the ampicillin resistance determinant (*AMP^R*), the ß-lactamase gene, from the bacterial plasmid pBR322. The *CEN4* sequence allows YCpMP2 to be maintained predominantly at a copy number of one (Newlon 1988) in haploid yeast cells. In addition, YCpMP2 carries *SUP4*-o, an ochre suppressor allele of a yeast tyrosine tRNA gene. In this study, *SUP4*-o is used as a mutational target. The replication origin from the filamentous phage M13, which permits the generation of single-stranded plasmid DNA (Dente *et al.* 1983; Levinson *et al.* 1984; Zagursky and Berman 1984), is also part of

Table 2. Strains Used in This Study

Strain	Genotype	Reference or Source
MKP-o	*MATα can1-100 ade2-1 lys2-1 ura3-52 his3-Δ200 leu2-3,112 trp1-Δ901*	Pierce *et al.* (1987)
MGK-d	as for MKP-o but also *LEU2::dcd1*	constructed by M. Glattke
SBT	as for MKP-o but also *tup$_{100}$*[a]	constructed for this study
SBT-T	as for SBT but also *LEU2::tmp1*	constructed for this study
SBT-TL	as for SBT-T but *tup$_{20}$*[a] instead of *tup$_{100}$*[a]	constructed for this study
gt12T	*MATα/MATa URA3/ura3-52 HIS4/his4-519 leu2-3,112/leu2-3,112 tup$_{100}$/tup$_{100}$*[a] *TMP1/LEU2::tmp1*	Taylor *et al.* (1987)
JF1754[b]	*Δlac gal metB leuB hisB436 hsdR rpsL*	Pierce *et al.* (1987)

[a] Subscripts indicate the concentration of dTMP (in µg/ml) required for normal growth.

[b] *Escherichia coli.*

Figure 8. **Structure of YCpMP2.** The 1.06 kb *Hind*III-*Bam*HI fragment carrying

SUP4-o is not drawn to scale.

this vector. pTL46 (Taylor *et al.* 1987) was used for constructing SBT-T as described in section 4.1.

3.2 Media Recipes

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

per liter: 20.0 g dextrose
              20.0 g Bacto peptone (Difco)
              10.0 g Bacto yeast extract (Difco)

For yeast strains carrying a *tup* mutation, 1.5 g potassium phosphate (monobasic) was added and the medium was then designated YPDP.

B. SD (Synthetic Minimal Dextrose):

per liter: 40.0 g dextrose
              6.7 g Bacto yeast nitrogen base w/o amino acids
                   (Difco)

Required nutrients were added at the concentrations suggested by Sherman *et al.* (1983). To enhance colouring due to the *ade2-1* allele, adenine was added at half (for SBT-TLp: at one third) the recommended concentration (Zimmermann 1973).

SD media for yeast strains carrying a *tup* mutation contained twice the normal concentration of standard media components and required nutrients. To prevent caramelization of the sugar, dextrose was prepared as a 0.33 g/ml solution, filter sterilized and added after autoclaving and cooling of the medium to 45°C.

C. MS (methotrexate/sulfanilamide):

per liter: 100.0 mg methotrexate (Sigma)
                5.0 g sulfanilamide (Sigma)

Methotrexate was added to water and concentrated NaOH was added drop-wise to increase the pH to 12.0. After the methotrexate dissolved, the pH was reduced to 7.0 by adding HCl drop-wise. Sulfanilamide was then added and dissolved by heating the mixture to 65°C. After cooling, components for YPDP or SD medium were added as described above.

D. All media used with SBT and SBT-T contained 100 μg/ml dTMP. The dTMP concentration in media for SBT-TLp was reduced to 20 μg/ml.

E. YT (Miller 1972):

per liter: 8.0 g Bacto tryptone (Difco)
                  5.0 g Bacto yeast extract (Difco)
                  5.0 g NaCl

When required, ampicillin (final concentration: 100 μg/ml) was added after autoclaving and cooling of the medium to 45°C. The medium was then designated YT+amp.

F. M9+amp (Miller 1972):

per liter: 500 μg thiamine
                  500 mg NaCl
                  246 mg $MgSO_4$
                  200 mg glucose
                  100 mg casamino acids (vitamin free)
                  100 mg ampicillin
                   10 mg $CaCl_2$
                    6 g Na_2HPO_4
                    3 g KH_2PO_4
                    1 g NH_4Cl

Ampicillin was added after autoclaving and cooling of the medium to 45°C.

G. For solid media, 20 g/l agar (Difco) was added.

3.3 Advantages of the *SUP4*-o System

There are several advantages to using the *SUP4*-o gene (Figure 9) as a mutational target. First, simple methods can be used to isolate mutations occurring in this gene (see section 3.4). Second, the two transcriptional promoters (A and B) are located within *SUP4*-o and only the gene itself, the first sixty 5′ and the first seven 3′ flanking base-pairs are necessary for normal expression *in vivo* (Hall *et al.* 1982; Shaw and Olson 1984; Allison and Hall 1985). The 5′ sequence apparently acts to fine tune

Figure 9. DNA Sequence of the *SUP4*-o Gene and its Flanking Regions. Shown

is the 242-base-pair *Bam*HI-*Eco*RI fragment carried on YCpMP2

which encompasses the *SUP4*-o gene and its 5′ and 3′ flanking

regions. The tRNA gene starts at position +1 and ends at

position +89. The internal promotors A and B are located at +8

through +19 and +68 through +78, respectively. The ochre

anticodon (AC) is at +36 through +38 and the 14 base-pair intron

(I) extends from +40 through +53. The transcription start site

(↑) is located at -5 and the transcription termination signal

(TTS) extends from +90 through +97.

```
          -80         -70         -60         -50         -40         -30         -20         -10          -1
5' GGATCC  GGGACCGGAT  AATTATTTGA  AATCTCTTTT  TCAATTGTAT  ATGTGTTATG  TAGTATACTC  TTTCTTCAAC  AATTAAATA 3'
3' CCTAGG  CCCTGGCCTA  TTAATAAACT  TTAGAGAAAA  AGTTAACATA  TACACAATAC  ATCATATGAG  AAAGAAGTTA  TTAATTTAT 5'

   +1     +10         +20         +30         +40         +50         +60         +70         +80     +89
5' CTCTCGGTAG  CCAAGTTGGT  TTAAGGCGCA  AGACTTTAAT  TTATCACTAC  GAAATCTTGA  GATCGGGCGT  TCGACTCGCC  CCCGGGAGA 3'
3' GAGAGCCATC  GGTTCAACCA  AATTCCGCGT  TCTGAAATTA  AATAGTGATG  CTTTAGAACT  CTAGCCCGCA  AGCTGAGCGG  GGGCCCTCT 5'
               ‾‾A‾‾                               ‾‾AC‾‾      ‾‾I‾‾                   ‾‾B‾‾

   +90    +100        +110        +120        +130        +140        +150
5' TTTTTTTGTT  TTTTATGTCT  CCATTCACTT  CCCAGACTTG  CAAGTTGAAA  TATTTCTTTC  AAGAATTC 3'
3' AAAAAAACAA  AAAATACAGA  GGTAAGTGAA  GGGTCTGAAC  GTTCAACTTT  ATAAAGAAAG  TTCTTAAG 5'
   ‾‾TTS‾‾
```

transcription and the 3' sequence provides a transcription termination signal. Thus, since *SUP4*-o is only 89 base-pairs long (Goodman *et al.* 1977), the entire gene and its flanking regions can be sequenced in one operation. Third, there are relatively few restrictions on the location within the gene where a mutation can be detected or on the type of DNA sequence alteration that can be recovered (Kunz *et al.* 1990a). This is most likely due to the involvement of various of the tRNA bases in: 1. pairing to maintain the tRNA secondary and tertiary structures; 2. interactions with RNA polymerase III, transcription factors, processing enzymes and the ribosome; or 3. codon recognition (Deutscher 1984; Sharp *et al.* 1985; Bjork *et al.* 1987). A list of all single base-pair substitutions that can be detected in *SUP4*-o is presented in Table 3. The data are the result of analyzing more than 5,000 spontaneous or induced mutants and using *in vitro* mutagenesis to make all of the substitutions not detected *in vivo* (L. Kohalmi and B. A. Kunz, in preparation). In addition to single base-pair substitutions, tandem and non-tandem double substitutions, single and multiple base-pair deletions and insertions, insertions of the yeast transposable element Ty, duplications, and other more complex changes have been detected (Kunz *et al.* 1990a). Finally, different mutagenic treatments or conditions have been found to produce distinctly different *SUP4*-o mutational spectra, reflecting the specific types of DNA damage and/or mechanisms involved. Consequently, this system is an extremely useful tool for the study of mutational mechanisms.

3.4 Detection of *SUP4*-o Mutants

Forward mutations in the *SUP4*-o gene are detected by scoring for reduced suppression of three ochre markers. The haploid yeast strains used

Table 3. Single Base-Pair Substitutions Detected in *SUP4*-o[a]

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

[a] Modified from Kunz *et al.* 1990a.

[b] For simplicity, only the base at each position on the transcribed strand is given.

in this study carry ochre mutations which confer either resistance to the arginine analog canavanine (*can1-100*), cause red pigmentation (*ade2-1*) or result in lysine auxotrophy (*lys2-1*). Since these mutations are suppressed by *SUP4*-o, cells harbouring YCpMP2 are canavanine-sensitive and form white, lysine-independent colonies. Selection for reduced suppression of all three ochre markers detects at least a 30% decrease in the production of functional suppressor tRNA (Wang and Hopper 1988) and is unlikely to bias mutant recovery significantly for several reasons. First, mutations that prevent suppression of only two of the ochre mutations are rare (<1% of mutations analyzed) at the chromosomal *SUP4*-o locus (Kurjan and Hall 1982). Second, sequence alterations have not been detected in the plasmid copy of *SUP4*-o from mutants isolated using less stringent selection methods (Kunz *et al*. 1987). Third, 178 different substitutions have been recovered at 68 of the 75 exon sites and at 2 of the 14 intron positions in the gene (Table 3) and a wide range of mutational classes has been identified using the selection protocol employed here (Kunz *et al*. 1990a,b; 1991).

3.5 Isolation of Spontaneous *SUP4*-o Mutants

To isolate spontaneous mutants, MKP-op or MGK-dp were grown from low titre inocula (100 cells/3 ml) in uracil omission medium and grown to stationary phase ($1-2 \times 10^7$ cells/ml) at 30°C with shaking. SBT-p, SBT-Tp and SBT-TLp were inoculated (100 cells/5 ml) in uracil-leucine omission medium containing dTMP (SBT-p and SBT-Tp: 100 μg/ml; SBT-TLp: 20 μg/ml) and grown at 30°C with shaking to exponential phase (3×10^6 cells/ml). Cell suspensions were diluted and plated on uracil or uracil-leucine omission medium to determine the titre of viable, plasmid-containing cells

and on fully supplemented medium to assay plasmid retention. To select for canavanine-resistant colonies, the cell suspensions were plated on uracil or uracil-leucine omission medium containing 30 μg/ml (MKP-op, MGK-dp, SBT-p, SBT-Tp) or 2 μg/ml (SBT-TLp) canavanine. All plates were scored after 6 days incubation at 30°C. Red colonies that emerged on canavanine-containing medium were transferred to uracil omission medium, grown for 2-3 days at 30°C, and replicated to uracil-lysine omission medium which was then incubated at 30°C for 2-3 days. Lysine auxotrophs were scored as *SUP4*-o mutants. Plasmid retention, mutation frequency and mutation rate per round of DNA replication were calculated as follows:

Plasmid Retention:

$$P = \frac{N_-}{N_+} \; X \; 100$$

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

Mutation Frequency:

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

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

Mutation Rate for Spontaneous Mutants (Drake 1991):

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

m = mutations in the target per DNA replication (solved by iteration using an algorithm)
N_m = the median population size at the time of sampling including residual growth on the selection plates (3.5 generations for MKP-op and 3 generations for MGK-dp on canavanine-containing medium)
f_m = the median mutation frequency

3.6 Induction of *SUP4*-o Mutants

3.6.1 dTMP Starvation

SBT-TLp was inoculated (100 cells/5 ml) in uracil-leucine omission medium containing 20 μg/ml dTMP and grown at 30°C with shaking to exponential phase (2 x 10^6 cells/ml). To increase the culture volume, whenever required, exponential phase cells were diluted and grown for approximately 14 to 16 h to exponential phase, using the same medium and growth conditions. Cells were then pelleted by centrifugation (3,020 x g, 5 min, room temperature), transferred to a microfuge tube and washed once by resuspension in 1 ml uracil-leucine omission medium. The cells were then pelleted a second time by centrifugation (Brinkmann 5415C) and resuspended in 1 ml uracil-leucine omission medium. The final cell titre (2 x 10^6 cells/ml) was achieved by transferring resuspended cells to culture flasks containing an appropriate volume of uracil-leucine omission medium. These cultures were then divided into two equal fractions. To one, dTMP was added to a final concentration of 20 μg/ml. Both cultures were then incubated with shaking at 30°C for a further 12 h. At the beginning of each experiment, and thereafter at 2 h intervals, the cell titres were determined and samples were removed, diluted or concentrated when necessary and plated to determine viability, plasmid retention and mutation frequencies and rates. The viability was calculated as follows:
Viability:

$$V = \frac{N_+}{N_T}$$

V = viability
N_+ = number of cells able to grow on uracil omission medium
N_T = total number of cells present (determined by Coulter counter)

Plasmid retention and mutation frequencies were determined as

described in section 3.5. The mutation rate was calculated according to Drake (1970):

Mutation Rate:

$$m = \frac{(f_s - f_o)}{(\ln N_s - \ln N_o)}$$

m = mutations in the target gene per DNA replication
f_s = mutation frequency at the time of sampling
f_o = mutation frequency at the beginning of the experiment
N_s = cell number at the time of sampling
N_o = cell number at the beginning of the experiment

3.6.2 Treatment with Excess dTMP

SBT-TLp was inoculated (100 cells/5 ml) in uracil-leucine omission medium containing 20 μg/ml dTMP and grown at 30°C with shaking to exponential phase (2 x 10^6 cells/ml). The culture was then divided into four equal fractions. One was used as a control, dTMP was added to the others at final concentrations of 0.25, 0.5 or 1 mg/ml and the cultures were incubated with shaking at 30°C for 6 h. At the beginning of each experiment, and thereafter at 1.5 h intervals, the cell titres were determined and samples were removed, washed and resuspended in sterile distilled water (2-4 x 10^6 cells/ml). Cell suspensions were diluted where necessary and viability, plasmid retention and mutation frequencies were determined as described in sections 3.5 and 3.6.1.

3.6.3 Treatment with Alkylating Agents

EMS: Ethyl methanesulfonate (Sigma). Stored at room temperature.

MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma). Stored at -20°C. MNNG (3 mg/ml) was dissolved in 100 mM sodium acetate, pH 4.8, immediately before use and kept on ice.

Phosphate buffer: 50 mM K_2HPO_4
                    50 mM KH_2PO_4
                    Equimolar dibasic and monobasic phosphate solutions were mixed in a 61:39 ratio to achieve a pH of 7.0. Stored at room temperature.

Cultures were grown in uracil (MKP-op) or uracil-leucine (MGK-dp) omission medium at 30°C with shaking to exponential phase (3×10^6 cells/ml). The cells were then washed and concentrated by centrifugation ($3,020 \times g$, 5 min, room temperature) to a final titre of 2×10^8 cells/ml in phosphate buffer. Subsequently, the cultures were divided into 1 ml fractions and transferred to sterile 13 mm tubes (Sarstedt). One fraction was used as a control while the others were treated with various concentrations of EMS or MNNG (the final volume for all treatments was 1.04 ml) for 45 min at 30°C with shaking. The treatments were terminated by washing with 5 ml of 5% (w/v) sodium thiosulphate and the cells were resuspended in 1 ml YPD medium and incubated for 1 h at 30°C with shaking. The cells in each fraction were then pelleted by centrifugation ($1,850 \times g$, 5 min, room temperature) and resuspended in 1 ml phosphate buffer. These cell suspensions were diluted when necessary and plated on uracil omission medium with, or without canavanine (30 μg/ml) to detect canavanine-resistant colonies or survivors, respectively. Plasmid retention and mutation frequencies were determined as described in section 3.5. The surviving fraction was determined as follows:

Surviving Fraction:

$$S_f = \frac{N_0}{N_A}$$

S_f = surviving fraction
N_0 = number of cells able to grow on uracil omission medium
N_A = number of cells able to grow on uracil omission medium after treatment with an alkylating agent

3.7 DNA Isolation

3.7.1 Large-Scale Yeast DNA Preparation

SE buffer:

900 mM sorbitol
100 mM ethylenediaminetetraacetic acid (Na$_2$EDTA), pH 7.5

Zymolyase:

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

TE-1 buffer:

50 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.4
20 mM Na$_2$EDTA, pH 7.4

TE-2 buffer:

10 mM Tris, pH 7.4
 1 mM Na$_2$EDTA, pH 7.4

RNase:

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

Total yeast DNA used for hybridization was isolated by a modification of a procedure described by Sherman et al. (1983). Yeast cultures were grown in 50 ml YPD to stationary phase (1-2 x 10^8 cells/ml) and the cells were pelleted by centrifugation (3,020 x g, 5 min, 4°C), washed and resuspended in 3 ml SE buffer. Zymolyase (250 μl) was added and the cell suspension was incubated for 45 min at 37°C with gentle shaking. The resulting spheroplasts were pelleted by centrifugation (755 x g, 5 min, 4°C) and resuspended in 5 ml TE-1 buffer. Sodium dodecyl sulfate (SDS, 500 μl, 10%, w/v) was added, the contents were mixed gently by inversion and the tube was incubated for 30 min at 65°C. Potassium acetate (1.5 ml, 5 M) was added and the suspension was chilled for 30 min on ice. The precipitate was pelleted by centrifugation (34,800 x g, 20 min, 4°C), the

supernatant transferred to a fresh tube and 14 ml of ice-cold ethanol (95%) was added. The precipitated nucleic acids were pelleted by centrifugation (3,020 x g, 5 min, 4°C) and the pellet was dried and resuspended in 3 ml TE-2 buffer. RNase (150 μl) was added and the suspension was incubated for 30 min at 37°C with shaking. Then, 3 ml isopropanol (room temperature) was added, the solution was mixed by inversion and the precipitate pelleted by centrifugation (1,085 x g, 4 min, 4°C). The pellet was dried, dissolved in 300 μl TE-2 buffer and stored at -20°C.

3.7.2 Yeast DNA Mini-Preparation

SCE buffer:	77 mM sorbitol 10 mM sodium citrate 6 mM Na$_2$EDTA, pH 8.0
SCEM buffer:	SCE buffer containing 10% (v/v) ß-mercaptoethanol (BME).
Zymolyase:	Zymolyase 100,000 (Seikagaku Kogyo Comp.) was dissolved at a concentration of 6 mg/ml in SCEM buffer.
TE buffer:	50 mM Tris, pH 7.5 20 mM Na$_2$EDTA, pH 7.5
RNase:	see section 3.7.1.

During the early stages of this research, total yeast DNA used for retrieval of SUP4-o was isolated by modifying and scaling down a procedure described by Sherman et al. (1983). Yeast cultures were grown in 5 ml of uracil omission medium at 30°C with shaking to a titre of 1 x 10^7 cells/ml and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 5 ml YPD medium and incubated overnight at 30°C with shaking. The cells were then collected by centrifugation

(1,850 x g, 10 min, room temperature), resuspended in 500 μl SCE buffer and transferred to a microfuge tube. After pelleting the cells for 30 s in a microfuge (Brinkmann 5415C), they were resuspended in 300 μl SCEM buffer. Zymolyase (20 μl) was added to the tube which was then vortexed to mix the contents and incubated at 37°C for 1 h. During this incubation time, the tubes were inverted at 15 min intervals to resuspend the cells. The resulting spheroplasts were pelleted by centrifugation for 20 s in a microfuge, resuspended in 357 μl TE buffer and 36 μl SDS (10%, w/v) by vortexing and incubated at 65°C for 30 min. Potassium acetate (107 μl, 5 M) was added to the tube which was inverted several times to mix the contents and then chilled on ice for 15 min. The precipitate was pelleted twice by centrifugation at 4°C for 15 min, the supernatant transferred to a fresh tube and 1 ml ice-cold 95% ethanol was added. The contents of the tube were mixed by inversion, and the tube was centrifuged briefly to pellet the nucleic acids which were then washed with 1 ml ice-cold 70% ethanol, dried by aspiration and resuspended in 300 μl TE buffer. RNase (1.5 μl) was added and the tube was incubated at 37°C for 30 min. The nucleic acid solution was then extracted once with 300 μl TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and twice with 150 μl TE-saturated chloroform. For each extraction the samples were centrifuged for 2 min at room temperature and the upper aqueous layer was transferred to a fresh tube. Following the last extraction, isopropanol (300 μl) was added, the contents of the tube were mixed by inversion, and the tube was centrifuged briefly to pellet the DNA which was then washed twice with ice-cold 70% ethanol, dried by aspiration and dissolved in 30 μl TE buffer. DNA samples were stored at -20°C.

3.7.3 Glass-Bead-Preparation for Yeast DNA Isolation

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

Triton solution: 100 mM NaCl
10 mM Tris, pH 8.0
1 mM Na_2EDTA, pH 8.0
2% (v/v) Triton X-100 (Fisher)
1% (w/v) SDS

TE buffer: 50 mM Tris, pH 8.0
20 mM Na_2EDTA, pH 8.0

The previous yeast DNA preparation was replaced midway through this study by a glass-bead technique (modified from: Hoffman and Winston 1987) which allowed more rapid processing of a large number of samples. This change had no obvious effects on the results. Putative *SUP4*-o mutants were grown to stationary phase (1-2 x 10^7 cells/ml) at 30°C with shaking in 7 ml uracil omission medium. Next, the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 200 μl Triton solution and transferred to a microfuge tube containing 300 mg acid-washed glass beads. Then, TE-saturated phenol and chloroform (100 μl each) was added to the tube which was vortexed for 2 min and spun for 5 min at room temperature in a microfuge (Brinkmann 5415C). Subsequently, 7.5 μl of the upper aqueous layer was transferred to a fresh tube, stored at -20°C and used for bacterial transformation within 4 days of isolation.

3.7.4 Large-Scale Plasmid DNA Preparation

GTE buffer: 50.0 mM glucose
25.0 mM Tris, pH 8.0
10.0 mM Na_2EDTA

```
NaOH/SDS:              200.0 mM NaOH
                         1.0% (w/v) SDS
                       Prepared just prior to use.

Tris/sodium acetate: 100.0 mM sodium acetate, pH 4.8
                      50.0 mM Tris, pH 8.0

TE buffer:             50.0 mM Tris, pH 8.0
                       50.0 mM Na₂EDTA, pH 8.0
```

Plasmid DNA used in strain constructions and for nick translation was prepared by a scaled up version of the alkaline procedure of Birnboim and Doly (1979). Bacterial cultures were grown overnight in 5 ml M9+amp medium at 37°C with shaking. The overnight culture was transferred to 1 1 M9+amp medium and grown to OD_{600} = 0.6. At this point chloramphenicol (250 μg/ml) was added and the culture was incubated for an additional 12-18 h at 37°C with shaking. Cells were pelleted by centrifugation (5,117 x g, 5 min, 4°C), resuspended in 4 ml of GTE buffer, 1 ml lysozyme (Boehringer Mannheim, 10 mg/ml dissolved in GTE buffer) was added and the cell suspension was chilled on ice for 45 min. NaOH/SDS (10 ml) was added and the mixture was incubated for 10 min on ice. Sodium acetate (7.5 ml, 3 M, pH 4.8) was added and the contents were mixed by inversion and incubated on ice for 60 min. The precipitate was pelleted by centrifugation (39,100 x g, 20 min, 4°C) and the supernatant was transferred to a fresh centrifuge tube. The nucleic acids were precipitated by adding 50 ml of ice-cold ethanol (95%) and incubating at -70°C for 20 min. The precipitate was pelleted by centrifugation (39,100 x g, 20 min, 4°C), the supernatant was removed and the pellet was dried and dissolved in 10 ml Tris/sodium acetate. The nucleic acids were precipitated by adding 25 ml ice-cold ethanol (95%) and incubating at -70°C for 20 min. The precipitate was pelleted by centrifugation (27,000 x g, 5 min, 4°C), the supernatant was

discarded and the pellet was dried and then dissolved in 8 ml TE buffer. Then, 8 g CsCl was also dissolved in the TE buffer and the solution was transferred to an ultra-centrifuge tube. Ethidium bromide (0.4 ml, 10 mg/ml) was added and the tube was sealed and centrifuged at 300,000 x g for 48 h at 4°C. The separated DNA bands were illuminated under a UV light and the band containing the plasmid DNA was carefully removed with a syringe and transferred to a sterile 13 mm tube (Sarstedt). An equal volume of CsCl-saturated isopropanol was added to extract the ethidium bromide and the top layer was removed. The remaining solution was transferred to a dialysis tube and incubated twice in 2 l TE buffer for 2 h at 4°C. The contents of the dialysis tube were transferred to a fresh 13 mm tube and centrifuged at 3,290 x g for 1 min at room temperature. The supernatant was then transferred to a sterile microfuge tube and the contents dehydrated by lyophilization. The pellet was dissolved in 100 μl of TE buffer and stored at 4°C.

3.7.5 Small-Scale Plasmid DNA Preparation

GTE buffer:	see section 3.7.4
NaOH/SDS:	see section 3.7.4
Ammonium acetate:	7.5 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C to obtain a pH below 6.0.
TE buffer:	see section 3.7.4

To isolate a large number of plasmid DNA samples for DNA sequencing, a modification of the procedure of Crouse *et al.* (1983) was used. Bacteria were grown overnight in 5 ml YT+amp medium at 37°C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room tempera-

ture), resuspended in 500 μl of GTE buffer and transferred to a microfuge tube. Cells were pelleted by centrifugation (Brinkmann 5415C), resuspended in 180 μl GTE buffer and 10 μl of lysozyme (Boehringer Mannheim, 25 mg/ml dissolved in GTE buffer) was added. The cell suspension was mixed gently and the tube was incubated at room temperature for 5 min. Next, the tubes were transferred to ice, 400 μl NaOH/SDS was added slowly in spirals starting at the bottom of the tube and the mixture was incubated on ice for 5 min. Sodium acetate (300 μl, 3 M, pH 4.8) was added drop-wise, the contents were mixed by inverting slowly and the tube was incubated on ice for 10 min. The precipitate was pelleted by centrifugation at 4°C for 15 min and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated two more times. Then, 450 μl isopropanol was added, the contents were mixed by inversion and the tube was spun for 2 min at room temperature to pellet the nucleic acids. The pellet was washed with 70% ice-cold ethanol and dried by aspiration. The nucleic acids were dissolved in 200 μl TE buffer and then extracted twice with 200 μl TE-saturated phenol and once with 200 μl TE-saturated chloroform. For each extraction the samples were centrifuged for 2 min at room temperature and the upper aqueous layer was transferred to a fresh microfuge tube. After the last extraction, an equal volume of ammonium acetate was added, the contents were mixed by inversion and the precipitate was pelleted by centrifugation for 15 min at 4°C. The supernatant was transferred to a fresh tube and the nucleic acids were precipitated by adding 1 ml of 95% ice-cold ethanol and pelleted by centrifugation for 1.5 min at room temperature. The pellet was washed with 1 ml 70% ice-cold ethanol, dried and dissolved in 100 μl TE buffer. DNA samples were stored at -20°C.

3.7.6 Rapid Alkaline Procedure for Plasmid DNA Isolation

Towards the end of this study, the alkaline extraction procedure was replaced by a more rapid version (modified from: Morelle 1989) which uses the same solutions as described in the previous protocol. Bacteria were grown overnight in 5 ml YT+amp medium at 37°C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 500 μl of GTE buffer and transferred to a microfuge tube. The cells were pelleted by centrifugation (Brinkmann 5415C) and resuspended in 180 μl GTE buffer. Lysozyme (10 μl) was added and the cell suspension was mixed gently and the tube was incubated at room temperature for 5 min. Next, the tubes were transferred to ice, 400 μl NaOH/SDS was added drop-wise and the mixture was incubated on ice for 5 min. Ammonium acetate (300 μl) was added drop-wise and the mixture was incubated on ice for 10 min. The precipitate was pelleted by centrifugation at 4°C for 15 min and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated two more times. Then, 500 μl isopropanol (room temperature) was added, the contents were mixed by inversion and the tube was held at room temperature for 10 min. Following centrifugation for 5 min at room temperature, the nucleic acid pellet was washed with 70% ice-cold ethanol, dried and dissolved in 70 μl TE buffer. DNA samples were stored at -20°C.

3.8 Transformation Procedures

3.8.1 Yeast Transformation

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

PEG: 44% (w/v) polyethylene glycol$_{4000}$ (Sigma). Dissolved just prior to use and filter sterilized.

Yeast cells were transformed using the lithium acetate procedure described by Ito *et al.* (1983). YPD (100 ml) was inoculated with 3×10^6 cells/ml of an overnight yeast culture and was then incubated with shaking at 30°C until the cell titre reached 1×10^7 cells/ml (approximately 2 h). The culture was centrifuged (3,020 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 (100 mM dissolved in TE buffer). The suspension was incubated at 30°C with shaking for 1 h and then 10 μl spermidine (1 mg/ml *N*-[3-aminopropyl]-1,4 butane diamine, Sigma) was added. For each transformation a 100 μl aliquot of the cell suspension was transferred to a sterile 13 mm tube (Sarstedt). DNA (1 μg) was added and the tube was incubated at 30°C for 30 min without shaking. Next, PEG (440 μl) was added gently and the tube was incubated for an additional 1 h at 30°C without shaking and then heated at 42°C for 5 min. Cells were then pelleted by centrifugation (1,850 x g, 5 min, room temperature), washed twice in 5 ml, and resuspended in 1 ml SD medium containing no supplements. Aliquots (200 μl) of the cell suspensions were plated on appropriately supplemented minimal medium and incubated at 30°C for 6 days.

3.8.2 Bacterial Transformation

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

Bacterial cells were transformed using a modification (Pierce *et al.* 1987) of the calcium chloride procedure of Mandel and Higa (1970). The *E. coli* strain JF1754 was grown overnight in 5 ml YT medium at 37°C with

shaking. The cells were diluted 1:100 in fresh YT medium (40 ml of YT medium for every 10 transformations), grown for 1 h 45 min at 37°C with shaking (OD_{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. The pellet was washed and resuspended in Buffer A (10 ml for every 40 ml of culture) and then chilled on ice for 20 min. The cells were then collected by centrifugation (3,020 x g, 10 min, 4°C) and resuspended in Buffer B (10 ml for every 40 ml of original culture). The cell suspension was chilled on ice for 1 h, pelleted by centrifugation (3,020 x g, 10 min, 4°C) and resuspended in 2 ml (for every 40 ml of culture) of the same solution. For each transformation, a 200 μl aliquot of the cell suspension was transferred to a sterile microfuge tube containing 5 μl (Mini-prep, section 3.7.2) or 7.5 μl (Glass-bead-prep, section 3.7.3) yeast DNA. Each tube was chilled on ice for 1 h, heated at 42°C for 2 min and then chilled on ice for 2 min. 2x YT (200 μl) was added to each tube which was then incubated at 37°C for 1 h. The cell suspensions were then plated on YT+amp medium and incubated overnight at 37°C.

3.9 DNA Sequencing

10x Buffer 3:	React®3 (BRL)
RNase:	see section 3.7.1
BamHI:	50 U/μl BamHI (BRL) was diluted to a concentration of 1 U/μl in 1x buffer 3 just prior to use.
RP primer:	0.1 A_{260} U/ml M13 reverse sequencing primer (Pharmacia). This 17 bp primer has the sequence 5'-d(CAGGAAACAGCTATGAC)-3' and binds at position +167 to +183 relative to the first base-pair of the SUP4-o gene on YCpMP2. Stored at -20°C.

[^{32}P] dATP: 3000 Ci/mmol alpha-[^{32}P] dATP (DuPont). Stored at -70°C.

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

Hin buffer: 240.0 mM dithiothreitol (BRL)
  60.0 mM Tris, pH 7.5
  60.0 mM NaCl
  60.0 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: 150.0 μM ddATP
  2.5 μM dATP
 50.0 μM dGTP
 50.0 μM dCTP
 50.0 μM dTTP
 25% (v/v) Hin buffer

ddG: 250.0 μM ddGTP
  2.5 μM dATP
 12.5 μM dGTP
 50.0 μM dCTP
 50.0 μM dTTP
 25% (v/v) Hin buffer

ddC: 250.0 μM ddCTP
  2.5 μM dATP
 50.0 μM dGTP
 12.5 μM dCTP
 50.0 μM dTTP
 25% (v/v) Hin buffer

ddT: 400.0 μM ddTTP
  2.5 μM dATP
 50.0 μM dGTP
 50.0 μM dCTP
  8.0 μM dTTP
 25% (v/v) Hin buffer

Deionized formamide: 2.5 g of Amberlite® MB-1 (Sigma) were mixed with 50 ml formamide (Fisher). The solution was covered with parafilm, stirred at 4°C for 2 h or overnight and filtered twice through Whatman filter paper #1. 1 ml aliquots were stored at -70°C.

Stop buffer: 10.0 mM Na$_2$EDTA, pH 8.0
95.0% (v/v) deionized formamide
 0.1% (w/v) xylene cyanol FF (Sigma)
 0.1% (w/v) bromophenol blue (Sigma)
Stored at 4°C.

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10x TEB buffer:	20.0 mM Na_2EDTA, pH 8.0
	1.0 M Tris
	0.9 M boric acid
	Stored at room temperature.

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

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

Sequencing gel: The Insta-gel was polymerized by the addition of ammonium persulfate and TEMED (*N,N,N',N'*-tetra-methylethylenediamine, Mallinckrodt) to final concentrations of 0.25% (w/v) and 0.1% (v/v), respectively. The polymerized gel was 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 linearized using the following enzyme digest: 1.2 μl of 10x buffer 3, 1.0 μl RNase, 7.8 μl plasmid DNA (approximately 1 μg) and 2 μl *Bam*HI were mixed in a screw cap microfuge tube and incubated for 30 min at 37°C. The microfuge tube was then heated for 3 min at 100°C and allowed to cool at room temperature for 5 min. Subsequently, 1 μl of RP primer was added. Then the tube was heated at 100°C for 3 min and immediately transferred to ice-water and allowed to cool for 1 min before proceeding. Successively, 1 μl dithiothreitol (100 mM), 1 μl DNA polymerase and 1 μl [^{32}P]dATP were added and the mixture was stirred with the Pipetman[R] tip. Two μl of this

mixture was added to the side of each of 4 microfuge tubes containing either the ddA, ddG, ddC, or ddT termination mix and the tubes were spun briefly (Brinkmann 5415C) to combine both solutions. These microfuge tubes were transferred to a 46°C waterbath and incubated for 20 min and the reactions were then terminated by adding 2.4 μl stop buffer. Finally, the microfuge tubes were heated for 3 min at 100°C, transferred immediately to ice and 2.5 μl of each reaction mixture was electrophoresed on a sequencing gel (electrolyte: 1x TEB buffer) at constant power, to heat the gel to 50°C, for about 3.5 h. The gel was then vacuum-dried at 80°C for 1 h and exposed to Kodak XAR-5 film at room temperature (the exposure time varied according to the radioactivity of the DNA in the gel).

3.10 Hybridization Analysis

3.10.1 Agarose Gel Electrophoresis

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

Stop buffer: 50.0 mM Na_2EDTA
50.0% (w/v) sucrose
0.1% (w/v) bromophenol blue (Sigma)
Stored at 4°C.

λ DNA: 1x buffer 3
50 μg λ DNA (Pharmacia)
200 U HindIII

The reaction mix was incubated for 1.5 h at 37°C and the reaction was terminated by adding stop buffer (1/4 of the reaction volume). The DNA was stored at 4°C and heated at 65°C for 5 min before use.

```
Loenings buffer:      40 mM Tris
                      20 mM sodium acetate
                       1 mM Na₂EDTA

                      pH was adjusted to 8.0 with glacial acetic acid.
                      Stored at room temperature.
```

Total yeast DNA was digested with the appropriate enzyme for 1.5 h at 37°C, stop buffer (1/4 of the reaction volume) was added to terminate the reaction and the resulting DNA fragments were separated by agarose gel electrophoresis (0.7%, w/v agarose (BRL) dissolved in Loenings buffer), for 18 h at 1 Volt/cm. A λ DNA standard accompanied the DNA samples each time. The gel was stained with ethidium bromide (final concentration: 10 μg/ml) and destained for 10 min in distilled water. A picture was taken for subsequent sizing of the hybridization bands. Then, the gel was sequentially incubated for 1 h in 0.5 M NaOH and 1 h in 1 M Tris (pH 8.0) at room temperature with slow shaking. Next, the gel was sandwiched between 3MM Chr Whatman Chromatography paper and a single layer of Saran Wrap and vacuum-dried at 60°C for 1 h (Tsao *et al.* 1983). Dried gels were stored at room temperature.

3.10.2 Nick Translation

```
10x NT buffer:        500 mM Tris, pH 7.2
                      100 mM MgSO₄
                        1 mM dithiothreitol (BRL)
                      500 $\mu$g/ml bovine serum albumin (BRL)
                      Stored at -20°C.

[³²P]dATP:            see section 3.9
```

Reaction mix: 1x   NT buffer

1 μg DNA

20 μM dATP (this final concentration includes the concentration of [^{32}P]dATP in the reaction mix)

20 μM dCTP

20 μM dGTP

20 μM dTTP

50 μCi [^{32}P]dATP

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

DNase: 0.1 U/ml DNase I (Pharmacia)

50% (v/v) glycerol

1x NT buffer

Prepared immediately prior to use and kept on ice.

DNA polymerase: 5 U/μl DNA polymerase I from *E. coli* (FPLC pure, Pharmacia). Stored at -20°C.

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

[^{32}P]-labelled probes for hybridization were prepared by nick translation (Maniatis *et al.* 1982). The reaction mix was added to a sterile screw cap microfuge tube and chilled to 11°C. DNase (1 μl) was added and the contents of the tube was gently mixed by tapping and incubated at 11-13°C for 10 min. Then, 1 μl DNA polymerase was added and the tube was kept at 16°C for 50 min. Successively, 2 μl Na$_2$EDTA (500 mM), 5 μl yeast tRNA (20 mg/ml, Boehringer Mannheim), 7 μl MgCl$_2$ (100 mM), 7 μl sodium acetate (3 M) and 200 μl ice-cold ethanol (95%) were added. The tube was then transferred to -70°C for 1 h and the precipitate was pelleted by centrifugation for 15 min at 4°C (Brinkmann 5415C). The supernatant was removed with a pasteur pipette and the pellet was washed with 1 ml ice-cold ethanol (70%), incubated at -70°C for 1 h and centrifuged for 15 min at 4°C. Again, the supernatant was removed with a

pasteur pipette and the pellet resuspended in 100 μl sterile water. To this, 100 μl salmon sperm DNA was added and the tube was heated at 100°C for 10 min and then rapidly transferred to ice-water.

3.10.3 Hybridization Procedure

20x SSC
        3.0 M NaCl
        0.3 M sodium citrate
        pH was adjusted to 7.0 with 10 N NaOH.
        Stored at room temperature.

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

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

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

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

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

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

Direct DNA hybridization in agarose gels was carried out as described by Tsao *et al.* (1983). The dried gel was transferred into a plastic bag

(Philips KB5936), prehybridization solution was added (2 ml/cm^2 of gel) and the bag was heat-sealed and incubated for 2-4 h at 65°C. The prehybridization solution was removed and replaced with hybridization solution (50 μl/cm^2 of gel), the nick translated DNA was added and the bag was heat-sealed and incubated overnight at 65°C. The gel was then removed carefully from the bag and the following washes were performed: 1. 250 ml of solution I for 5 min at room temperature (twice). 2. 250 ml of solution II for 15 min at room temperature (twice). 3. 250 ml of solution III for 2 h, 1 h and 0.5 h, respectively, at 65°C. The gel was then transferred to 3MM Chr Whatman Chromatography paper, air-dried, covered with a single layer of Saran Wrap and exposed to Kodak XAR-5 film with an intensifying screen at -70°C.

3.11 Labelling Yeast Cells with [^{14}C] Arginine

[^{14}C] arginine: 1 μCi; 323.5 mCi/mmol (DuPont)

Exponentially growing yeast cells (2 x 10^6 cells/ml) were concentrated by centrifugation (3,020 x g, 5 min, room temperature) to a final titre of 1 x 10^8 cells/ml in appropriately supplemented, prewarmed (30°C) SD medium. The culture was divided into two 1 ml fractions and 10 μl of [^{14}C] arginine and unlabelled arginine (final concentration: 115 μM) was added to each fraction.

To examine the influence of the arginine analog canavanine (Figure 10) on [^{14}C] arginine uptake, canavanine (final concentration: 11.5 mM - approximately 100 times the final arginine concentration) was added to one of the fractions prior to the addition of labelled arginine while the other fraction was used as a control (the canavanine volume was replaced by sterile water). The final volume of all cultures was 1.03 ml. The

Figure 10. Chemical Structures of Arginine and Canavanine.
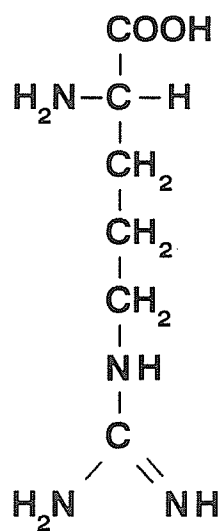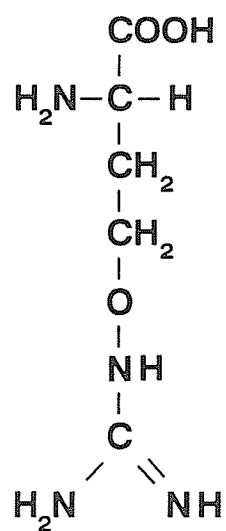
Arginine   Canavanine

cultures were then incubated with shaking at 30°C. Aliquots (150 μl) were removed at regular intervals and immediately transferred into 2 ml ice-cold, sterile water. The cell samples were collected on nitrocellulose filters (Millipore, 47 mm, 0.45 μm) and washed 10 times with 4 ml ice-cold, sterile water. After drying at 50°C for 30 min, the filters were transferred to scintillation vials, scintillation fluid (Scintiverse I, Fisher) was added and radioactivity was determined (Beckman LS-230, [^{14}C]-window).

To determine the influence of dTMP on [^{14}C] arginine uptake, the experiment was performed as described above except that the canavanine was replaced by 0.062 mM dTMP (= 20 μg/ml - the concentration required for growth of SBT-TLp and approximately 50% of the final arginine concentration) or 11.5 mM dTMP (approximately 100 times the final arginine concentration).

3.12 Inhibition of Cell Growth by Canavanine

To examine the effect of canavanine concentration on the recovery of spontaneous canavanine-resistant mutants in SBT-TLp, cells were grown in uracil-leucine omission medium to exponential phase (3 x 10^6 cells/ml) and were then spread (1 x 10^6 cells/plate) on uracil-leucine omission medium containing different concentrations of canavanine.

The effect of canavanine concentration on the growth of MKP-o, SBT-TL, SBT-T and SBT was determined by growing cells in uracil omission medium (MKP-o), MS-uracil omission medium containing 100 μg/ml dTMP (SBT) or uracil-leucine omission medium containing 100 μg/ml (SBT-T) and 20 μg/ml (SBT-TL) dTMP to exponential phase (2 x 10^6 cells/ml). The cultures were then divided into two (MKP-o, SBT, SBT-T) or five (SBT-TL) equal

fractions. One fraction was used as a control, canavanine was added to the other(s). The cultures were then grown with shaking at 30°C for 12 h and cell titres were determined at 3 h intervals.

To determine the effect of inhibiting the general amino acid permease on growth inhibition by canavanine, SBT-TL was grown to exponential phase (2×10^6 cells/ml) and the culture was then divided into seven equal fractions. One fraction was used as a control, canavanine (30 μg/ml, final concentration) was added to the other six. In addition, to five of these six cultures, alanine, phenylalanine, histidine, valine or a mixture of all four amino acids (final concentration of each amino acid: 100 μg/ml) was added. All cultures were then grown with shaking at 30°C for 12 h and cell titres were determined at 0 and 12 h.

3.13 Measurements of dNTP Pools

3.13.1 Preparation of Cell Extracts

0.5 M TNO in Freon®:   2.19 ml tri-n-octylamine (TNO, Sigma)
                        7.81 ml 1,1,2-trichloro-1,2,2-trifluoroethane
                        (Freon®, Mallinckrodt)
                        Solutions were mixed just prior to use and were
                        kept on ice.

The cell extracts for dNTP measurements were prepared by a modified version of the method of Sargent and Mathews (1987). Cultures of strains MKP-op, MGK-dp and SBT-TLp were each grown in appropriately supplemented minimal medium (20 ml) to exponential phase (3×10^6 cells/ml for MKP-op and MGK-dp, 2×10^6 cells/ml for SBT-TLp if the cells were then treated with, or starved for, dTMP; sections 3.6.1 and 3.6.2). If extracts were prepared from EMS or MNNG-treated cells, the alkylation treatment that preceded sampling was performed as described (section 3.6.3) and the cells were harvested for extraction immediately after the final wash and

resuspension in KPO$_4$ buffer. For each strain or treatment, cells were harvested by rapid filtration using 0.45 μm membrane filters (Gelman GN-6 filters, 47 mm) which were immediately immersed in 3 ml ice-cold 5% (w/v) trichloroacetic acid (TCA, Mallinckrodt) and incubated for 30 min on ice (extracts from dTMP-treated cells gave the same results whether the cells were washed prior to transferring the filter to TCA or not). Next, the extract was centrifuged at 17,000 x g for 15 min at 4°C and the supernatant was transferred to a 15 ml Corning disposable centrifuge tube (No. 2531315; modified polystyrene). The pH of the supernatant was increased (acids interfere with the subsequent procedures) to between 5.5 and 6.0 by vortexing with 1.5 volume of ice-cold 0.5 M TNO in Freon® for 1 min (Khym 1975). In contrast to other neutralization processes, this ensures that no salts (high salt extracts inhibit the DNA polymerase, Sargent 1987) are added to the cell extract and that the nucleotides are fully recovered in the aqueous phase (Khym 1975). To separate the phases, samples were spun for 2 min at 1,850 x g at room temperature. pH values between 5.5 and 6.0 were confirmed by using pH indicator stickers (pHast: pH 0-14, Merck) to test 2.5 μl of the aqueous layer. (If the pH value of the aqueous layer was below 5.5, the TNO/freon extraction was repeated). Subsequently, the aqueous layer was carefully transferred to a fresh tube and the volume recovered was recorded. The samples were stored overnight at -70°C and dried by lyophilization. The residue was dissolved in 0.1 ml sterile distilled water and stored at -20°C.

3.13.2 dNTP Pool Assay

    1.5x dNTP buffer: 75.0 mM Tris, pH 8.3
                                    7.5 mM MgCl$_2$
                                    Stored at room temperature.

BME: 1.0% (v/v) ß-mercaptoethanol
                    The dilution was prepared just prior to use.

BSA: 10.0 mg/ml bovine serum albumin (BRL)
                    Stored at -20°C.

AT template: 0.75 mg/ml poly [dAdT]·poly [dAdT] (Pharmacia)
                    Stored at -20°C.

IC template: 0.75 mg/ml poly [dIdC]·poly [dIdC] (Pharmacia)
                    Stored at -20°C.

DNA polymerase dilution buffer:
                    100 mM KPO$_4$, pH 7.0
                      1 mM dithiothreitol (BRL)
                     50% (v/v) glycerol
                    Stored at -20°C.

DNA polymerase: 50 U/μl DNA polymerase I (Kornberg polymerase,
                    endonuclease free; Boehringer Mannheim: stored at
                    -20°C) from *E. coli* was diluted to a concentration
                    of 0.1 U/μl in DNA polymerase dilution buffer
                    just prior to use and kept on ice.

[^{3}H]dNTP: All [^{3}H]dNTP were stored at -20°C.

    [^{3}H]dATP   1 mCi/ml [8-^{3}H(N)]- deoxyadenosine 5'triphos-
                        phate, tetrasodium salt (DuPont);
                        specific activity: 20.0 Ci/mmol

    [^{3}H]dTTP   1 mCi/ml [methyl-^{3}H]- deoxythymidine 5'triphos-
                        phate, tetrasodium salt (DuPont);
                        specific activity: 20.5 Ci/mmol

    [^{3}H]dCTP   1 mCi/ml [5,5'-^{3}H]- deoxycytidine 5'triphosphate,
                        tetrasodium salt (DuPont); specific
                        activity: 23.7 Ci/mmol

    [^{3}H]dGTP   1 mCi/ml [8-^{3}H]- deoxyguanosine 5'triphosphate,
                        tetrasodium salt (DuPont); specific
                        activity: 11.1 mCi/mmol

TCA/PPi: 5.0% (v/v) trichloroacetic acid
                    2.0% (w/v) sodium pyrophosphate (Mallinckrodt)
                    Prepared just prior to use.

An enzymatic assay (North *et al.* 1980) was used to measure the dNTP pools. This method determines incorporation of a limiting dNTP from a cell extract into an alternating copolymer template-primer (poly [dAdT]·poly [dAdT] or (poly [dIdC]·poly [dIdC]) by *E. coli* DNA polymerase I in the presence of an excess of the labelled complementary dNTP. By measuring the amount of labelled nucleotides incorporated into the newly synthesized DNA (acid precipitable counts), the concentration of the complementary nucleotide can be established. The reaction conditions are as described by Sargent (1987) and Sargent and Mathews (1987).

For each cell extract to be analyzed, and for each dNTP to be measured, a separate reaction mixture was required. The reaction mixture consisted of 10 μl of cell extract and 90 μl of a reaction buffer formulated specifically for the particular dNTP to be measured (Table 4). The reaction buffers were prepared prior to the experiment and were kept on ice until needed. DNA polymerase and [^{3}H]dNTP were the last components added immediately before the assay was started. Deoxyadenine monophosphate (dAMP) was present in the buffers to inhibit the exonuclease activity of DNA polymerase I (Hunting and Henderson 1981). In addition, an endonuclease free polymerase preparation was used. These steps were taken to prevent degradation of newly synthesized DNA which would artificially lower the measured incorporation rate of the labelled nucleotides. Unlabelled dNTP was included in each reaction buffer to ensure that in the reaction mix there was a vast excess of the nucleotide complementary to that being measured.

Prior to initiating the assay, 10 μl of diluted or undiluted cell extract was added to a microfuge tube and kept on ice. The reaction was then started by adding 90 μl of the appropriate reaction buffer and

Table 4. Reaction Buffers for dNTP Pool Assay

	dNTP to be measured[a]			
	dATP	dTTP	dCTP	dGTP
1.5 x dNTP buffer	60	60	60	60
BME	0.6	0.6	0.6	0.6
BSA	2	2	2	2
100 mM cold dNTP	1 (dTTP)	1 (dATP)	1 (dGTP)	1 (dCTP)
100 mM dAMP	10	10	10	10
H_2O	11	10	11	13
Template	3	3	1	1
Polymerase	2	3	2	2
^3H dNTP	1 (dTTP)	1 (dATP)	3 (dGTP)	1 (dCTP)

[a] Values are given in μl and are the volumes required for one reaction. Multiples of these volumes were used if several samples were analyzed.

immediately transferring the microfuge tube to a 37°C waterbath. The incubation time for the AT and IG templates was 90 min and 110 min, respectively, which allows unrestricted incorporation of the nucleotide to be measured (Sargent 1987). To ensure the exact timing of each reaction, the individual reactions were started at 30 s intervals. The reactions were stopped by spotting 25 μl onto dried TCA/PPi-presoaked filters (see section 3.13.4 for details). The filters were placed vertically in a rack to prevent cross contamination and the spots were air-dried before being processed further.

To ensure optimal accuracy of the dNTP measurements, the following steps were observed: 1. at least two different dilutions of each cell extract were used and the incorporation of radioactive dNTP's was compared to the dilutions used and checked for linearity. 2. every reaction was spotted twice and the results were averaged. 3. for each strain or treatment analyzed, results were obtained from three independent cultures.

3.13.3 dNTP Standards

Each time dNTP pool assays were performed, a separate standard curve for each of the four nucleotides involved was determined to allow the conversion of the radioactive counts into pmol of dNTPs present. The standard reactions were set up as described above but instead of 10 μl of cell extract, 10 μl of a dNTP standard solution was mixed with the reaction buffer. The dNTP standard solutions used, 0 (distilled water), 0.5, 1, 2 and 4 μM, gave standard values for 0, 5, 10, 20 and 40 pmol, respectively. Examples of the standard curves are shown in Figure 11.

3.13.4 Chromatography and Scintillation Counts

TCA/PPi: see section 3.13.2

Figure 11. **dNTP Standard Curves.** A: dATP. B: dTTP. C: dCTP. D: dGTP.

Standard curves were fitted by regression analysis.
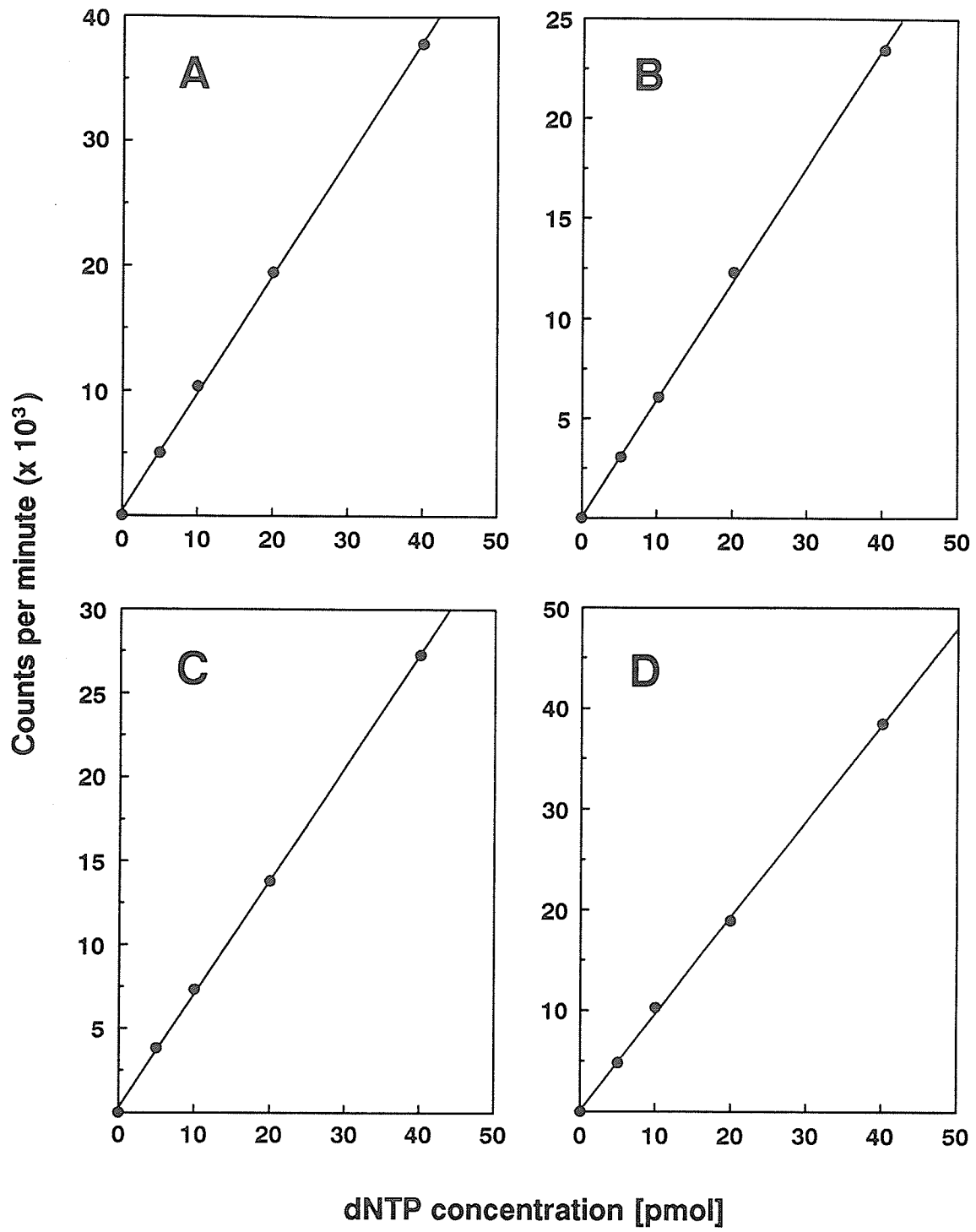
Scintillation cocktail:

> 12.0 g 2,5-diphenyloxazole (Scintanalyzed, Fisher)
> 0.4 g 1,4-bis-[4-methyl-5-phenyl-2-oxazolyl] benzene (Scintanalyzed, Fisher)
> 4.0 l toluene (Scintanalyzed, Fisher)
> Stored at room temperature.

To separate the newly synthesized DNA from the excess radioactive dNTP in the reaction mixes, an ascending chromatographic procedure was used. Chromatography sheets (19 x 19 cm) were prepared from 3 MM Chr Whatman Chromatography paper. Nine sampling squares (2 x 2 cm) were drawn with a soft pencil and labelled with sample numbers as shown in Figure 12. Before using the sheets for sampling, each square was soaked in 100 μl TCA/PPi and air-dried overnight. Radioactive samples (25 μl) were spotted to the centre of each square and allowed to air-dry, 500 ml of TCA/PPi solution was added to a 21 x 21 cm glass dish in a chromatography tank and up to 6 chromatograms were suspended in the solution so that the bottom of each sheet was submerged to a depth of 1 cm. Chromatography took place at room temperature. The sheets were removed when the solvent front ran within 1 cm of the top margin (the running time was approximately 90 min). For each chromatogram, the strip containing the sample squares was cut out, washed twice for 15 min in 95% ethanol at room temperature and air-dried overnight or at 50°C for 1 h. Next, the strip was cut into the individual sampling squares, each square was placed in a scintillation vial and covered with 5 ml of scintillation cocktail. Samples were counted (Beckman LS-230) for 5 min (5% error) using a [^{3}H] window. Figure 13 illustrates the separation of the labelled DNA from the unincorporated labelled dNTPs.

3.13.5 Calculations

To calculate the final concentration of dNTP's in an individual cell

Figure 12. Chromatogram Design.
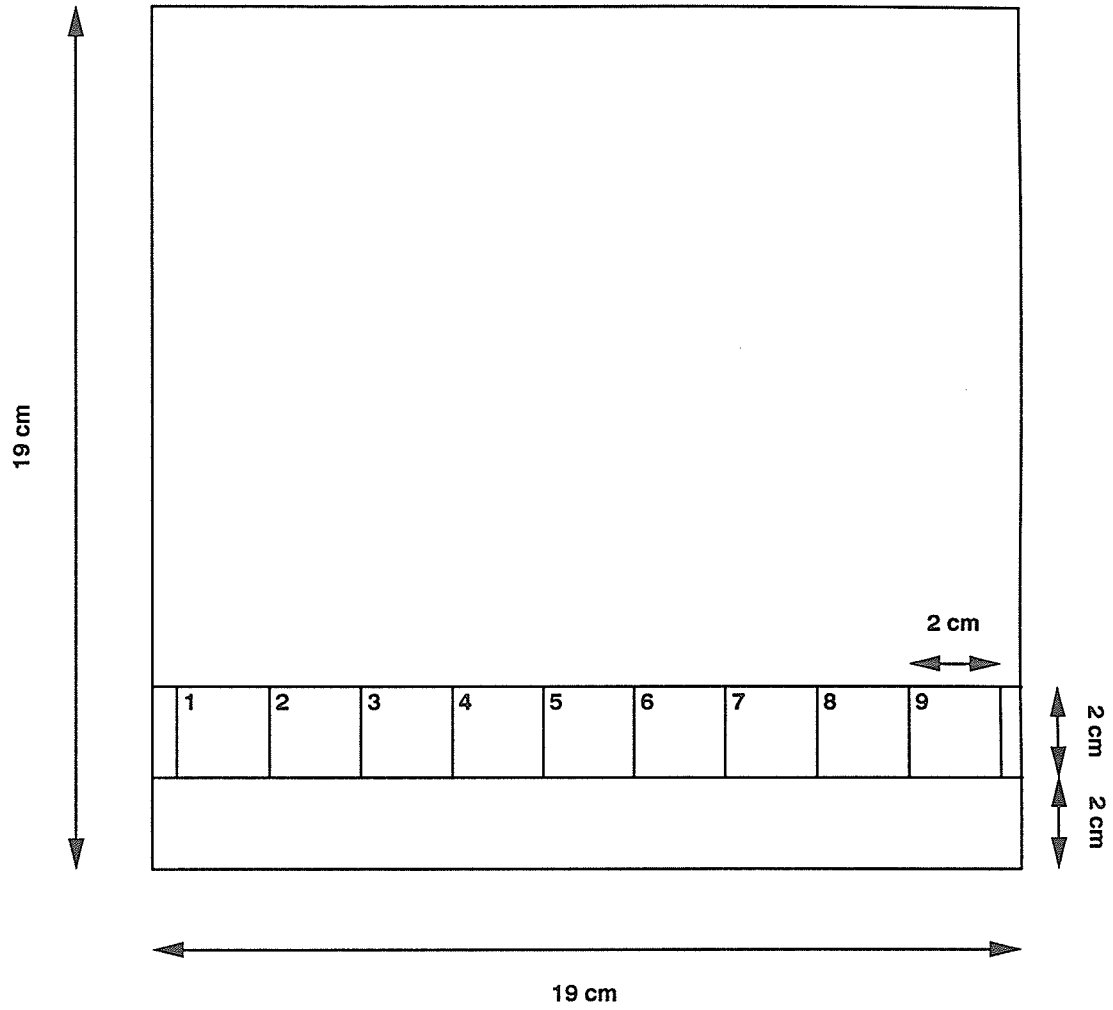
Figure 13. Separation of Acid Precipitated DNA from Nucleotides. A 2 cm wide strip extending above the sampling square(s) was cut into 2 x 2 cm squares (as indicated below the graph) after completion of chromatography and the radioactive counts per square were determined. (●): the sampling square was spotted with a control sample; (▲) the sampling square was spotted with labelled DNA obtained by performing the nucleotide pool assay with a 40 pmol dTTP standard.

Direction of solvent movement
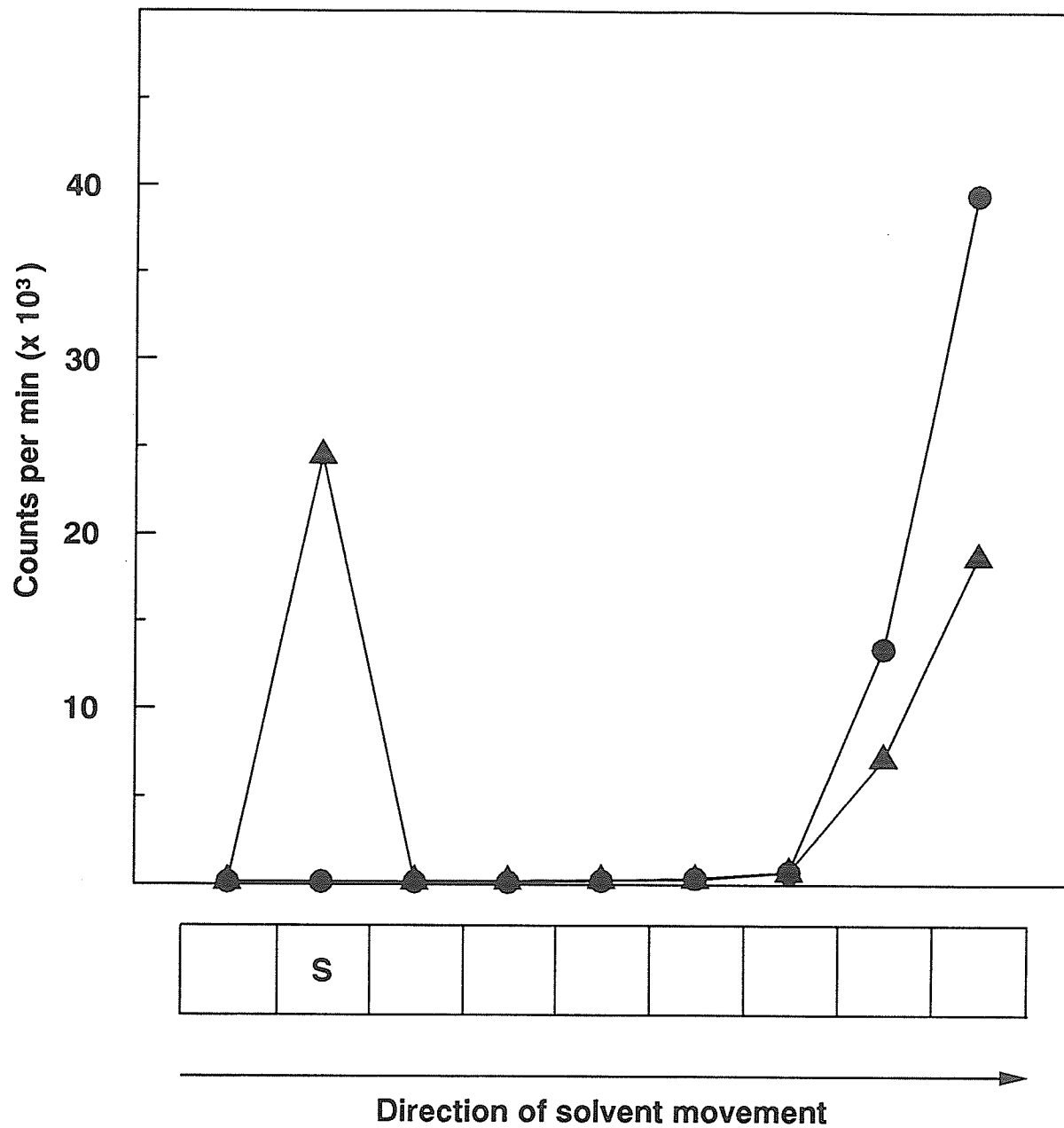
extract, the following protocol was used:

1. The values obtained for the double spotting of each extract or standard were averaged.

2. Standard curves were plotted using linear regression analysis.

3. The standard curves were used to convert all radioactive counts into pmol.

4. Although each cell sample was extracted in 3 ml of TCA, only a fraction of the initial extract was recovered after neutralization. Thus, the values in pmol were corrected to the actual volume of extract recovered.

5. Since all four dNTPs were present in each cell extract, the final values had to take into consideration the dilution of the labelled dNTP by the corresponding cold dNTP present in the cell extract. To calculate the corrected concentration of a particular dNTP, the following values have to be known: 1. the uncorrected concentration of the dNTP assayed (using dTTP as an example: $[dTTP_{uc}]$), 2. the concentration of the complementary labelled dNTP present in the reaction buffer ($[dATP_l]$), 3. the uncorrected concentration for the complementary dNTP determined in a separate assay for that dNTP ($[dATP_{uc}]$) and 4. the concentration of the labelled dNTP present in the reaction buffer for assaying the complementary dNTP ($[dTTP_l]$). The correction factor for dTTP reads as follows (corrected concentrations for the three other dNTPs are calculated similarly):

$$[dTTP_c] = \frac{[dATP_{uc}][dTTP_l]([dATP_l] + [dTTP_{uc}])}{[dATP_{uc}][dTTP_{uc}] - [dTTP_l][dATP_l]}$$

6. All corrected values were normalized to pmol per 10^6 cells.

7. The results obtained from the three different cell extracts prepared for each strain or treatment were averaged and the standard

deviation (Sokal and Rohlf 1969) was determined.

3.14 Other Methods

Cell titres were determined using a Coulter electronic particle counter (Coulter Electronics, Hialeah, Florida).

DNA concentrations were determined by measuring absorbance at 260 nm (Milton Roy, Spectronic 601).

3.15 Statistical Analysis

Chi-square contingency tests (Sokal and Rohlf 1969) were used to evaluate differences in a variety of parameters. When small values were compared, Yates' correction for continuity was applied. The Monte Carlo estimate of the P value of the hypergeometric test (Adams and Skopek 1987) was calculated to assess the significance of differences in the distributions of base-pair substitutions (1,500 simulations were run for each comparison, the program was run on a Digital Equipment Corporation VAX/VMS Version V4.5 computer located at the Chemical Institute of Industrial Toxicology in Research Triangle Park, North Carolina). For both types of test, values of $P < 0.05$ were considered significant.

RESULTS

4 RESULTS

4.1 Isolation of a Nucleotide-Permeable dTMP Auxotroph

Yeast lacks the enzyme thymidine kinase (Grivell and Jackson 1968). Thus, yeast cells with a defective thymidylate synthase gene (*TMP1*) require thymidylate (dTMP) rather than thymine or thymidine (Brendel and Fäth 1974; Bisson and Thorner 1973; Little and Haynes 1979). Yeast, in common with most organisms, normally can take up DNA bases and nucleosides but not nucleotides. To overcome this barrier, yeast mutants which are permeable to dTMP (*tup*) have been isolated (Wickner 1974). Thus, in a dTMP-permeable thymidylate auxotroph, dTTP levels can be modulated merely by varying the concentration of dTMP in the growth medium. For the purposes of this study, isogenic derivatives of MKP-o which are permeable to dTMP, or permeable to and auxotrophic for dTMP, were isolated.

4.1.1 Isolation of a dTMP-Permeable Derivative of MKP-o

To isolate a dTMP-permeable derivative of MKP-o, cells of MKP-o were plated on MS medium containing dTMP (100 μg/ml). Treatment with methotrexate and sulfanilamide blocks dTMP biosynthesis (Jannsen *et al.* 1973; Laskowski and Lehmann-Brauns 1973; Little and Haynes 1973, 1979; Fäth *et al.* 1974; Wickner 1974; Barclay and Little 1977), presumably by inhibiting synthesis of tetrahydrofolate thereby reducing the level of N^5, N^{10}-methylene tetrahydrofolate, the methyl donor for the thymidylate synthase reaction (Figure 3). Since the biosynthetic pathways of adenine, histidine and methionine are also folate-dependent (Barclay and Little 1977), methionine had to be added as well (adenine and histidine are necessary supplements for MKP-o and so were present in the medium). Colonies which were able to grow on this medium were checked on MS medium

lacking dTMP. Isolates that were able to grow only in the presence of dTMP had to be able to take up the nucleotide (*tup*). A suitable *tup* derivative of MKP-o was selected and designated SBT.

4.1.2 Disruption of the *TMP1* Gene

A yeast strain deficient for thymidylate synthase was constructed by disrupting the *TMP1* gene in SBT. This was accomplished by transforming SBT with the 4.2 kb *Hind*III-*Ava*I DNA fragment from plasmid pTL46 (Taylor *et al*. 1987). This fragment consists of the yeast *LEU2* gene inserted into *TMP1* so that *LEU2* is flanked by *TMP1* sequences (Figure 14A). Transformants that emerged on leucine omission medium supplemented with dTMP were tested for thymidylate auxotrophy on medium lacking dTMP. An appropriate strain showing the correct growth pattern was chosen and designated SBT-T. Insertion of a single copy of the 4.2 kb *Hind*III-*Ava*I fragment at the *TMP1* locus was confirmed by DNA hybridization analysis. The rationale for the hybridization analysis is as follows. The yeast *Hind*III chromosomal DNA fragment carrying the thymidylate synthase gene is 9.6 kb in size (Figure 14B). Replacement of the 2.7 kb *Pst*I-*Ava*I fragment of *TMP1* with the pTL46 *Pst*I-*Ava*I 4.1 kb fragment carrying *LEU2* gene should increase the size of the *Hind*III chromosomal fragment carrying *TMP1* from 9.6 kb to 11 kb (Figure 14C). Total yeast DNA was isolated from Leu$^+$ Tmp$^-$ transformants and digested with *Hind*III. The resulting DNA fragments were separated by gel electrophoresis and the dried gels were hybridized with radioactive *TMP1* or *LEU2* DNA. Hybridization of *TMP1* or *LEU2* to total yeast DNA isolated from a wild-type strain resulted in a single band of 9.6 kb or 16.5 kb, respectively, the sizes of the *Hind*III fragments encompassing the wild-type *TMP1* or *LEU2* genes (Figures 15A and B, lane 1). As antici-

Figure 14. Disruption of the Chromosomal *TMP1* Gene with *LEU2* (modified
from Taylor *et al*. 1987). (A) pTL46; (B) chromosomal *Hind*III
fragment carrying *TMP1*. (C) chromosomal *Hind*III fragment
carrying *TMP1* disrupted with the *Hind*III-*Ava*I fragment of pTL46
including *LEU2*. ▓▓▓ *TMP1*; ☐ *LEU2*; ═══ bacterial vector
DNA; ─── yeast chromosomal DNA. The restriction sites are
indicated with the following letters: A: *Ava*I; B: *Bam*HI;
Bg: *Bgl*II; H: *Hind*III; P: *Pst*I.
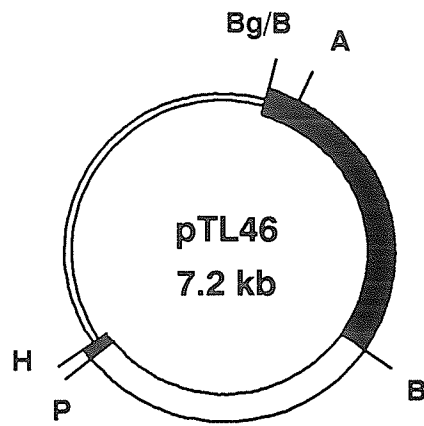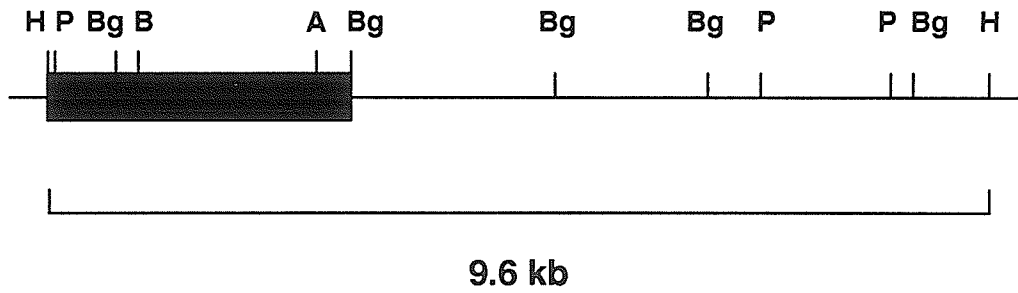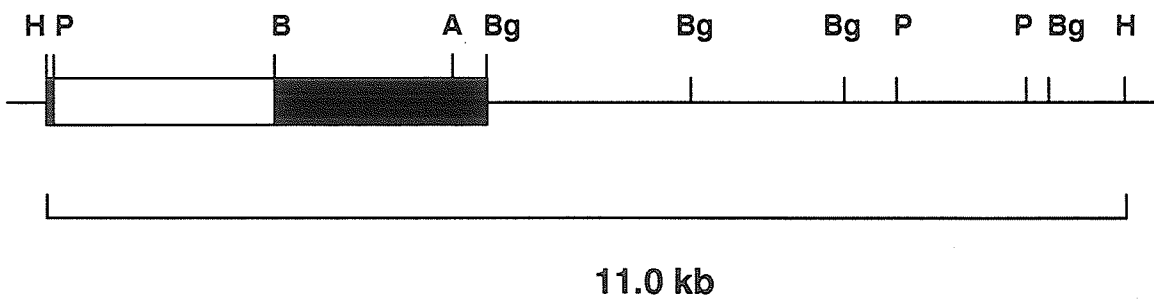
Figure 15. **Hybridization Analysis of Total Yeast DNA.** (A) Hybridization with a *TMP1* probe; (B) Hybridization with a *LEU2* probe. *Hind*III-digested genomic DNA of the following strains was used for the hybridization analysis: Lane 1: wild-type (*leu2, TMP1*); Lane 2: SBT-TL (*leu2, tmp1::LEU2*); Lane 3: gt12T (*leu2/leu2, TMP1/tmp1::LEU2*).

23.1-

9.4-

6.5-

1   2   3

-11.0
- 9.6

A

23.1-

9.4-

6.5-

1   2   3

-16.5
-11.0

B
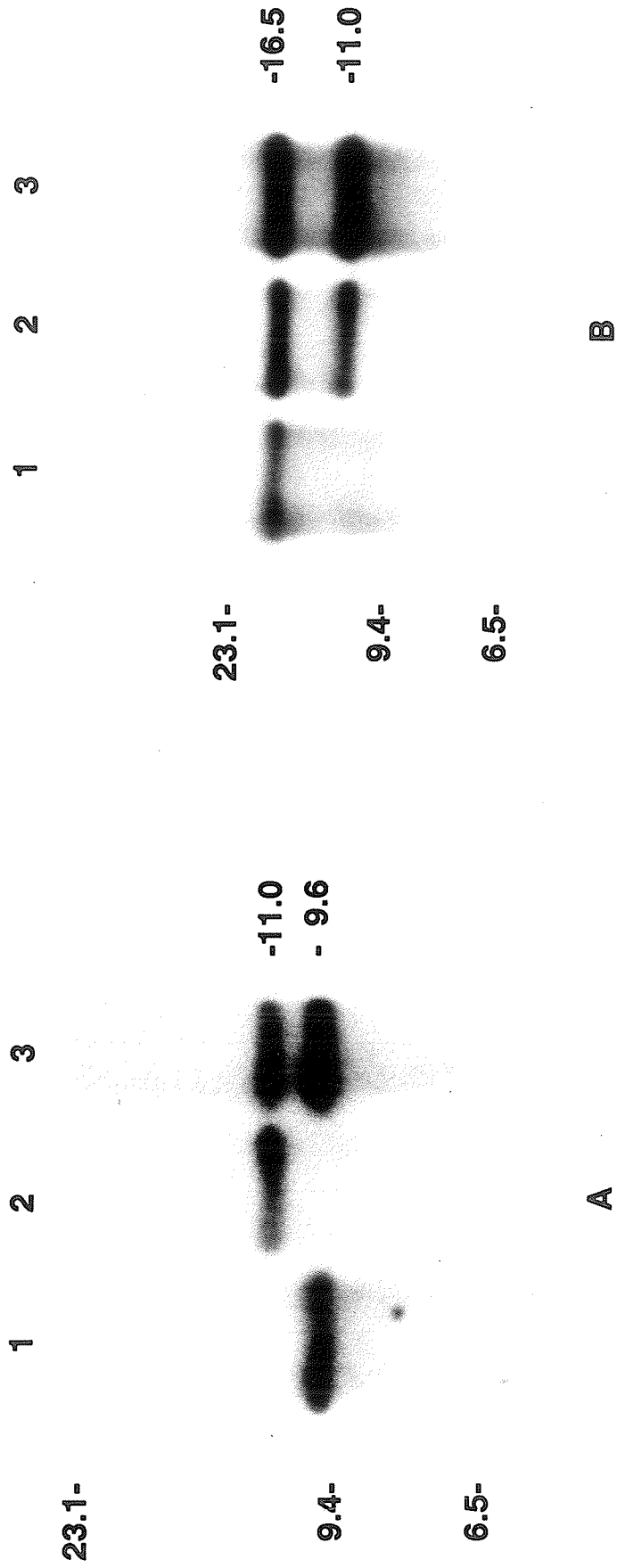
pated, a single hybridization band of 11 kb was detected for SBT-T DNA probed with the *TMP1* fragment (Figure 15A, lane 2). Hybridization with the *LEU2* probe resulted in an 11 kb band and the 16.5 kb band observed for DNA from the wild-type strain (Figure 15B, lane 2). For DNA from a diploid strain having one of the two copies of *TMP1* disrupted with *LEU2*, the 9.6 kb and 11 kb bands are detected when hybridizing with the *TMP1* probe (Figure 15A, lane 3) whereas the 11 kb and 16.5 kb bands appear for hybridization with the *LEU2* probe (Figure 15B, lane 3). This pattern is consistent with replacement of the wild-type *TMP1* gene with the disrupted *TMP1* gene.

4.1.3 Isolation of a Thymidylate Auxotroph Able to Grow on 20 μg/ml dTMP

Preliminary experiments indicated that SBT-T, which requires 100 μg/ml dTMP for growth, was not greatly affected by excess dTMP concentrations in the growth medium. Therefore, SBT-TL, a derivative of SBT-T able to grow at a lower dTMP concentration, was isolated by screening for growth on medium containing only 20 μg/ml dTMP. This reduced dTMP requirement could be due to the acquisition of a second *tup* mutation which increases dTMP uptake (Bisson and Thorner 1982b), to a more efficient utilization of intracellular dTMP or to a decrease in the breakdown of excess dTMP (Toper *et al.* 1981)

SBT, SBT-T and SBT-TL were transformed with the plasmid YCpMP2 to generate SBT-p, SBT-Tp and SBT-TLp.

Together with MGK-d, in which the dCMP deaminase gene (*DCD1*) is disrupted with *LEU2* (Kohalmi *et al.* 1991), SBT, SBT-T and SBT-TL are isogenic derivatives of MKP-o. Consequently, any observed differences between MKP-o and these strains can be attributed to the *tup* mutation and/or to

disrupting the *TMP1* or *DCD1* gene instead of variations in the genetic background.

4.2 Association of Nucleotide Permeability with Enhanced Uptake of Canavanine

In preliminary studies, it was found that when SBT-TLp was plated on medium containing 30 μg/ml canavanine, the concentration normally used to select canavanine-resistant (Can^R) mutants with the *SUP4*-o system, the spontaneous Can^R frequency was more than 100-fold lower than for MKP-op. To investigate this unexpected result in more detail, the following experiments were carried out.

4.2.1 Effect of Canavanine on Mutant Recovery

Selection for canavanine resistance on media having decreased levels of the drug revealed that even small changes in canavanine concentration dramatically influenced the recovery of Can^R mutants in SBT-TLp (Figure 16). This was not due to a general reduction in spontaneous mutagenesis since the frequencies of locus reversion and suppression of the *ade2-1* and *lys2-1* alleles were similar for MKP-o and SBT-TL (Table 5). However, when the canavanine concentration was increased from 2.5 to 30 μg/ml, the frequencies of both Can^R and *SUP4*-o mutants (each type of mutant is scored on the basis of canavanine resistance) were reduced by more than 600-fold (Table 6). Thus, while the effect was not limited to events at the *CAN1* locus, it appeared to be restricted to the Can^R phenotype.

As described previously, SBT-TLp is permeable to and auxotrophic for thymidylate. To determine whether either of these factors played a role in reducing the frequency of Can^R mutants in this strain, we examined the

Figure 16. Recovery of Spontaneous Canavanine-Resistant Mutants in SBT-TLp as a Function of Canavanine Concentration. The plates shown contain different concentrations of canavanine (from bottom to top: 1, 2, 3, 4 or 5 μg/ml). The results for two independent experiments are shown.
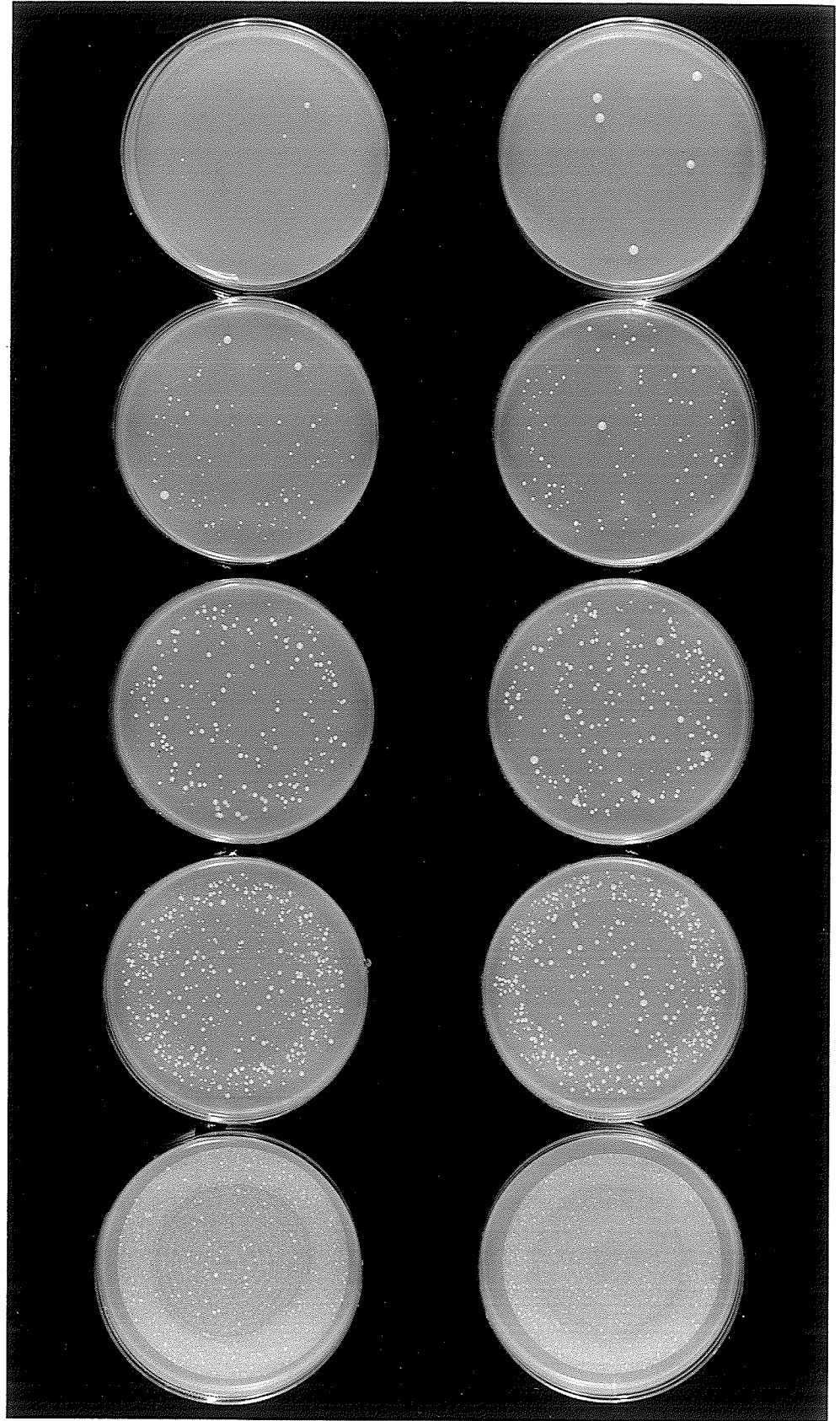
Table 5. Reversion Frequencies of the *ade2-1* and *lys2-1* Alleles

Strain	Allele	Viable cells plated $(x\ 10^9)$	Suppressor mutants[a]		Locus revertants[b]	
			No. detected	Frequency $(x\ 10^{-7})$	No. detected	Frequency $(x\ 10^{-8})$
MKP-o	*lys2-1*	3.2	505	1.6	16	0.5
	ade2-1	3.3	567	1.7	99	3.0
SBT-TL	*lys2-1*	5.5	435	0.9	74	1.3
	ade2-1	4.3	377	0.9	111	2.6

[a] Phenotype: Lys^+, Ade^+, Can^S.

[b] Phenotype: Lys^+, Ade^-, Can^R for reversion of *lys2-1* or Ade^+, Lys^-, Can^R for reversion of *ade2-1*.

Table 6. Effect of Canavanine Concentration on the Frequencies of Spontaneous Can^R and *SUP4*-o Mutants in SBT-TLp

Canavanine (μg/ml)	Viable cells plated (x 10^7)	Total Can^R mutants		*SUP4*-o mutants	
		No. detected	Frequency (x 10^{-6})	No. detected	Frequency (x 10^{-8})
2.5	3.6	13,755	380	69	190
5.0	28	1,443	5.1	317	110
10.0	31	652	2.1	123	39
20.0	70	620	0.88	1	0.14
30.0	70	434	0.63	0	-

influence of canavanine on the recovery of spontaneous Can^R colonies in MKP-op, SBT-p, SBT-Tp and SBT-TLp, strains carrying different combinations of *TUP* and *TMP1* alleles (Table 7). The frequencies of the total Can^R mutants were identical for SBT-p and SBT-Tp but were 40-fold lower than the value for MKP-op. In addition, the total Can^R frequency for SBT-TLp, which requires less dTMP for growth than SBT-p or SBT-Tp, was six-fold lower than for these strains. These results demonstrated that the effect of canavanine on the recovery of Can^R mutants was dependent on the *tup* mutation(s) and suggested that it might be correlated with the efficiency of dTMP uptake.

4.2.2 Effect of Canavanine on Cell Growth

The frequencies of the total Can^R and *SUP4*-o mutants selected in SBT-TLp at 2.5 μg/ml canavanine were very close to the corresponding values found for MKP-op at 30 μg/ml canavanine (compare Tables 6 and 7). This suggested that the mutations responsible occurred at the normal frequencies in SBT-TLp but the *tup* allele(s) caused growth of this strain to be more efficiently inhibited by canavanine so that fewer mutants were detected at the higher canavanine concentrations. To test this possibility, the effect of canavanine on the growth of Can^R cells was assayed using MKP-o, SBT-TL, SBT and SBT-T, strains that do not carry YCpMP2 so that the *can1-100* allele is not suppressed. The growth of MKP-o was unaffected by a canavanine concentration of 30 μg/ml (Figure 17A). On the other hand, the growth of SBT-TL was progressively inhibited with increasing canavanine concentrations (Figure 17B). For both SBT and SBT-T, growth was also inhibited by 30 μg/ml canavanine (Figure 17C and 17D). Thus, the reduced recovery of Can^R mutants in SBT-TLp was associated with

Table 7. Effect of Canavanine on the Recovery of Spontaneous Can[R] and *SUP4*-o Mutants in MKP-op and Its Derivatives[a]

Strain	Viable cells plated (x 10^7)	Total Can[R] mutants		*SUP4*-o mutants	
		No. detected	Frequency (x 10^{-6})	No. detected	Frequency (x 10^{-8})
MKP-op (*TUP*, *TMP1*)	7.2	10,833	150	201	300
SBT-p (*tup*$_{100}$, *TMP1*)	1.8	68	3.8	1	5.6
SBT-Tp (*tup*$_{100}$, *tmp1*)	2.8	105	3.7	0	-
SBT-TLp (*tup*$_{20}$, *tmp1*)	70	434	0.63	0	-

[a] Medium for mutant selection contained 30 μg/ml canavanine.

Figure 17. Effect of Canavanine Concentration on the Growth of MKP-o and its Derivatives. (A) MKP-o; (B) SBT-TL; (C) SBT; (D) SBT-T. Canavanine: (■) 0 μg/ml; (▲) 5 μg/ml; (◆) 10 μg/ml; (▼) 20 μg/ml; (●) 30 μg/ml.

127
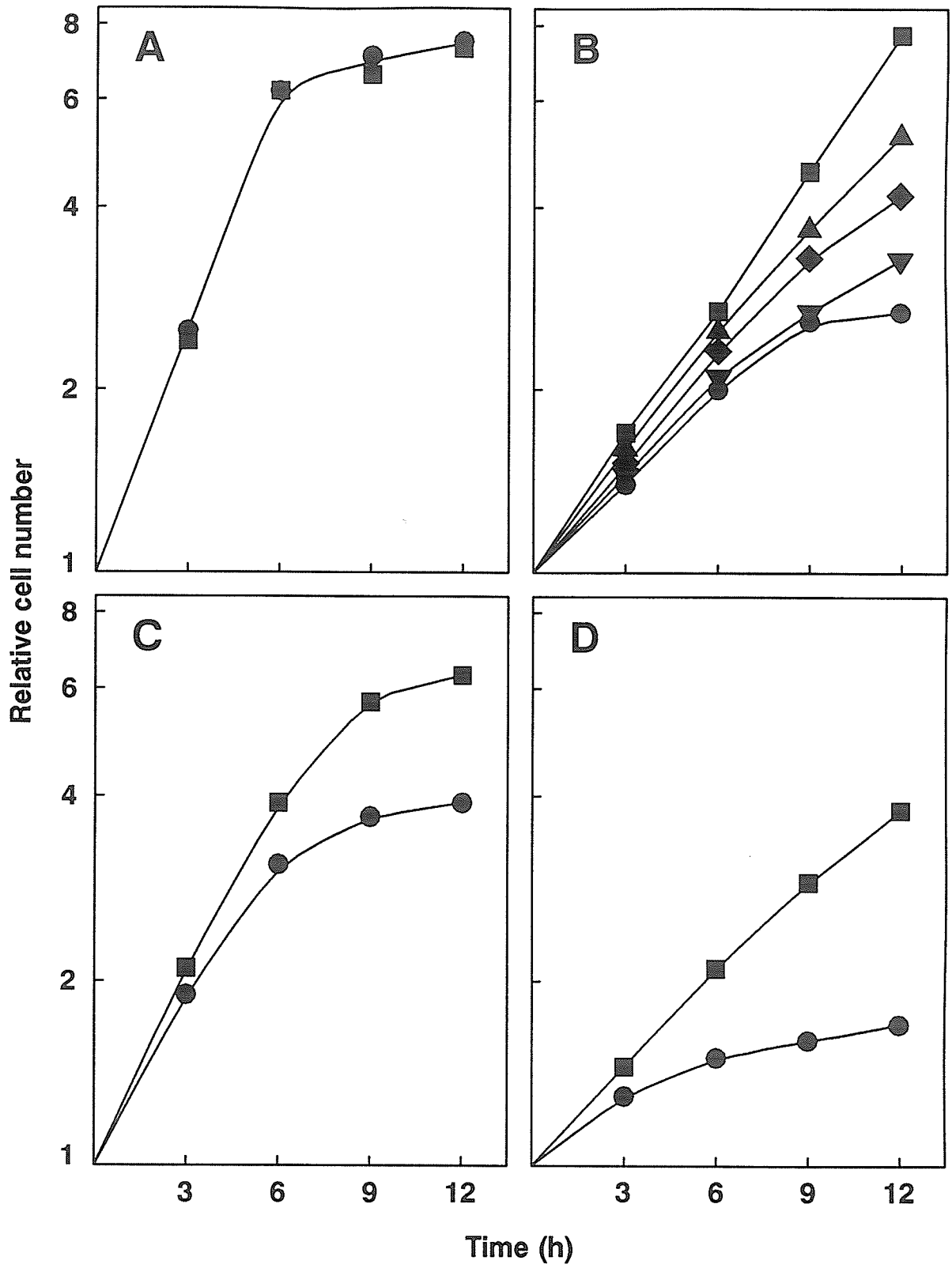
apparent sensitization to growth inhibition by canavanine and both effects were related to dTMP permeability rather than the *TMP1* deficiency.

4.2.3 Influence of Nucleotide Permeability on Uptake of Labelled Arginine

The most reasonable explanation for the enhanced growth inhibition by canavanine seemed to be that the *tup* mutation(s) present in the dTMP permeable strains somehow increased uptake of canavanine. This was tested by comparing the uptake of radioactively labelled arginine (both, canavanine and arginine are transported into the cell by the arginine permease which is encoded by *CAN1* gene) by strains MKP-o and SBT-TL and their derivatives carrying YCpMP2 (Table 8). The measurements showed that the amount of labelled arginine incorporated by MKP-o increased slowly over the treatment period. This might have reflected leakiness of the *can1-100* allele. Under identical conditions, SBT-TL also took up labelled arginine but by two hours, had incorporated approximately four times as much as MKP-o. When the *can1-100* alleles in MKP-op and SBT-TLp were suppressed by *SUP4*-o, both strains rapidly incorporated arginine as expected, but SBT-TLp took up two-fold more at each time point than MKP-op. A 100-fold excess of canavanine (11.5 mM) substantially inhibited arginine uptake in all of the strains tested. These results argued that the *tup* mutation(s) was promoting the uptake of arginine and canavanine by the same mechanism.

To show that the *tup* mutation does not enhance uptake of canavanine via the same mechanism responsible for uptake of dTMP, the effect of dTMP on the uptake of radioactive arginine was measured in SBT-TL and SBT-TLp. The results, presented in Table 9, indicate that dTMP, at the normal growth concentration (0.062 mM = 20 μg/ml) or at a concentration exceeding

Table 8. [^{14}C] Arginine Uptake in Canavanine-Resistant or Sensitive Strains[a]

Time (min)	MKP-o (Can[R])			SBT-TL (Can[R])		
	-Canavanine	+Canavanine		-Canavanine	+Canavanine	
0	275	141	(49)	394	161	(59)
15	1,062	369	(65)	2,155	603	(72)
30	2,176	294	(86)	4,566	544	(88)
60	3,776	422	(89)	14,288	1,558	(89)
120	9,139	1,509	(83)	35,432	3,184	(91)

Time (min)	MKP-op (Can[S])			SBT-TLp (Can[S])		
	-Canavanine	+Canavanine		-Canavanine	+Canavanine	
0	824	174	(79)	1,607	125	(92)
1	3,378	379	(89)	7,118	462	(94)
2.5	7,088	584	(92)	16,080	992	(94)
5	15,321	1,115	(93)	35,874	1,537	(96)
10	30,253	1,838	(94)	68,454	3,333	(95)
20	60,802	2,935	(95)	147,781	9,239	(94)

[a] The values for uptake are given in cpm. Plasmid retentions in MKP-op and SBT-TLp are 90% and 57%, respectively. Cpms for these strains were normalized to take plasmid retention into account. Numbers in parentheses are the per cent decreases in uptake of [^{14}C] arginine in the presence of canavanine (11.5 mM).

Table 9. Effect of dTMP on [^{14}C] Arginine Uptake in Canavanine-Resistant or Sensitive Strains[a]

Strain	Time (min)	dTMP concentration (mM)		
		0	0.062	11.5
SBT-TL (Can^R)	0	270	265	218
	1	484	354	364
	2.5	618	717	504
	5	1,354	1,232	1,231
	10	2,709	2,761	2,096
	20	5,643	5,325	4,612
SBT-TLp (Can^S)	0	2,250	2,152	1,395
	1	9,253	7,157	5,857
	2.5	22,388	19,393	15,860
	5	39,557	37,938	35,186
	10	87,279	76,418	66,502
	20	161,115	162,731	141,422

[a] The values for uptake are given in cpm. Cpms were normalized to take plasmid retention into account (plasmid retention in SBT-TLp is 57%).

that of labelled arginine by 100-fold (11.5 mM), had little effect on the uptake of arginine. Although the uptake of radioactive arginine in the presence of 11.5 mM dTMP seemed to be somewhat lower than the control values, the decrease was small compared to the more dramatic reduction caused by canavanine (at 20 min: 18% for dTMP vs. 91% for canavanine in SBT-TL and 12% for dTMP vs. 94% for canavanine in SBT-TLp; compare Tables 8 and 9). Furthermore, the values at time 0 were almost the same for cells kept at 0 or 0.062 mM dTMP while the value for 11.5 mM dTMP was reduced. Consequently, the lower uptake observed in the presence of 11.5 mM dTMP was probably an experimental artifact. In any event, these results do not seem to support the possibility that the same mechanism enhances the uptake of canavanine and dTMP in the *tup* strains.

4.2.4 Canavanine Sensitivity vs. the GAP System

In addition to arginine permease, the general amino acid permease (GAP) can also transport arginine and canavanine into yeast (Grenson *et al.* 1970). Conceivably, the *tup* mutation(s) might promote canavanine uptake by increasing GAP activity. The GAP system can be inhibited by several amino acids that do not inhibit arginine uptake by arginine permease (alanine, methionine, phenylalanine, valine). However, when SBT-TLp was grown in medium containing canavanine (30 μg/ml) and these amino acids (100 μg/ml), inhibition of cell growth was not alleviated as would be expected if increased canavanine uptake was due to increased GAP activity (Table 10). This suggests that the increase in canavanine uptake is not correlated with activity of the GAP system.

4.2.5 Selection of Can^R Mutants in SBT-TLp

The potential relationships between the *tup* mutation(s) and canavanine

Table 10. Effect of Amino Acids That Inhibit the General Amino Acid Permease on Growth Inhibition of SBT-TLp by Canavanine

Amino acid[a]	Canavanine[b]	Cells per ml ($\times 10^6$)	
		0 hours	12 hours
none	-	2.0	15.0 (7.5)[c]
none	+	2.1	6.8 (3.2)
ala	+	2.1	7.6 (3.6)
met	+	2.1	6.7 (3.2)
phe	+	2.0	6.4 (3.2)
val	+	2.1	7.1 (3.4)
ala, met, phe, val	+	2.0	6.7 (3.3)

[a] ala: alanine; met: methionine; phe: phenylalanine; val: valine. The concentration for each amino acid was 100 μg/ml.

[b] Concentration: 30 μg/ml.

[c] Number in parentheses indicates the increase in the cell titre between the 0 and 12 h time points.

uptake are considered in greater detail in the Discussion. For now, it is sufficient to realize that recovery of Can^R mutants in the nucleotide-permeable strain is inhibited by the canavanine concentration (30 μg/ml) routinely used to select these mutants in MKP-op. However, the results presented in Figure 16 and Table 6 indicated that in SBT-TLp, spontaneous Can^R and *SUP4*-o mutants could be recovered at frequencies very similar to those for MKP-op by reducing the canavanine concentration to 2 or 2.5 μg/ml. Consequently, a lower canavanine concentration (2 μg/ml) was used in all further mutation experiments with SBT-TLp.

4.3 Analysis of Spontaneous Mutations in MKP-op

The isolation and characterization of *SUP4*-o mutations occurring spontaneously in MKP-op was a collaborative effort in which I participated. MKP-op is the parental strain for all the other yeast strains used for analysis of mutational specificity in this study and so the analysis of spontaneous mutagenesis in MKP-op serves as the basis for other comparisons. Therefore, data for MKP-op were used as control data throughout this study.

In a culture of MKP-op grown under conditions selective for YCpMP2, 88% of the cells retained the plasmid (37,299 colonies scored on medium selective for YCpMP2 vs. 42,231 colonies which formed on medium that allows cells with or without the plasmid to grow). To isolate spontaneous mutants, cultures were grown from low titre inocula to stationary phase in medium selective for YCpMP2. Among the total canavanine-resistant colonies detected, 3.1% were red, lysine auxotrophs and so were classified as *SUP4*-o mutants (Table 11). On this basis, the mean *SUP4*-o mutation frequency was determined to be 2.0 x 10^{-6}, corresponding to a spontaneous

Table 11. Spontaneous Mutation Frequencies in MKP-op

Colony types	No. examined[a] (% of total)	Frequency[a] $(x\ 10^{-6})$
Total[b]	31,923 (100.0)	65
Red, Lys⁻	984 (3.1)	2.0

[a] Colonies were isolated from, and frequencies are the means for, 45 independent cultures.

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

mutation rate of 4.7 x 10^{-7} events per round of replication. To ensure the independence of each mutant analyzed by DNA sequencing, not more than two mutants were selected from an individual culture. If the same sequence alteration was detected in both mutants, one was excluded from further analysis. A collection of 334 spontaneous *SUP4*-o mutants was characterized by DNA sequencing.

The majority (81%) of spontaneous changes were single base-pair substitutions and these were recovered at a mutation frequency of 1.6 x 10^{-6} (Table 12). The rate of mutation was calculated to be 4.3 x 10^{-9} per base-pair per generation. Both types of transition and all four transversions were identified (Table 13), but there was a slight excess of transversions (59% vs. 41%), and four times as many events occurred at G·C sites as at A·T sites (79% vs. 21%). In addition to single base-pair substitutions, two non-tandem, double-base-pair changes were detected (Table 12). Each involved A·T to G·C and G·C to A·T transitions, in one case at positions +8 and +10 (for correlation of position numbers with *SUP4*-o sites see Figure 18) and in the other at positions +14 and +12, respectively. While the spontaneous base-pair substitutions were distributed throughout the *SUP4*-o gene, mutations occurred at some locations more often than others with positions +18, +51, +83, +84, and +88 being the most frequently mutated (Figure 18). No base-pair substitutions were detected 5′ or 3′ to the region encoding *SUP4*-o and base-pair changes were found at only one position (+51) within the tRNA intron.

Deletions, ranging from the loss of a single base-pair to the elimination of up to 807 base-pairs, were also detected (Table 14). These events (including 4 deletions recovered as part of complex changes)

Table 12. Spontaneous Sequence Alterations in *SUP4*-o in MKP-op

Sequence alteration	No. detected (% of total)		Frequency (x 10^{-7})
Substitution			
Single change	272	(81.4)	16.4
Non-tandem double	2	(0.6)	0.1
Deletion			
1 bp	22	(6.6)	1.3
>1 bp	7	(2.1)	0.4
Insertion			
1 bp	1	(0.3)	0.06
Ty element	26	(7.8)	1.6
Duplication	1	(0.3)	0.06
Complex change	3	(0.9)	0.2
Total	334		20.12

Table 13. Spontaneous Base-Pair Substitutions in MKP-op

Substitution	No. detected[a] (% of total)	Frequency (x 10^{-7})
Transitions		
G·C to A·T	72 (26.1)	4.3
A·T to G·C	42 (15.2)	2.5
Total	114 (41.3)	6.8
Transversions		
G·C to T·A	91 (33.0)	5.5
G·C to C·G	55 (19.9)	3.3
A·T to C·G	5 (1.8)	0.3
A·T to T·A	11 (4.0)	0.6
Total	162 (58.7)	9.7

[a] Including double substitutions.

Figure 18. Distribution of Spontaneous Base-Pair Substitutions in the *SUP4*-o Gene of MKP-op. For simplicity, only the region of the transcribed strand encoding the tRNA is shown. Double base substitutions are indicated by lower-case letters. The intron extends from +40 through +53.

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

   AGA AAAgCt  Aa AA  AA              CCAGAACG  AACAGTGCA               A CTAGCAC  TC CAGACA  AC CAGCC  TTAAGAAAA
   A T AAAG T  C  AA  AT              CAGCAC    GGCAG GCC              A C A  A    C CGTACG   AC A CT   AAGG AA
   A   AAA     C      A               CAGTAC    G AG GC               A C A  A    C G GTT    G  A      AAGG TA
   A   CAT     C      A               TT  C     TT A  C               A C A  T    C C GTT    G  C      CA G TA
   C   TA      C      A               T   C     T  A                  A A G  T T  C C  T     G  C      CA G TC
   T   A       C      A               T   C     T  A                  A A    T T           G  C      CG T TC
   T   A               A                          A C                 A A    T              CG T TC
   T   G               A                          C                   A A                   CG T
   T   G               A                                              A A                   CG T
       T               A                                              A A                   TT T
       T               A                                              A                     TT T
                       A                                                                    TT T
                       A                                                                    T  T
                       G                                                                    T  T
                       T                                                                    T  T
                       T
```

Table 14. Spontaneous Deletions[a]

Site	No. of base-pairs involved	No. detected (% of total)		Frequency (x 10^{-8})
-724 to +83	807[b]	1	(3.2)	0.6
-555 to +70	625	1	(3.2)	0.6
-248 to -1	248[b]	1	(3.2)	0.6
-122 to +13	135	1	(3.2)	0.6
+15	1	1	(3.2)	0.6
+18 to +19	1	1	(3.2)	0.6
+58 to +64[c]	7	2	(6.5)	1.2
+63 to +83	27	1	(3.2)	0.6
+79 to +83	1	17	(55.0)	10.3
+84 to +86	1	2	(6.5)	0.6
+88 to +119[d]	32	1	(3.2)	0.6
+89 to +96	8	1	(3.2)	0.6
+89 to +97	9	1	(3.2)	0.6
Total		31		18.7

[a] Includes deletions in complex changes.

[b] Values are only estimates because sequence repeats are present at the deletion termini.

[c] A 5-base-pair insertion in the deleted region accompanied the deletion.

[d] A single-base-pair deletion in +79 to +83 accompanied the multiple-base-pair deletion.

accounted for 9.0% of the total mutations identified, corresponding to a frequency of 1.7 x 10^{-7}. In 20 of the mutants, single G·C pairs were deleted from runs of G·C pairs extending from +18 to +19, +79 to +83 or +84 to +86. In one mutant, an insertion of a single G·C pair in the run of G·C pairs extending from +79 to +83 occurred. Slipped mispairing of nascent and template strands during replication in runs of repeated base-pairs (Streisinger et al. 1966) could have been responsible for these events (gain or loss of a single base-pair) and the loss of 8 and 9 base-pairs from +89 to +96 and +89 to +97, respectively. Two mutants, containing very large deletions (248 or 807 base-pairs) retained single copies of short, repeated sequences (4 or 2 base-pairs, respectively) originally present at both deletion termini and so may have been generated by illegitimate recombination occurring between the repeats (Nalbantoglu et al. 1986). Three other mutants featured more complex alterations. In one mutant, a deletion of 32 base-pairs (+88 to +119) was accompanied by the removal of a single G·C pair (+79 to +83). The two other mutants both had the sequence 5'-GATCTCA-3' at +58 to +64 replaced by 5'-CCGGG-3'. Base-pairing within a quasi palindromic sequence could have been involved in the formation of this mutation as detailed in the Discussion.

Non-plasmid DNA extending 5' from position +17, +37 or +43, with position +37 being a hotspot for this type of insertion, was found in 7.8% of the total spontaneous mutants analyzed, corresponding to a frequency of 1.6 x 10^{-7} (Table 15). Sequencing of the first 100 to 200 nucleotides of the insertions showed that they had conserved features of the 330 base-pair delta sequences that are long terminal repeats for transposable Ty elements in S. cerevisiae (Roeder and Fink 1983; Rothstein et al. 1987; Boeke 1989; Garfinkel et al. 1989). In particular, the insertions either

Table 15. Spontaneous Ty Insertions

Location	No. detected (% of total)		Frequency (x 10^{-8})
+17 <> +18[a]	2	(7.7)	1.3
+37 <> +38	23	(88.5)	14.4
+43 <> +44	1	(3.8)	0.6
	26		16.3

[a] The symbol <> means that the Ty insertion is presumed to have occurred between the two indicated positions.

began with the sequence 5'-TGTTGGAATA-3' or started with 5'-TG-3' and had a *Hinf*I recognition site at position +34. Computer-facilitated comparisons of the sequenced ends of twelve of these inserts with established sequences for delta elements revealed striking homologies (up to 100%). Of the twelve mutants involved, eleven contained plasmids which were larger than YCpMP2 by 6 kb, the approximate size of a Ty element (Roeder and Fink 1983), and so likely carried an intact transposon. The remaining plasmid was only slightly larger than YCpMP2, consistent with the presence of a single delta sequence. Thus, this insertion was most likely the remnant of a transposon that had excised by homologous recombination between its flanking delta sequences (Roeder and Fink 1983; Rothstein *et al.* 1987).

4.4 Mutational Specificity of dTTP Depletion

dTTP depletion is known to cause mutations in a large number of organisms (Kunz 1982, 1988). To gain a better understanding of the processes which might be involved, dNTP pools were measured in a thymidylate auxotroph following starvation for dTMP. Mutants recovered under the same conditions were analyzed by DNA sequencing.

4.4.1 Measurement of dNTP Pool Imbalances Induced by dTMP Starvation

To determine the effect of dTMP starvation on dNTP pools in yeast, an enzymatic assay (Sargent and Mathews 1987) was used to measure the concentrations of all four DNA precursors in exponential phase cells of MKP-op and cells of SBT-TLp starved for dTMP for 12 h. In MKP-op, the levels of dATP, dCTP and dGTP were similar ($0.6 - 0.7$ pmol/10^6 cells) with the concentration of dTTP being about 2-fold greater (Table 16). The removal of dTMP from the growth medium of the *tmp1* strain SBT-TLp reduced dTTP levels to such an extent (<0.001 pmol per 10^6 cells) that calculations

Table 16.   Intracellular dNTP Concentrations for MKP-op and SBT-TLp[a]

Strain	dATP	dTTP	dGTP	dCTP
MKP-op	0.68 ± 0.04[a]	1.55 ± 0.04	0.58 ± 0.02	0.61 ± 0.04
SBT-TLp[b]	81.46 ± 7.34	BDL[c]	4.93 ± 0.65	2.21 ± 0.22

[a] Concentrations are given in pmol/10^6 cells. Each value is the mean $\pm$ S.D. of three independent measurements.

[b] Treatment: 0 μg/ml dTMP, 12 h.

[c] Below detection level.

of dTTP concentrations were not reliable (Table 16). On the other hand, the pools of dATP, dGTP and dCTP were increased, although· to different extents (120-, 8.5- and 3.6-fold, respectively). Changes in the latter three pools are considered in the Discussion.

4.4.2 Induction of *SUP4*-o Mutants by dTTP Depletion

To evaluate the effect of dTTP depletion on cell viability, plasmid retention and *SUP4*-o mutation frequency, cells of the *tmp1* strain SBT-TLp were grown to exponential phase in medium supplemented with dTMP and then transferred to medium lacking dTMP. The cells were plated on medium selective and non-selective for YCpMP2, to determine the extent of cell killing and plasmid loss, and on medium containing canavanine to select for *SUP4*-o mutants. The effects of 12 h of dTMP starvation are shown in Table 17. With increasing treatment times, an increasing number of cells were killed and a successively greater fraction of cells lost YCpMP2.

The initial increase in mutation frequency did not occur until after cell viability had started to decrease (6 h of dTMP starvation). In addition, there was no increase in mutation yield regardless of the treatment time (the lower values calculated for yield compared to the mutation frequency at 0 time are a consequence of the low plasmid retention in SBT-TLp). Thus, it seemed that the apparent increase in mutation frequency might be an artifact related to cell killing. However, although cell number did not increase after 4 h of dTMP starvation, the yield did not decrease as would have been expected in the absence of a net induction of mutants and a continued reduction in viability. Furthermore, partial starvation with 4 or 6 μg/ml dTMP caused a small increase in the *SUP4*-o mutation frequency (2- and 2.5-fold, respectively) without

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Table 17. Effect of Thymidylate Starvation on Viability, Plasmid Retention
and *SUP4*-o Mutation[a]

Time (h)	Surviving fraction[b]	Plasmid retention[b] (%)	Mutation frequency ($\times 10^{-6}$)	Mutation yield ($\times 10^{-6}$)
0	1.00	100	4.0	3.1
2	0.97	93	5.1	3.5
4	0.89	90	4.7	3.2
6	0.62	80	8.4	1.9
8	0.45	73	11	3.9
10	0.21	55	22	4.0
12	0.10	35	60	3.6

[a] Data are the means for 4 independent cultures.

[b] Data have been normalized to time 0. Plasmid retention at time 0 = 57%.

affecting cell viability and increased the mutation yield by 3.2-fold (Table 18). These findings argued that there was a net induction of mutants and the increase in the *SUP4*-o mutation frequency was genuine. Overall, the magnitudes of the effects of partial dTMP depletion on cell viability, plasmid retention and *SUP4*-o mutation depended on the exogenous dTMP concentration (Table 18).

dTTP depletion for 12 h increased the *SUP4*-o mutation frequency 30-fold compared to the spontaneous frequency for MKP-op (Table 19). In MKP-op, mutants were selected after twenty generations of growth. However, growth was restricted to less than one generation during the 12 h of dTMP starvation. Consequently, and assuming that the mutations were randomly distributed among viable and non-viable cells, the rate of *SUP4*-o mutation per round of DNA replication was calculated to be 171-fold (8.1×10^{-5}) greater for dTMP starvation than the spontaneous rate for MKP-op (4.7×10^{-7}).

4.4.3 Characterization of *SUP4*-o Mutations Induced by dTTP Depletion

To determine the mutational specificity of dTTP depletion, 89 *SUP4*-o mutants induced by dTMP starvation for 12 h were analyzed by DNA sequencing. To ensure that the isolated mutants were due to independent mutational events, not more than 5 mutants were selected from each culture. In addition, any member of a group of 5 mutants that was found to have a sequence alteration identified in another member of the same group was excluded from further analysis. The results for dTTP depletion were then compared to those for 334 spontaneous mutants selected in MKP-op. Fewer mutational classes were detected in SBT-TLp (Table 20), but this could reflect the smaller number of mutants analyzed for dTTP depletion

Table 18. Effect of Low dTMP Concentrations (12 h) on Viability, Plasmid
Retention and *SUP4*-o Mutation[a]

dTMP concentration (μg/ml)	Surviving fraction[b]	Plasmid retention[b] (%)	Mutation frequency (x 10^{-6})	Mutation yield (x 10^{-6})
0[c]	0.10	35	60	3.6
0.5	0.19	40	48	7.1
1.0	0.40	49	17	5.5
2.0	0.55	61	19	8.8
4.0	1.00	100	10	10.0
6.0	1.00	100	8.5	7.7
20.0[c]	1.00	100	4.0	3.1

[a] Data are the means for 3 independent cultures.

[b] Data have been normalized to values obtained for cells growing with 20 μg/ml dTMP. Plasmid retention at 20 μg/ml dTMP = 57%.

[c] Data are taken from Table 17.

Table 19. Effect of dTMP Starvation on Can^R and *SUP4*-o Mutation

Strain	Colony types	No. examined[a] (% of total)		Frequency[a] (x 10^{-6})
MKP-op[b]	Total[c]	31,923	(100.0)	65
	Red, Lys$^-$	984	(3.1)	2.0
SBT-TLp[d]	Total	99,469	(100.0)	9600
	Red, Lys$^-$	620	(0.6)	60

[a] Colonies were isolated from, and frequencies are the means for, 45 (MKP-op) or 42 (SBT-TLp) independent cultures.

[b] Data for spontaneous mutation in MKP-op are taken from Table 11.

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

[d] Treatment: 0 μg/ml dTMP, 12 h.

Table 20. Sequence Alterations Induced by dTTP Depletion

Sequence alteration	MKP-op[a]		SBT-TLp[b]	
	No. detected (% of total)	Frequency ($\times 10^{-7}$)	No. detected (% of total)	Frequency ($\times 10^{-7}$)
Substitution				
Single change	272 (81.4)	16.4	76 (85.4)	512
Non-tandem double	2 (0.6)	0.1	-	
Deletion				
1 bp	22 (6.6)	1.3	9 (10.1)	61
>1 bp	7 (2.1)	0.4	3 (3.4)	20
Insertion				
1 bp	1 (0.3)	0.06	1 (1.1)	7
Ty element	26 (7.8)	1.6	-	
Complex change	3 (0.9)	0.2	-	
Duplication	1 (0.3)	0.06	-	
Total	334	20.12	89	600

[a] Data for MKP-op are taken from Table 12.

[b] Treatment: 0 μg/ml dTMP, 12 h.

rather than being a significant change in the types of sequence alteration recovered. In both cases, the majority of alterations were single base-pair events but these accounted for significantly more of the total mutations in SBT-TLp (97%) than in MKP-op (88%) ($P < 0.05$). A detailed analysis of the base-pair substitutions is present below.

Deletions and insertions constituted approximately 11% of the total mutations characterized following dTTP depletion and 7% of the spontaneous events for MKP-op (Table 20). This difference was not significant ($P > 0.2$). For dTTP depletion, the majority of the deletions (7/9) and one insertion were detected within the run of 5 G·C pairs at +79 through +83. The other deletions (2/9) occurred in the run of 3 G·C pairs at +84 through +86 or at +65 to +67. Formation of these mutations could have involved slipped mispairing of the template and nascent strands during replication through these runs as described by Streisinger *et al.* (1966). Mechanisms potentially responsible for the single-base-pair deletions are further addressed in the Discussion.

Three multiple base-pair deletions were also detected after dTTP depletion (Table 20): 1. the elimination of 17 bases encompassing positions -6 to +11; 2. the loss of 5 bases from positions +25 through +29 and 3. the removal of 5 bases from positions +35 to +43.

4.4.4 Analysis of Single Base-Pair Substitutions Induced by dTTP Depletion

Analysis of the base-pair substitutions showed that the relative fraction of transversions was 4-fold greater than that for transitions in SBT-TLp (Table 21). By comparison, there was only a slight excess (1.4-fold) of transversions among the spontaneous substitutions recovered in MKP-op. This difference was significant ($P < 0.001$) and was due

Table 21. Base-Pair Substitutions Induced by dTTP Depletion

Substitution	MKP-op[a]		SBT-TLp[c]	
	No. detected[b] (% of total)	Frequency (x 10^{-7})	No. detected (% of total)	Frequency (x 10^{-7})
Transitions				
G·C to A·T	72 (26.1)	4.3	11 (14.5)	74
A·T to G·C	42 (15.2)	2.5	4 (5.3)	27
Total	114 (41.3)	6.8	15 (19.8)	101
Transversions				
G·C to T·A	91 (33.0)	5.5	26 (34.2)	175
G·C to C·G	55 (19.9)	3.3	16 (21.0)	108
A·T to C·G	5 (1.8)	0.3	3 (4.0)	20
A·T to T·A	11 (4.0)	0.6	16 (21.0)	108
Total	162 (58.7)	9.7	61 (80.2)	411

[a] Data for MKP-op are taken from Table 13.

[b] Including double substitutions.

[c] Treatment: 0 μg/ml dTMP, 12 h.

primarily to a decrease in the relative fractions of both types of transition (G·C to A·T: $P < 0.05$; A·T to G·C: $P < 0.025$) and an increase in the proportion of A·T to T·A transversions ($P < 0.001$) in SBT-TLp compared to MKP-op. The relative fractions of the other three transversions were similar for the two strains. Interestingly, the largest increases in mutation frequency following dTTP depletion were observed for A·T to T·A and A·T to C·G transversions (178- and 67-fold) consistent with T (the dNTP having the smallest pool) being replaced by A or G (the dNTPs having the largest pools).

The distributions of the substitutions are compared in Figure 19. No changes were detected 5′ or 3′ to the *SUP4*-o coding sequence and substitutions were recovered at only one site within the tRNA intron (position +51). Compared to MKP-op, the distribution of base-pair substitutions in SBT-TLp appeared quite different. All sites mutated in SBT-TLp were detected in MKP-op. However, only 30 of the 64 different sites recovered in MKP-op were mutated in SBT-TLp. Again, this observation has to be interpreted cautiously due to the small number of mutations analyzed for SBT-TLp. Despite the elevated dATP pool in SBT-TLp (120-fold), substitutions that could have involved a replacement with an A occurred at only 52% (31/60) of the positions where such changes can be detected on either strand in *SUP4*-o. In addition, changes were not recovered with equal frequency at all sites, rather there were hotspots and coldspots indicating that the mutations did not occur at random. Only one of the sites (+51) mutated most frequently in both distributions coincided. However, the relative proportions of the specific substitutions detected at this site differed considerably for the two distributions. The hypergeometric test developed by Adams and Skopek (1987) was used to

Figure 19. Distribution of *SUP4*-o Base-Pair Substitutions Induced by dTTP Depletion. For simplicity, only the coding region of the transcribed strand is shown. Double base substitutions are indicated by lower-case letters. The intron extends from +40 through +53. (A) MKP-op: spontaneous; (B) SBT-TLp: 0 μg/ml dTMP, 12 h.

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A

B

compare the distribution of substitutions induced in SBT-TLp to that for changes occurring spontaneously in MKP-op. The result indicated that the probability of random sampling error being responsible for differences in the two distributions was less than 1 in 333 (the upper limit of the 90% confidence interval on the estimate of P was 0.003).

4.4.5 Sequence Context and Strand Specificity of Base-Pair Substitutions Induced by dTTP Depletion

As outlined in the Introduction, dNTP imbalance might inhibit proof-reading by DNA polymerases *in vitro* by enhancing the rate of polymerization when the next correct nucleotide to be inserted is one present in excess. Clearly, one manifestation of this next-nucleotide effect *in vivo* would be a preference for mutations to occur at sites where the next nucleotide to be inserted is present in excess. To check for such a bias, the DNA sequence context of base substitutions induced by dTTP depletion was analyzed. For the purpose of the analysis, it was assumed that the majority of mutations induced by dTTP depletion might be due to mis-incorporation of dATP since the dATP pool was increased 120-fold by dTMP starvation and 70% of the resulting substitutions involved replacements with A·T pairs. There was no obvious preference for dATP misincorporation to occur at *SUP4*-o sites preceded or followed by a particular 5' or 3' base, respectively (Table 22). An even more detailed analysis of replacements did not reveal any preference for sites followed by an A (Table 23). The number of G·C to A·T transitions was too small to attach any statistical significance to the apparent bias associated with these events.

To determine whether the production of mutations by dTTP depletion

Table 22. Nearest Neighbours Flanking Sites of Base Substitutions Induced
by dTTP Depletion[a]

Sequence[b] 5′ to 3′	No. of available sites[c]	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site
A X	20	8	10	0.5
C X	31	13	24	0.8
T X	21	6	9	0.4
G X	23	7	10	0.4
X A	21	7	8	0.4
X C	27	6	18	0.7
X T	22	11	13	0.6
X G	25	10	14	0.6

[a] Misinserted base: A.

[b] X: site of misinsertion.

[c] Sites where A misinsertions can be detected in *SUP4*-o.

Table 23. Bases 3′ to Sites of Specific Substitutions Induced by dTTP
Depletion

Change	Sequence[a] 5′ to 3′	No. of available sites[b]	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site
C to A	X A	4	1	1	0.3
	X C/G/T	34	18	25	0.7
G to A	X A	12	6	7	0.6
	X C/G/T	23	4	4	0.2
T to A	X A	5	0	0	-
	X C/G/T	17	5	16	0.9

[a] X: site of misinsertion.

[b] Sites where the particular substitutions can be detected in *SUP4*-o.

featured a strand bias, the strand specificity of base-pair substitutions presumed to result from dATP misinsertion was analyzed (Table 24). No preference for such changes to occur on either strand of *SUP4*-o was detected, whether or not the next base incorporated 3′ to the misinsertion site was an A.

4.5 Mutational Specificity of Deoxycytidylate Deaminase Deficiency or Treatment with Excess dTMP

Elevated dCTP and dTTP pools caused by deficiency in the dCMP deaminase gene or by treatment with excess thymidine, respectively, have also been reported to be mutagenic in bacteriophage T4 and mammalian cells (Williams and Drake 1977; Weinberg *et al.* 1985; Aronow *et al.* 1984; Sargent and Mathews 1987). To probe the relationships between mutation induction and these dNTP pool imbalances, dNTP levels in a dCMP deaminase deficient strain or in SBT-TLp treated with excess dTMP were measured and *SUP4*-o mutations induced by these conditions were characterized by DNA sequencing.

4.5.1 Measurement of dNTP Pools in MGK-dp and SBT-TLp Treated with Excess dTMP

To determine whether dCMP deaminase deficiency and dTMP treatment led to dNTP imbalances in yeast, an enzymatic assay (Sargent and Mathews 1987) was used to determine the concentrations of all four DNA precursors in exponential phase cells of MGK-dp and SBT-TLp (measurements for the latter strain were made following two different treatments with dTMP). Compared to MKP-op, the dCTP concentration was markedly enhanced (76-fold) in MGK-dp and the dTTP pool was diminished by 40% resulting in a 127-fold increase in the dCTP:dTTP ratio (Table 25). In addition to alterations in

Table 24. Strand Specificity of Substitutions Induced by dTTP Depletion[a]

Sequence[b] 5′ to 3′	No. of available sites[c]		No. of sites detected		No. of mutants recovered		Average no. of mutants per available site	
	TS[d]	NTS[d]	TS	NTS	TS	NTS	TS	NTS
X A	11	10	3	4	3	5	0.3	0.5
X C/G/T	34	30	16	11	26	19	0.8	0.6

[a] Misinserted base: A.

[b] X: site of misinsertion.

[c] Sites where A misinsertions can be detected in *SUP4*-o.

[d] TS: transcribed strand; NTS: non-transcribed strand.

Table 25. Intracellular dNTP Concentrations for MKP-op, MGK-dp and SBT-TLp
Treated with Excess dTMP[a]

Strain	dATP	dTTP	dGTP	dCTP
MKP-op[b]	0.68 ± 0.04	1.55 ± 0.04	0.58 ± 0.02	0.61 ± 0.04
MGK-dp	1.19 ± 0.12	0.93 ± 0.08	0.24 ± 0.02	46.53 ± 2.76
SBT-TLp[c]	1.69 ± 0.16	16.94 ± 2.01	5.06 ± 1.13	0.51 ± 0.17
SBT-TLp[d]	2.86 ± 0.37	214.80 ± 27.52	13.58 ± 1.27	0.69 ± 0.20

[a] Concentrations are given in pmol/10^6 cells. Each value is the mean
± S.D. of three independent measurements.

[b] Data for MKP-op are taken from Table 16.

[c] Treatment: 250 μg/ml dTMP, 1.5 h.

[d] Treatment: 1 mg/ml dTMP, 6 h.

the concentrations of the pyrimidine dNTPs, a 75% increase in the dATP concentration and a 50% decrease in the level of dGTP in MGK-dp relative to MKP-op were noted.

Growth of SBT-TLp in medium containing excess dTMP led to increases in the dTTP, dGTP and dATP pools (Table 25) with the degree of increase depending on the severity of the treatment (11-, 9-, and 2.5-fold, respectively, after exposure to 250 μg/ml dTMP for 1.5 h; 139-, 23- and 4-fold, respectively, after treatment with 1 mg/ml dTMP for 6 h). The dCTP concentration was not altered by either treatment and so the dTTP:dCTP ratio was increased by as much as 122-fold over that for MKP-op (see Discussion for comments on these results).

4.5.2 Induction of *SUP4*-o Mutants by Elevated dCTP or dTTP Levels

Cultures of MGK-dp were grown to stationary phase from low titre inocula and plated to measure plasmid retention and to select canavanine-resistant colonies. Plasmid retention was not affected by the dCMP deaminase deficiency (Table 26) since 86% of the MGK-dp cells tested carried YCpMP2 and this value is typical for maintenance of the plasmid in MKP-op. However, disruption of the *DCD1* gene increased the *SUP4*-o mutation frequency by 2.7-fold relative to that for MKP-op ($P < 0.001$) (Table 27). This corresponded to a doubling of the *SUP4*-o mutation rate per round of DNA replication in the *dcd1* strain (9.5×10^{-7} vs. 4.7×10^{-7} for MKP-op). Prolonged treatment of growing SBT-TLp cells with dTMP concentrations above 250 μg/ml diminished plasmid retention which was reduced to 52% by 6 h exposure to 1 mg/ml dTMP (Table 26). dTMP treatment was also lethal and mutagenic with the magnitudes of these effects increasing with the severity of dTMP stress (Figure 20). For example, the mutation frequencies

Table 26. Effect of Elevated dCTP and dTTP Levels on Plasmid Retention

Strain	No. of colonies on non-selective medium	No. of colonies on selective medium	Plasmid retention (%)
MKP-op[a]	42,231	37,299	88
MGK-dp	12,787	11,027	86
SBT-TLp[b]	23,909	7,089	52[c]

[a] Data for MKP-op are taken from section 4.3.

[b] Treatment: 1 mg/ml dTMP, 6 h.

[c] Value has been normalized to time 0. Plasmid retention at time 0 = 57%.

Table 27. Effect of dCMP Deaminase Deficiency on Spontaneous Mutation

Strain	Colony types	No. examined[a] (% of total)		Frequency[a] $(\times 10^{-6})$
MKP-op[b]	Total[c]	31,923	(100.0)	65
	Red, Lys⁻	984	(3.1)	2.0
MGK-dp	Total	67,666	(100.0)	257
	Red, Lys⁻	1,446	(2.1)	5.4

[a] Colonies were isolated from, and frequencies are the means for, 45 (MKP-op) or 26 (MGK-dp) independent cultures.

[b] Data for MKP-op are taken from Table 11.

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

Figure 20. Cell Killing and *SUP4*-o Mutagenesis Induced by Treatment with Excess dTMP. For each dTMP concentration, the data points are the averages for 3 independent experiments. (A) surviving fraction; (B) induced mutation frequency. The mutation frequencies have been corrected for the spontaneous value. dTMP: (○) 250 μg/ml; (■) 500 μg/ml; (▲) 1 mg/ml.
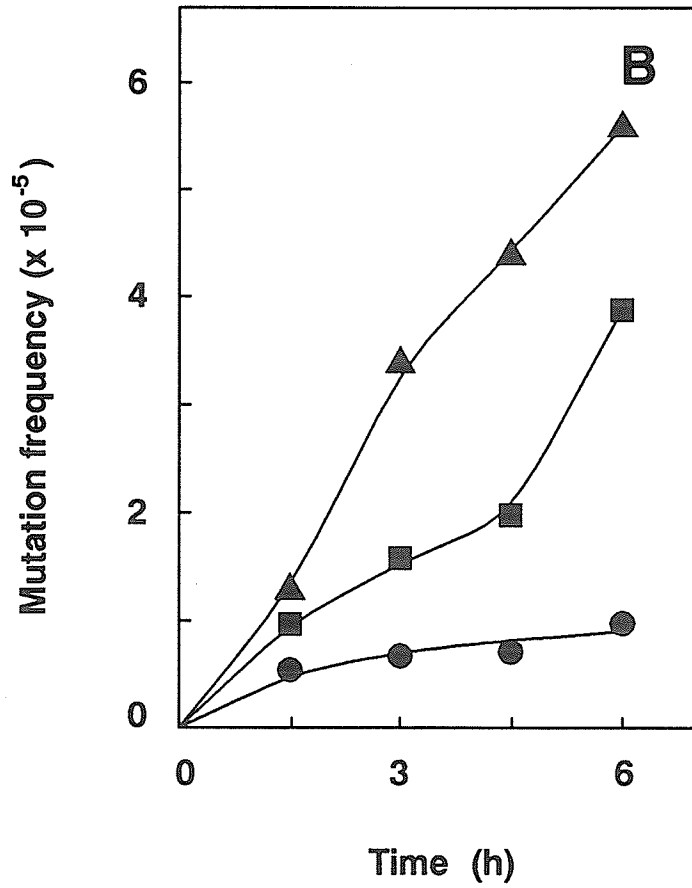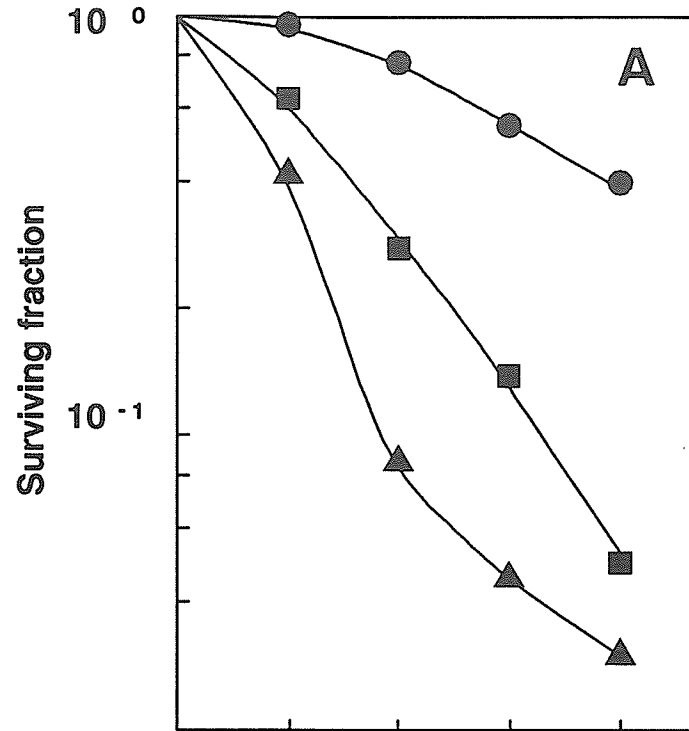
for 6 h treatment with 0.25, 0.5 or 1 mg/ml dTMP were 5-, 19- or 28-fold higher, respectively, than the spontaneous frequency for MKP-op. To fully appreciate the significance of these increases, it must be realized that the spontaneous frequency for MKP-op reflects mutation selection after growth for approximately 20 generations. In contrast, less than two rounds of DNA replication occurred during the 6 h exposure to excess dTMP. On this basis, and assuming that the mutations were randomly distributed among viable and non-viable cells, the rates of *SUP4*-o mutation per round of DNA replication during treatment with 0.25, 0.5 or 1 mg/ml dTMP for 6 h were calculated to be 8.5- (4.0×10^{-6}), 64- (3.0×10^{-5}) or 104-fold (4.9×10^{-5}) greater, respectively, than the corresponding rate for MKP-op (4.7×10^{-7}).

4.5.3 Characterization of *SUP4*-o Mutations Induced by Elevated dCTP or dTTP Levels

The mutational specificities of the dNTP imbalances were determined by characterizing 207 *SUP4*-o mutants arising in the *dcd1* strain and 200 mutants induced by excess dTMP (1 mg/ml dTMP for 6 h). Then the results were compared to those for 334 spontaneous mutants selected in MKP-op. For MGK-dp, each mutant was selected from a separate culture to ensure that the *SUP4*-o mutations characterized by DNA sequencing originated independently. For the same reason, only five mutants were selected from each culture of SBT-TLp treated with excess dTMP and any member of a group of five mutants that was found to have a sequence change identified in one other member of the same group was not considered further. Henceforth, in this section (4.5) reference to results obtained with SBT-TLp will mean SBT-TLp treated with 1 mg/ml dTMP for 6 h. Table 28

Table 28. Sequence Alterations in *SUP4*-o Mutants Arising in a *dcd1* Strain or Induced by Excess dTMP in SBT-TLp

Sequence alteration	MKP-op[a]			MGK-dp			SBT-TLp[b]		
	No. detected	(% of total)	Frequency (x 10^{-7})	No. detected	(% of total)	Frequency (x 10^{-7})	No. detected	(% of total)	Frequency (x 10^{-7})
Substitution									
Single change	272	(81.4)	16.4	183	(88.4)	47.7	175	(87.5)	490
Tandem double	-			-			1	(0.5)	2.8
Non-tandem double	2	(0.6)	0.1	-			-		
Deletion									
1 bp	22	(6.6)	1.3	19	(9.2)	5.0	20	(10.0)	56
>1 bp	7	(2.1)	0.4	-			1	(0.5)	2.8
Insertion									
1 bp	1	(0.3)	0.06	1	(0.5)	0.3	2	(1.0)	5.6
Ty element	26	(7.8)	1.6	1	(0.5)	0.3	-		
Duplication	1	(0.3)	0.06	-			-		
Complex change	3	(0.9)	0.2	3	(1.4)	0.7	1	(0.5)	2.8
Total	334		20.12	207		54.0	200		560

[a] Data for MKP-op are taken from Table 12.

[b] Treatment: 1 mg/ml dTMP, 6 h.

shows that fewer mutational classes were detected under conditions of dNTP imbalance with single base-pair events accounting for significantly more of the total mutations than in MKP-op (>98% vs. 89%, respectively, $P < 0.001$ in both cases). A detailed analysis of the substitutions and single base-pair deletions and insertions is presented below.

Three other classes of mutation were identified. A multiple base-pair deletion induced by excess dTMP eliminated the 183 base-pairs encompassing positions +76 in *SUP4*-o through -107 in YCpMP2 (see Figure 21 for correlation of position numbers with *SUP4*-o sites). There was no sequence homology at the deletion endpoints and so this event might have resulted from some form of illegitimate recombination. Although the fractions of mutations due to insertion of Ty elements were markedly decreased (>15-fold) in MGK-dp and SBT-TLp, one such event was found to have occurred immediately 5′ to position +38 in MGK-dp (this is the trans-position hotspot in MKP-op). Recovery of 5 Ty insertions in MGK-dp and only 1 after treatment with excess dTMP would have given transposition frequencies similar to that for MKP-op. Thus, it might be that the preferential increase in single base-pair events under conditions of dNTP imbalance effectively reduced the probability of detecting Ty insertions. The three complex changes characterized in MGK-dp each featured the same sequence replacement (5′-GATCTCA-3′ at +58 to +64 converted to 5′-CCGGG-3′) recovered twice in MKP-op. Furthermore, one complex change involving the complementary sequence replacement (5′-CCCGG-3′ at +82 to +86 converted to 5′-TGAGATC-3′) was detected after treatment with excess dTMP.

4.5.4 Analysis of Single Base-Pair Events Induced by Elevated dCTP or dTTP Levels

The majority (78%) of base-pair substitutions recovered in the dCMP deaminase deficient strain could have involved replacement with C compared to 37% of the substitutions detected in MKP-op (Table 29). Similarly, 87% of the substitutions induced by treatment with excess dTMP could have involved replacement with T relative to 63% for MKP-op. These differences were significant ($P < 0.001$ in both cases) and were consistent with the majority of substitutions occurring spontaneously in MGK-dp or induced by excess dTMP being due to misincorporation of the nucleotide in excess. Since the dATP and dGTP pools were also increased by treatment with excess dTMP, it is conceivable that all the substitutions recovered in SBT-TLp were promoted by dNTP imbalance.

Given the inverse relationship of the dCTP:dTTP ratios in MGK-dp and SBT-TLp, one might have expected a reduction in the proportion of G·C to A·T events with an associated increase in the proportion of A·T to G·C events in MGK-dp and vice versa for SBT-TLp. Although the fractions of G·C to A·T transitions in MGK-dp and A·T to G·C transitions in SBT-TLp decreased compared to the corresponding values for MKP-op ($P < 0.01$), the fractions of A·T to G·C transitions in MGK-dp and G·C to A·T transitions in SBT-TLp did not change significantly relative to those for MKP-op ($P > 0.30$ in both cases). Instead, the presumptive biases for replacement with C in MGK-dp and with T in SBT-TLp were primarily associated with 3-fold increases in the fractions of G·C to C·G and A·T to C·G transversions in MGK-dp ($P < 0.02$) and a 10-fold increase in the fraction of A·T to T·A transversions in SBT-TLp ($P < 0.01$) compared to MKP-op. For both strains, these features resulted in a significantly higher ratio of

Table 29. Base-Pair Substitutions Arising in a *dcd1* Strain or Induced by Excess dTMP in SBT-TLp

Substitution	MKP-op[ab]		MGK-dp		SBT-TLp[bc]	
	No. detected (% of total)	Frequency ($\times 10^{-7}$)	No. detected (% of total)	Frequency ($\times 10^{-7}$)	No. detected (% of total)	Frequency ($\times 10^{-7}$)
Transitions						
G·C to A·T	72 (26.1)	4.3	24 (13.1)	6.2	53 (29.9)	148
A·T to G·C	42 (15.2)	2.5	25 (13.7)	6.5	3 (1.7)	8
Total	114 (41.3)	6.8	49 (26.8)	12.7	56 (31.6)	156
Transversions						
G·C to T·A	91 (33.0)	5.5	10 (5.5)	2.6	26 (14.7)	72
G·C to C·G	55 (19.9)	3.3	107 (58.4)	27.9	21 (11.9)	59
A·T to C·G	5 (1.8)	0.3	10 (5.5)	2.6	-	
A·T to T·A	11 (4.0)	0.6	7 (3.8)	1.9	74 (41.8)	206
Total	162 (58.7)	9.7	134 (73.2)	35.0	121 (68.4)	337

[a] Data for MKP-op are taken from Table 13.

[b] Including double substitutions.

[c] Treatment: 1 mg/ml dTMP, 6 h.

transversions to transitions than for MKP-op ($P < 0.05$).

The distributions of the substitutions that occurred in the three strains, including double events, are shown in Figure 21. No substitutions were detected 5' or 3' to the *SUP4*-o coding sequence and changes were recovered only at one site within the tRNA intron (position +51). The three distributions appeared to be quite different. Altogether, 67 distinct sites were mutated but substitutions occurred at markedly fewer sites in MGK-dp (47) and SBT-TLp (34) than in MKP-op (64). This did not simply reflect a preference for replacement with C in the *dcd1* strain, or T after treatment with excess dTMP, because such substitutions occurred at only 67% (44/66) or 52% (31/60) of the positions, respectively, where they can be detected on either strand in *SUP4*-o. The presence of hotspots and coldspots indicated that the mutations did not occur at random. There was no overlap in the sites that were mutated most frequently in MGK-dp (+64, +79, +89) and MKP-op (+6, +18, +51, +83, +84, +88). Although three of the sites mutated most often in SBT-TLp (+2, +6, +55, +83, +84) and MKP-op coincided, the relative proportions of the specific substitutions at these sites varied considerably between the two strains. The hypergeometric test (Adams and Skopek 1987) was used to statistically compare the distributions of substitutions occurring in MGK-dp and SBT-TLp to each other, and to that for MKP-op. For all three comparisons, the probability of random sampling error being responsible for differences in the distributions was less than 1 in 500 (the upper limit of the 90% confidence interval on each estimate of P was 0.002).

Single base-pair deletions and insertions accounted for approximately 10% of the total mutations characterized in MGK-dp and SBT-TLp (Table 28). The majority of deletions in MGK-dp (18/19) and SBT-TLp (18/20) and one

Figure 21. Distribution of Base-Pair Substitutions Arising in a *dcd1* Strain or Induced by Excess dTMP. For simplicity, only the coding region of the transcribed strand is shown above each distribution. Double base substitutions are indicated by lowercase letters. The intron extends from +40 through +53. (A) MKP-op; (B) MGK-dp; (C) SBT-TLp treated with 1 mg/ml dTMP for 6 h.

A

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

        AGA AAAGCt  Aa AA AA    CCAGAACG              A CTAGCAC   TC CAGACA   AC CAGCC   TTAAGAAAA
        AT AAAG T   C  AA AT    CAGCAC                A C A A    C CGTACG    AC A CT    AAGG AA
        A AAA       A   A       CAGTAC     GCACC G    A C A A    C  G GTT    G A        AAGG TA
        A CAT       C   A       TT  C      G GC       A C A T    C  C GTT    G C C      CA G TA
        C TA        C   A       T C        G          A C A T    C  T        G C C      CA G TC
        T A         C   A       T A        G          A C A GT T    T        G C        CG T TC
        T A         A   A       T C        A          A T        T           CG T       CG T TC
        T G         G   A                  A          A                                 CG T
        T G         G   A                  C          A                                 TT T
        T T         T                                 AAA                               TT T
        T T         T                                                                   TT T
                    AAAAAA                                                              TTTTTTTT  TTTT
                    AAAAGTT
```

B

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

        AGC AGGGCG  CC C  A      GA C       GCACC G    A         AGGG       C AA GC C   GCG CA CC   CCAGG GA
        CGC AGGG    C  G         GA C       G GC       A         C G        C CA GT C   GCG A CC    CGGG GA
        CGC CGT     C  G         GA C       G G        A         C G        C CG G G    GCG C C     CGGG GA
                    C  C         A C        G          A         C G        C CT G C    G G T C     G T A
                    C            A                               C          C CT G C    G G         G T
                                 A                               C          C CT G G    G           T T A
                                 A                               C          C           G           T A
                                                                            C                       T C
                                                                            C                       C
                                                                            C                       C
                                                                            C                       C
                                                                                        CCCC        C
```

C

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

        AT AAT  A  C  AA AA       AA        T AG  A    A         TTG        AT  AT      A  T        AAGTAaa
        AT AA   A  T    A AA      AT        AT A       T         TT G       TT          A  G        CA TAAA
        TT AT           A  T      TT        AT         T         T                      G           CG AAA
        TT T                      T         AT         T         T                                  CG ATA
        T  T                      T         AT         T                                            CT TA
        TT T                                AT         T                                            CT
        TTTTTT  T                           AT                                                      CT
        TTTTTT T                                                                                    CT
        TTTTTT T                                                                                    CT
        TTTTTT T                                                                                    CT
        TTTTTT T
        TTTTTT T                                                           TTTTTTT     TCCCCCCC
```

insertion in each strain were detected within the run of 5 G·C pairs at +79 through +83. Another insertion occurred after treatment with excess dTMP in the run of 3 G·C pairs at +65 through +67. The remaining deletions involved the loss of G·C pairs at site +64 (MGK-dp) and site +6 or +7 (SBT-TLp) and the loss of an A·T pair at position +89 (SBT-TLp). Thus, the formation of most of these events could have involved slipped mispairing of template and nascent strands during replication through the runs (Streisinger *et al.* 1966), a process independent of nucleotide misinsertion (but see Discussion).

4.5.5 Sequence Context and Strand Specificity of Base-Pair Substitutions Induced by Elevated dCTP or dTTP Levels

As emphasized above, mutations induced by dNTP imbalance in eukaryotic cells might be due, in part, to inhibition of proofreading by a next-nucleotide effect. An indication of such inhibition would be a preference for mutations to occur at sites where the next nucleotide to be inserted is the one present in excess. To probe for such a bias, the sequence context of substitutions recovered in MGK-dp and SBT-TLp was examined. There was no obvious preference for dCTP misincorporation in MGK-dp, or dTTP misinsertion in SBT-TLp, to occur at *SUP4*-o sites preceded by a particular 5′ base (Table 30). The same appeared to be true with regard to the 3′ base for dCTP misinsertion. Yet, Table 31 shows that T to C transitions, and possibly A to C transversions, were 5-fold more likely to occur at sites flanked by a 3′ C (5′-XC-3′ where X is the site of mis-insertion) than at sites followed by A, G or T (T to C: $P < 0.02$; for the transversion, the small number of events involved makes the statistical significance uncertain). This bias was not observed for G to C trans-

Table 30. Bases Flanking Sites of Substitutions Occurring in a *dcd1* Strain or Induced by Excess dTMP in SBT-TLp

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Sequence[b] 5' to 3'	MGK-dp (misinserted base: C)				SBT-TLp (misinserted base: T)[a]			
	No. of available sites[c]	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site	No. of available sites[c]	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site
A X	21	11	39	1.9	22	14	34	1.5
C X	23	15	41	1.8	25	12	30	1.2
T X	14	7	16	1.1	21	7	17	0.8
G X	22	13	46	2.1	27	9	72/35[d]	2.7/1.3[d]
X A	22	15	53	2.4	21	5	7	0.3
X C	19	13	57	3.0	23	6	10	0.4
X T	20	6	8	0.4	20	14	47	2.4
X G	19	12	24	1.3	31	17	89/52[d]	2.9/1.7[d]

[a] Treatment: 1 mg/ml dTMP, 6 h.
[b] X: site of misinsertion.
[c] Sites where misinsertion of the particular base can be detected in *SUP4*-o.
[d] Data for A to T hotspot at position +2 included/excluded.

Table 31. Analysis of Bases 3′ to Sites of Substitutions Occurring in a
dcd1 Strain or Induced by Excess dTMP in SBT-TLp

Strain	Change	Sequence[a] 5′-> 3′	No. of available sites[b]	No. of sites detected	No. of mutants recovered	Average no. of mutants per site
MGK-dp	T to C	X C	7	5	17	2.4
		X A/G/T	16	6	8	0.5
	A to C	X C	3	1	5	1.7
		X A/G/T	15	4	5	0.3
	G to C	X C	9	7	35	3.9
		X A/G/T	30	22	72	2.4
SBT-TLp[c]	C to T	X T/G	23	15	47	2.0
		X A/C	12	3	7	0.6
	A to T	X T/G	11	7	73/36[d]	6.6/3.3[d]
		X A/C	11	1	1	0.1
	G to T	X T/G	17	9	17	1.0
		X A/C	21	8	9	0.4

[a] X: site of misinsertion.

[b] Sites where the particular substitutions can be detected in *SUP4*-o.

[c] Treatment: 1 mg/ml dTMP, 6 h.

[d] Data for A to T hotspot at position +2 included/excluded.

versions which explains the apparent lack of preference for 5'-XC-3' sites in Table 30 because G to C changes constituted 75% of the substitutions considered there.

Mutations potentially due to misincorporation of dTTP were, on average, at least 7-fold more likely to occur at sites immediately followed by thymine or guanine, than by adenine or cytosine ($P < 0.001$ for each comparison) and 95% (136/143) of the substitutions were at the former sites (Table 30). If the hotspot for dTTP misinsertion at the second position in *SUP4*-o is excluded from this comparison, the preference for 5'-XT/G-3' sites is still 4- to 5-fold greater than for 5'-XC-3' or 5'-XA-3' sites ($P < 0.005$ in both cases). A more detailed analysis revealed that C to T and A to T events were, respectively, 3-fold and at least 33-fold more likely to occur at sites followed by T or G than by A or C (Table 29, C to T: $P < 0.02$; A to T: $P < 0.001$). G to T events also were recovered 2-fold more often at 5'-XT/G-3' sites but this preference was not significant ($P > 0.05$). It can be determined from Figure 18 that most (22/24) of the substitutions that could have resulted from misinsertion of dGTP occurred at 5'-XG-3' sites. The majority of these changes (16/22), however, were at a single position (+83).

The distributions of the substitutions presumed to result from misinsertion of dCTP or dTTP were also examined to determine whether mutation induction by dNTP excess featured a strand bias. Potential dCTP misinsertion events did not exhibit a preference for either strand of *SUP4*-o whether or not the next base incorporated 3' to the misinsertion site was C (Table 32, $P > 0.20$ or 0.50, respectively). On the other hand, it appeared that dTTP misincorporation was 2.5-fold more likely to occur on the transcribed strand when dTTP or dGTP was the next correct nucleo-

Table 32. Strand Specificity of Substitutions Occurring in a *dcd1* Strain or Induced by Excess dTMP in SBT-TLp

Strain	Misinserted base	Next base inserted	No. of available sites[a]		No. of sites detected		No. of mutants recovered		Average no. of mutants per available site	
			TS[b]	NTS[b]	TS	NTS	TS	NTS	TS	NTS
MGK-dp	C	C	12	7	8	5	41	16	3.4	2.3
		A/T/G	27	34	16	17	43	42	1.6	1.2
SBT-TLp[c]	T	T/G	24	27	15	16	95/58[d]	41	4.0/2.4[d]	1.5
		A/C	26	18	6	5	10	7	0.4	0.4

[a] Sites where misinsertion of the particular base can be detected in *SUP4*-o.

[b] TS: transcribed strand; NTS: non-transcribed strand.

[c] Treatment: 1 mg/ml dTMP, 6 h.

[d] Data for A to T hotspot at position +2 included/excluded.

tide to be inserted. When the A to T transversion hotspot at position +2 was omitted from this comparison, there also was no significant strand preference for dTTP misinsertion ($P > 0.10$).

4.6 Influence of Elevated dCTP Levels on EMS and MNNG Mutagenesis

O^6-alkylguanine is thought to be major the premutational lesion for EMS- and MNNG-induced changes. It is believed to mispair with thymidine during DNA synthesis *in vivo* leading to an increase in G·C to A·T transitions (Abbott and Saffhill 1979; Dodson *et al.* 1982; Eadie *et al.* 1984; Loechler *et al.* 1984; Burns *et al.* 1986, 1987; Singer 1986). Elevated dCTP levels have been found to reduce EMS-induced cell killing and mutagenesis (Meuth 1981b), possibly through competition with dTTP for pairing opposite O^6-alkylguanine. To investigate whether such competition can offset the mutagenic effects of alkylating agents, the wild-type strain MKP-op and MGK-dp, which has an elevated dCTP pool, were treated separately with EMS and MNNG and the resulting mutants were analyzed by DNA sequencing.

4.6.1 EMS and MNNG Mutagenesis in MKP-op

To be able to evaluate the influence of elevated dCTP pools on EMS and MNNG mutagenesis, it was first necessary to determine the mutational specificity of EMS and MNNG in MKP-op, a strain with normal dNTP levels.

4.6.1.1 Effect of EMS or MNNG Treatment on Intracellular dNTP Pools in MKP-op

Earlier studies indicated that treatment with alkylating agents could affect the balance of dNTP pools (Das *et al.* 1983). To determine if the treatment conditions employed in this study for alkylation mutagenesis cause dNTP pool alterations, the concentrations of all four dNTPs were

measured in MKP-op after exposure to EMS or MNNG. Extracts for dNTP measurements were prepared after treating cells in buffer with an alkylating agent for 45 min and then incubating the treated cells in YPD medium for 1 h. During this period, no cell growth occurred. Control values were obtained for these treatment conditions by replacing the alkylating agent with an equivalent volume of water. Following this mock treatment, the concentrations of all four DNA precursors were reduced by 55% to 72% when compared to values obtained for exponentially growing cells (Table 33). However, the dNTP pools still retained the same relationships to each other with dTTP having the highest levels. The dTTP:dATP:dCTP:dGTP ratio for exponentially growing cells was 1:0.44:0.39:0.37 while the ratio calculated for the mock-treated cells was 1:0.34:0.32:0.23. In comparison to mock-treated cells, cells exposed to EMS or MNNG had increased dNTP levels (2- to 6-fold) with the increases being slightly greater for MNNG treatment.

4.6.1.2 Effects of EMS and MNNG on Viability, Plasmid Retention and Mutation in MKP-op

To determine the effects of EMS and MNNG on cell viability, retention of the plasmid YCpMP2 and the induction of SUP4-o mutants in MKP-op, cells were exposed to increasing concentrations of either one of the alkylating agents. Cell killing was similar for each agent over the dose ranges used, but the frequencies and yields of induced SUP4-o mutants were greater for MNNG than EMS (Figure 22). Neither treatment had any effect on plasmid retention (Table 34). Even for the highest doses used in this study (3% for EMS, 90 μg/ml for MNNG) at least 87% of the cells plated to select canavanine-resistant colonies retained YCpMP2. To minimize the contribu-

Table 33. Intracellular dNTP Concentrations after Alkylation Treatment of
MKP-op[a]

Strain	dATP	dTTP	dGTP	dCTP
MKP-op[b]	0.68 ± 0.04	1.55 ± 0.04	0.58 ± 0.02	0.61 ± 0.04
MKP-op[c]	0.23 ± 0.07	0.68 ± 0.17	0.16 ± 0.04	0.22 ± 0.03
MKP-op + EMS[d]	0.77 ± 0.23	1.83 ± 0.64	0.48 ± 0.17	0.48 ± 0.09
MKP-op + MNNG[e]	1.32 ± 0.38	2.74 ± 0.62	0.74 ± 0.24	0.70 ± 0.15

[a] Concentrations are given in $pmol/10^6$ cells. Each value is the mean ± S.D. of three independent measurements.

[b] Data for MKP-op (exponential phase) are taken from Table 16.

[c] Mock-treated cells.

[d] Treatment: 1% EMS.

[e] Treatment: 30 μg/ml MNNG.

Figure 22. EMS- and MNNG-Induced Cell Killing and *SUP4*-o Mutagenesis. For
each agent, the data points are the means for four independent
experiments. (A) surviving fraction; (B) induced mutation
frequency; (C) induced mutation yield. The mutation frequencies
and yields have been corrected for spontaneous values. (•) EMS;
(▪) MNNG.
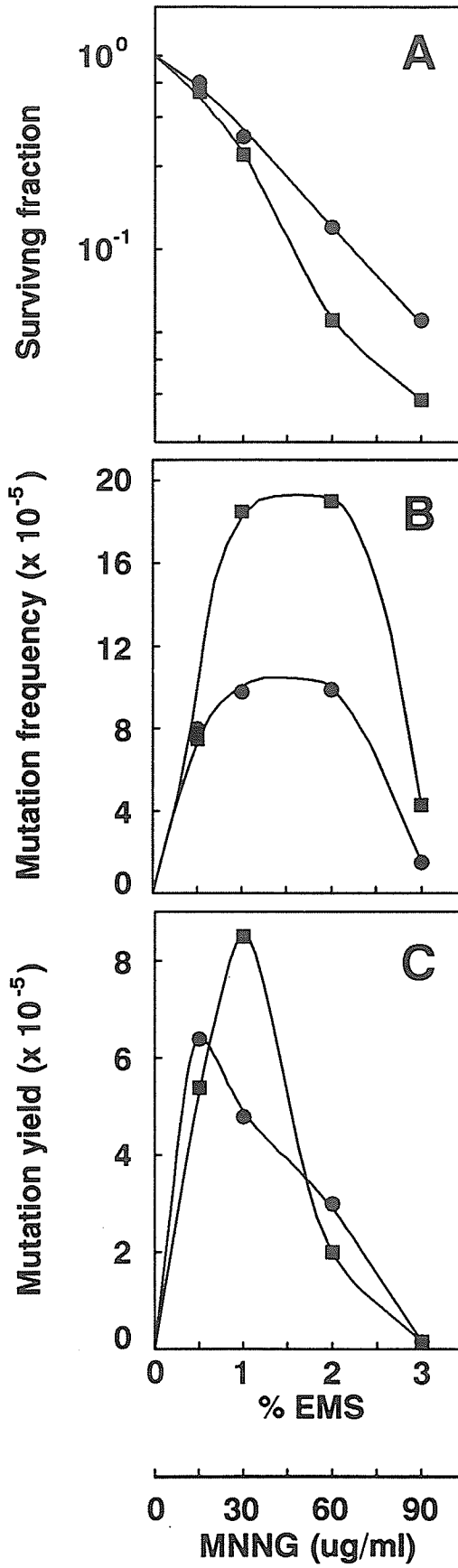
Table 34. Effect of EMS and MNNG Treatment on Plasmid Retention in MKP-op

Strain	No. of colonies on non-selective medium	No. of colonies on selective medium	Plasmid retention (%)
Control[a]	42,231	37,299	88
EMS (1%)	5,831	5,101	87
EMS (3%)	4,852	4,198	87
MNNG (30 μg/ml)	6,994	6,244	89
MNNG (90 μg/ml)	6,612	6,012	90

[a] Spontaneous data for MKP-op are taken from section 4.3.

tion of spontaneous events, *SUP4*-o mutants were selected for sequence analysis following doses of EMS (1%) or MNNG (30 μg/ml) that increased the *SUP4*-o mutation frequency by 33- or 60-fold, respectively. At these doses, both agents reduced cell survival to approximately 50% while the induced mutation frequencies and yields were at, or near, the maximum values (Figure 22). Characterization of the EMS- and MNNG-induced canavanine-resistant colonies revealed that for each agent the fraction of *SUP4*-o mutants (red, Lys⁻ isolates) was approximately 9% (Table 35). Thus, the fraction of *SUP4*-o mutants induced by EMS or MNNG was about 3-fold higher than the corresponding spontaneous value (*P* < 0.001 for both agents).

4.6.1.3 Characterization of EMS- and MNNG-Induced Mutants in MKP-op

A collection of 152 EMS-induced and 166 MNNG-induced mutations in the *SUP4*-o gene was analyzed by DNA sequencing. In both cases, exclusively base-pair substitutions were recovered and in each case G·C to A·T transitions accounted for over 96% of the mutations detected (Table 36). This contrasts markedly with the spectrum of spontaneous *SUP4*-o mutations where only 23% of the total base-pair substitutions were G·C to A·T transitions and where transversions outnumbered transitions by 50% (Table 13). The remaining mutations detected after EMS or MNNG treatment were A·T to G·C transitions and G·C to C·G and G·C to T·A transversions. In addition, two double base-pair changes were recovered after treatment with EMS, each involving a G·C to A·T transition (at +7 and +65, respectively) with a G·C to T·A (+11) or a G·C to C·G (+67) transversion at a second nearby G·C site.

The distributions of the EMS- and MNNG-induced mutations within the *SUP4*-o gene are compared in Figure 23 and summarized in Table 37. For both

Table 35. EMS- or MNNG-Induced Canavanine-Resistant Colonies in MKP-op

Treatment	Colony types	No. examined[a] (% of total)		Frequency[a] $(x\ 10^{-4})$
Control[b]	Total[c]	31,923	(100)	0.65
	Red, Lys⁻	984	(3)	0.02
EMS (1%)	Total	7,429	(100)	10.9
	Red, Lys⁻	674	(9)	1.0
MNNG (30 μg/ml)	Total	10,804	(100)	20.5
	Red, Lys⁻	976	(9)	1.8

[a] Colonies were isolated from, and frequencies are the means for, 45 (spontaneous), 6 (EMS) or 4 (MNNG) independent cultures.

[b] Spontaneous data for MKP-op are taken from Table 11.

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

Table 36. EMS- or MNNG-Induced Base-Pair Substitutions in MKP-op

Substitution	EMS[a] (1%)		MNNG (30 μg/ml)	
	No. detected (% of total)	Frequency (x 10^{-7})	No. detected (% of total)	Frequency (x 10^{-7})
Transitions				
G·C to A·T	150 (97.4)	97.4	160 (96.4)	173.4
A·T to G·C	1 (0.6)	0.6	2 (1.2)	2.2
Total	151 (98.0)	98.0	162 (97.6)	175.6
Transversion				
G·C to T·A	1 (0.6)	0.6	2 (1.2)	2.2
G·C to C·G	2 (1.4)	1.4	2 (1.2)	2.2
A·T to C·G	-		-	
A·T to T·A	-		-	
Total	3 (2.0)	2.0	4 (2.4)	4.4

[a] Including double substitutions.

Figure 23. Distribution of *SUP4*-o Base-Pair Substitutions Induced by EMS or **MNNG** in MKP-op. For simplicity, only the region of the strand encoding the tRNA is shown. Double base substitutions are indicated by lower-case letters. The intron extends from +40 through +53. (A) EMS (1%); (B) MNNG (30 μg/ml).

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A

B

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

Table 37. G·C to A·T Transitions Induced in MKP-op by EMS or MNNG

Position	Sequence[a] 5′ to 3′	EMS (1%)		MNNG (30 μg/ml)	
		No. detected[b]	Frequency (x 10^{-6})	No. detected[b]	Frequency (x 10^{-6})
3	AGA	10	6.5	1	1.1
32	AGA	4	2.6	13	14.3
56	AGA	11	7.2	10	11.0
61	AGA	1	0.65	0	-
88	AGA	4	2.6	4	4.4
10	AGC	0	-	0	-
25	AGG	3	1.9	5	5.5
1	AGT	15	9.8	53	58.5
15	AGT	1	0.65	1	1.1
34	AGT	15	9.8	15	16.6
86	GGA	5	3.2	6	6.6
11	GGC	4	2.6	3	3.3
26	GGC	3	1.9	13	14.3
67	GGC	2	1.3	9	9.9
79	GGC	1	0.65	3	3.3
66	GGG	10	6.5	7	7.7
7	GGT	8	5.2	3	3.3
19	GGT	2	1.3	1	1.1
5	CGA	10	6.6	2	2.2
51	CGA	5	3.2	2	2.2
64	CGA	1	0.65	0	-
72	CGA	0	-	0	-
77	CGA	7	4.5	1	1.1
27	CGC	10	6.5	3	3.3
78	CGC	0	-	1	1.1
6	CGG	1	0.65	2	2.2
65	CGG	4	2.6	0	-
83	CGG	3	1.9	0	-
84	CGG	0	-	0	-
69	CGT	4	2.6	0	-
59	TGA	2	1.3	0	-
18	TGG	4	2.6	2	2.2

[a] In each trinucleotide, the central G is the mutated base.

[b] G·C to A·T events can be detected at all the sites listed (see Table 3).

agents, base-pair substitutions occurred throughout the gene, with the exception of the tRNA intron where mutations were recovered only at one position (+51). Furthermore, there was considerable overlap in the sites that were mutated by both agents. With regard to G·C to A·T transitions, EMS-induced events occurred at 22 of the 23 G·C sites detected for MNNG. In addition, mutation frequencies were similar at a number of sites (+7, +11, +15, +18, +19, +27, +51, +56, +86, +88), many sites were mutated often by EMS and MNNG and the two sites most frequently mutated (+1, +34) were the same for both agents. EMS missed 7 (+10, +12, +68, +72, +78, +80, +84) of the 32 G·C sites where transitions can be detected and MNNG missed 6 of the same sites. However, some differences were noted. EMS mutated 6 sites (+59, +61, +64, +65, +69, +83) that were not targets for MNNG and MNNG mutated 1 site (+78) not found for EMS. A pronounced hotspot was found only for MNNG (position +1) and at several sites mutated by each agent (+1, +3, +26, +32, +34, +67), the mutation frequencies differed considerably for EMS and MNNG (Table 37). By using the hypergeometric test of Adams and Skopek (1987) to statistically compare the two distributions to each other, it was established that the probability of random sampling error being responsible for any differences was less than 1 in 500 (the upper limit of the 90% confidence interval on the estimate of P was 0.002).

4.6.1.4 Sequence Context and Strand Specificity of Substitutions Induced by EMS or MNNG in MKP-op

Earlier studies of *E. coli* and bacteriophage λ indicated that mutations induced by MNNG occurred more frequently at sites where the guanine of a G·C pair was preceded (5′) by a guanine or where the guanine

was located on the non-transcribed strand (Burns *et al.* 1987; Reed and Hutchinson 1987; Richardson *et al.* 1987a). The presence of hotspots and coldspots in the distributions of EMS- or MNNG-induced base-pair substitutions in *SUP4*-o indicated that these mutations did not occur at random. Of the 32 sites where G·C to A·T transitions can be detected in *SUP4*-o, 20 have the guanine preceded (5') by a purine (5'Pur-G) and the remainder by a pyrimidine (5'Pyr-G) (Table 38). Interestingly, EMS- and MNNG-induced mutations were recovered at the same 16 5'Pur-G sites although an additional site was found after EMS mutagenesis. Despite this similarity, EMS and MNNG mutagenesis differ with regard to the frequencies at which mutations were recovered at 5'Pur-G or 5'Pyr-G sites (Table 38). Sites having a guanine preceded by an adenine or a guanine were 11- or 6-fold more likely to be mutated by MNNG, respectively, than if preceded by a pyrimidine (Table 38). If the MNNG hotspot at position +1 is excluded from this comparison, the preference for 5'A/G sites is still 6-fold greater than for 5'Pyr-G sites. All of these differences are highly significant ($P < 0.001$). On the other hand, EMS mutagenesis exhibited no significant preference for guanines flanked by a 5' purine. The base 3' to the guanine had no influence on EMS mutagenesis or MNNG mutagenesis when the hotspot at position +1 was excluded from the analysis (Table 39).

From Table 40, it appears that both EMS and MNNG mutagenesis were 2-fold more likely to occur at sites having the guanine on the transcribed strand. However, if the MNNG hotspot at position +1 is omitted from this analysis, then MNNG mutagenesis exhibits no preference for either strand. Moreover, even with the hotspot data included for MNNG, the apparent strand preferences are not significant for either agent ($P > 0.05$).

Table 38. Influence of the 5' Base on EMS- or MNNG-Induced G·C to A·T Transitions in MKP-op

5' Base	No. of available sites	EMS (1%)			MNNG (30 µg/ml)		
		No. of sites detected	No. of mutants recovered	Average no. of mutants per site available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available
A	10	9	64	6.4	8	102/49[a]	10.2/4.9[a]
G	8	8	35	4.4	8	45	5.6
Pur	18	17	99	5.5	16	147/94[a]	8.2/5.2[a]
C	12	9	45	3.7	6	11	0.9
T	2	2	6	3.0	1	2	1.0
Pyr	14	11	51	3.6	7	13	0.9

[a] Data for MNNG hotspot at position +1 included/excluded.

Table 39. Influence of the 3' Base on EMS- or MNNG-Induced G·C to A·T Transitions in MKP-op

3' Base	EMS (1%)				MNNG (30 µg/ml)		
	No. of sites available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available
A	12	11	60	5.0	8	39	3.2
G	7	6	25	3.6	4	16	2.3
Pur	19	17	85	4.5	12	55	2.9
C	7	5	20	2.9	6	32	4.6
T	6	6	45	7.5	5	73/20[a]	12.1/3.3[a]
Pyr	13	11	65	5.0	11	105/52[a]	8.1/4.0[a]

[a] Data for MNNG hotspot at position +1 included/excluded.

Table 40. Strand Specificity of EMS- or MNNG-Induced G·C to A·T Transitions in MKP-op

Strand	EMS (1%)				MNNG (30 µg/ml)		
	No. of available sites	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available
TS[a]	12	10	80	6.7	8	90/37[b]	7.5/3.1[b]
NTS[a]	20	18	70	3.5	15	70	3.5

[a] TS: transcribed strand; NTS: non-transcribed strand.

[b] Data for MNNG hotspot at position +1 included/excluded.

4.6.2 EMS and MNNG Mutagenesis in MGK-dp

To determine the influence of elevated dCTP on EMS and MNNG mutagenesis, cells of MGK-dp were treated with each alkylating agent and their effects on dNTP pool levels, cell survival, plasmid retention and mutation induction were examined.

4.6.2.1 Intracellular dNTP Levels in EMS- or MNNG-Treated MGK-dp

As observed previously for MKP-op (Table 33), the experimental conditions used for alkylation treatment caused about a 3.0-fold reduction in the dATP, dTTP and dCTP concentrations in MGK-dp (Table 41). The dGTP concentration dropped below the level which can be reliably detected with the dNTP pool assay (the standard deviation was greater than the value calculated for the concentration of dGTP). The relative proportions of the different dNTPs in mock-treated cells (dCTP:dATP:dTTP = 1:0.023:0.015) were quite similar to those observed for exponentially growing MGK-dp (1:0.025:0.020). After alkylation treatment, dATP and dTTP levels were about 2-fold higher than in the mock-treated cells. The dCTP levels were slightly less than in the mock-treated cells but were still much greater (EMS: 30-fold; MNNG: 18-fold) than the corresponding pools in MKP-op treated with the alkylating agents (compare Tables 33 and 41).

4.6.2.2 Effects of Elevated dCTP Levels on Viability, Plasmid Retention and Mutation in EMS- or MNNG-Treated Cells of MGK-dp

Table 42 compares the effects of elevated dCTP levels on the toxicity and mutagenicity of EMS and MNNG. Cells (MGK-dp) having elevated dCTP pools survived EMS treatment better than cells (MKP-op) with 'normal' dCTP levels (84% vs. 50%, respectively; $P < 0.001$). In contrast, MNNG-induced cell killing was slightly more severe for MGK-dp than MKP-op cells (62%

Table 41. Intracellular dNTP Concentrations after Alkylation Treatment of MGK-dp[a]

Strain	dATP	dTTP	dGTP	dCTP
MGK-dp[b]	1.19 ± 0.12	0.93 ± 0.08	0.24 ± 0.02	46.53 ± 2.76
MGK-dp[c]	0.39 ± 0.10	0.26 ± 0.13	BDL[d]	16.94 ± 1.00
MGK-dp + EMS[e]	0.59 ± 0.29	0.50 ± 0.27	0.11 ± 0.06	14.33 ± 3.40
MGK-dp + MNNG[f]	0.73 ± 0.17	0.65 ± 0.11	0.27 ± 0.10	12.85 ± 1.62

[a] Concentrations are given in pmol/10^6 cells. Each value is the mean ± S.D. of three independent measurements.

[b] Data for MGK-dp (exponential phase) are taken from Table 25.

[c] Mock-treated cells.

[d] Below detection level.

[e] Treatment: 1% EMS.

[f] Treatment: 30 μg/ml MNNG.

Table 42. Effect of Elevated dCTP Levels on Survival and Mutant Recovery Following EMS or MNNG Treatment

Treatment	Strain	Surviving fraction	Colony types	No. examined[a] (% of total)		Frequency[a] (x 10^{-4})
EMS (1%)	MKP-op[b]	0.51	Total[c]	7,429	(100)	10.9
			Red, Lys⁻	674	(9)	1.0
	MGK-dp	0.84	Total	3,673	(100)	4.6
			Red, Lys⁻	416	(11)	0.5
MNNG (30 μg/ml)	MKP-op[b]	0.44	Total	10,804	(100)	20.5
			Red, Lys⁻	976	(9)	1.8
	MGK-dp	0.38	Total	4,323	(100)	12.6
			Red. Lys⁻	343	(8)	1.0

[a] Colonies were isolated from, and frequencies are the means for, 8 (EMS: MKP-op) or 6 (MNNG: MKP-op and MGK-dp, EMS: MGK-dp) independent cultures.

[b] Data for MKP-op are taken from Table 35 and Figure 22.

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

vs. 56%, respectively; $P < 0.001$). Although the relative fractions of
SUP4-o mutations were unaffected, the frequencies of the Can^R and *SUP4*-o
mutations were both reduced by about 60% for EMS treatment and 40% for
MNNG treatment of MGK-dp compared to MKP-op ($P < 0.001$ for treatments with
either alkylating agent). Thus, elevated dCTP levels decreased the
mutagenicity of both EMS and MNNG, but the magnitude of the effect was
smaller for the latter agent. Elevated dCTP levels did not influence
plasmid retention in EMS- or MNNG-treated cells (Table 43).

4.6.2.3 Characterization of *SUP4*-o Mutants Induced by EMS or MNNG in MGK-dp

To evaluate the influence of elevated dCTP levels on the types of
sequence alteration induced by alkylating agents, *SUP4*-o mutants induced
by EMS (203) or MNNG (202) in MGK-dp were analyzed. The resulting spectra
were then compared to those for *SUP4*-o mutants induced by EMS or MNNG in
MKP-op or occurring spontaneously in MGK-dp (Tables 44 and 45). As for
MKP-op, the majority (>98%) of EMS- and MNNG-induced sequence alterations
recovered in MGK-dp were base-pair substitutions. One tandem (EMS) and one
non-tandem (MNNG) double base-pair change were also recovered, each
involving two G·C to C·G transversions (EMS: sites +84 and +85; MNNG:
sites +78 and +83). Two mutants recovered after EMS treatment each had a
deletion of a single G·C pair, one in the run of 5 G·C pairs extending
from +79 to +83, the other at position +68. In addition, three complex
changes were identified. One, for EMS treatment, featured the same se-
quence replacement (5'-GATCTCA-3' at +58 to +64 converted to 5'-CCGGG-3')
found to occur spontaneously in MGK-dp (section 4.5.3). A complex change,
in an MNNG treated cell, combined a two base-pair deletion in the G·C run

Table 43. Plasmid Retention in MGK-dp Treated with EMS or MNNG

Strain	No. of colonies on non-selective medium	No. of colonies on selective medium	Plasmid retention (%)
Control[a]	12,787	11,027	86
EMS (1%)	7,909	7,063	89
MNNG (30 μg/ml)	5,484	5,118	93

[a] Spontaneous data for MGK-dp are taken from Table 26.

Table 44. Influence of Elevated dCTP Levels on EMS-Induced Sequence Alterations

Sequence alteration	MKP-op + EMS[a]		MGK-dp + EMS[a]		MGK-dp[b]	
	No. detected (% of total)	Frequency (x 10^{-6})	No. detected (% of total)	Frequency (x 10^{-6})	No. detected (% of total)	Frequency (x 10^{-6})
Substitution						
Single change	150 (98.7)	98.7	199 (98.0)	33.3	183 (88.4)	4.8
Tandem double	-		1 (0.5)	0.2	-	
Non-tandem double	2 (1.3)	1.3	-		-	
Deletion						
1 bp	-		2 (1.0)	0.4	19 (9.2)	0.5
Insertion						
1 bp	-		-		1 (0.5)	0.03
Ty element	-		-		1 (0.5)	0.03
Complex change	-		1 (0.5)	0.4	3 (1.4)	0.07
Total	152	100.0	203	34.3	207	5.43

a Treatment: 1% EMS.
b Data for MKP-op (EMS) and MGK-dp (spontaneous) are taken from section 4.6.1.3 and Table 28, respectively.

Table 45. Influence of Elevated dCTP Levels on MNNG-Induced Sequence Alterations

Sequence alteration	MKP-op + MNNG[a]		MGK-dp + MNNG[a]		MGK-dp[b]	
	No. detected (% of total)	Frequency (x 10^{-6})	No. detected (% of total)	Frequency (x 10^{-6})	No. detected (% of total)	Frequency (x 10^{-6})
Substitution						
Single change	166 (100)	180	199 (98.5)	93.6	183 (88.4)	4.8
Non-tandem double	-		1 (0.5)	0.5	-	
Deletion						
1 bp	-		-		19 (9.2)	0.5
Insertion						
1 bp	-		-		1 (0.5)	0.03
Ty element	-		-		1 (0.5)	0.03
Complex change	-		2 (1.0)	0.9	3 (1.4)	0.07
Total	166	180	202	95	207	5.43

[a] Treatment: 30 µg/ml MNNG.

[b] Data for MKP-op (MNNG) and MGK-dp (spontaneous) are taken from section 4.6.1.3 and Table 28, respectively.

from +65 to +67 with an A·T to T·A transversion at +76. The third change, also for MNNG treatment, resulted in a run of 8 Gs (with respect to the transcribed strand) between the sites +78 and +84 which increased the length of the *SUP4*-o coding sequence from 89 to 90 bp. This could be explained either by a G·C to C·G transversion at position +78 accompanied by a single base-pair insertion in the run from +79 to +83 or by a single base-pair deletion at +78 and a double base-pair insertion in the run from +79 to +83. The frequencies of the single base-pair deletions and the complex changes were similar to those for the same events occurring spontaneously in MGK-dp. Thus, these two classes of mutation detected in the EMS- or MNNG-treated cells could be spontaneous in origin.

4.6.2.4 EMS- or MNNG-Induced Base-Pair Substitutions in MGK-dp

G·C to A·T transitions were responsible for the majority (>96%) of all EMS- and MNNG-induced base-pair substitutions in MKP-op (Table 36). Increased levels of dCTP would be expected to successfully compete with guanine for incorporation across from O^6-alkylguanine during DNA synthesis. Consequently, a decrease in the fraction of G·C to A·T transitions among EMS- or MNNG-induced mutations would be anticipated. In agreement with this expectation, the frequencies of EMS- and MNNG-induced G·C to A·T transitions in MGK-dp were reduced by 90% and 70%, respectively, relative to the corresponding values for MKP-op (Tables 46 and 47). However, these reductions were considerably larger than the decreases observed for the overall base-pair substitution frequencies (compare Tables 44 and 46 for EMS and Tables 45 and 47 for MNNG). The differences were due to 35- and 12-fold increases in the frequencies of EMS- and MNNG-induced G·C to C·G transversions, respectively, in MGK-dp. Although this transversion was the

Table 46. Influence of Elevated dCTP Levels on EMS-Induced Base-Pair Substitutions

Substitution	MKP-op + EMS[ab]		MGK-dp + EMS[a]		MGK-dp[b]	
	No. detected (% of total)	Frequency ($\times 10^{-6}$)	No. detected (% of total)	Frequency ($\times 10^{-6}$)	No. detected (% of total)	Frequency ($\times 10^{-6}$)
Transitions						
G·C to A·T	150 (97.4)	97.4	54 (26.9)	9.0	24 (13.1)	0.6
A·T to G·C	1 (0.6)	0.6	9 (4.4)	1.5	25 (13.7)	0.7
Total	151 (98.0)	98.0	63 (31.3)	10.5	49 (26.8)	1.3
Transversions						
G·C to T·A	1 (0.6)	0.6	5 (2.5)	0.8	10 (5.5)	0.3
G·C to C·G	2 (1.4)	1.4	125 (62.2)	20.9	107 (58.4)	2.8
A·T to C·G	-		2 (1.0)	0.3	10 (5.5)	0.3
A·T to T·A	-		6 (3.0)	1.0	7 (3.8)	0.2
Total	3 (2.0)	2.0	138 (68.7)	23.0	134 (73.2)	3.5

[a] Treatment: 1% EMS. Including double substitutions.

[b] Data for MKP-op (EMS) and MGK-dp (spontaneous) are taken from Tables 36 and 29, respectively.

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Table 47. Influence of Elevated dCTP Levels on MNNG-Induced Base-Pair Substitutions

Substitution	MKP-op + MNNG[ab]		MGK-dp + MNNG[ac]		MGK-dp[b]	
	No. detected (% of total)	Frequency (x 10⁻⁶)	No. detected (% of total)	Frequency (x 10⁻⁶)	No. detected (% of total)	Frequency (x 10⁻⁶)
Transitions						
G·C to A·T	160 (96.4)	173.4	112 (55.2)	52.4	24 (13.1)	0.6
A·T to G·C	2 (1.2)	2.2	19 (9.3)	8.9	25 (13.7)	0.7
Total	162 (97.6)	175.6	131 (64.5)	61.3	49 (26.8)	1.3
Transversions						
G·C to T·A	2 (1.2)	2.2	7 (3.4)	3.3	10 (5.5)	0.3
G·C to C·G	2 (1.2)	2.2	59 (29.1)	27.6	107 (58.4)	2.8
A·T to C·G	-		-		10 (5.5)	0.3
A·T to T·A	-		6 (3.0)	2.8	7 (3.8)	0.2
Total	4 (2.4)	4.4	72 (35.5)	33.7	134 (73.2)	3.5

[a] Treatment: 30 µg/ml MNNG.
[b] Data for MKP-op (MMNG) and MGK-dp (spontaneous) are taken from Tables 36 and 29, respectively.
[c] Including double substitutions and substitutions occurring in complex changes.

most frequent spontaneous base-pair substitution in this strain, it was at least 7.5-fold more frequent after EMS or MNNG treatment of MGK-dp (Tables 46 and 47). This indicated that the largest portion of the increases in the frequencies of induced G·C to C·G events was related to the alkylation treatments. The frequencies of A·T to G·C, G·C to T·A and A·T to T·A events detected after EMS or MNNG treatment of MGK-dp were similar to the corresponding spontaneous values.

Figures 24 and 25 show the distributions of *SUP4*-o base-pair substitutions induced in MGK-dp by EMS or MNNG, respectively. To facilitate the comparisons, the distributions for alkylation treatment of MKP-op and spontaneous mutagenesis in MGK-dp are also presented. In no case were substitutions detected in the 5′ or 3′ flanking regions of the gene but changes were always recovered at intron site +51, the only position within the intron that was mutated. G·C to A·T transitions and G·C to C·G transversions accounted for more than 85% of the changes induced by alkylation treatment in MGK-dp and so these two classes of substitution are considered here separately.

For both, EMS and MNNG treatment, 60% of the total sites where G·C to A·T transitions occurred in MGK-dp and MKP-op were common to both strains (EMS: 18/29; MNNG: 17/28) (Table 48). However, EMS induced these events at fewer sites in MGK-dp than MKP-op (19 vs. 28) whereas they occurred at similar numbers of sites in the two strains following MNNG treatment (22 vs. 23, respectively). With one exception for MNNG treatment (position +27), the EMS- and MNNG-induced transition frequencies were lower at all sites where these events were detected in both MGK-dp and MKP-op (Table 48). However, the site frequencies were not uniformly reduced with the decreases ranging from 31% to 98% for EMS treatment and from 10% to

Figure 24. Distribution of *SUP4*-o Base-Pair Substitutions Induced in **MGK-dp by EMS.** For simplicity, only the region of the strand encoding the tRNA is shown above each distribution. Double base substitutions are indicated by lower-case letters. The intron extends from +40 through +53. (A) MKP-op: EMS (1%); (B) MGK-dp: EMS (1%); (C) MGK-dp: spontaneous.

A

```
      +1         +10        +20        +30        +40        +50        +60        +70        +80        +89
3' GAGAGCCATC GGTTCAACCA AATTCCGCGT TCTGAAATTA AATAGTGATG CTTTAGAACT CTAGCCCGCA AGCTGAGCGG GGGCCCTCT 5'
   A A ATt    A  T  TT    TTA         T A                 T        T  AtTT T            A  AC    A  TT
   A A A T    A     T     TTA         T A        A        T           TTT  T               A     A  TT
   A A A T    A     T     TTA         T A        A        T           TTg  T               A     A  TT
   A A A T    t                       T A        A        T              T  T              A     TT  T
   A A A T                   A         A         A        T              T                 A         T
   A A A                     A         A         A                       T                 A
   A A A                     A         A         A                       T
   A A A                     A         A         A                       T
   AAA A A                   A         A         A
   AAA                       A         AAA       A
                                       AAA
```

B

```
      +1         +10        +20        +30        +40        +50        +60        +70        +80        +89
3' GAGAGCCATC GGTTCAACCA AATTCCGCGT TCTGAAATTA AATAGTGATG CTTTAGAACT CTAGCCCGCA AGCTGAGCGG GGGCCCTCT 5'
   AGC AGGG G  A  G  G    G  GGA C    G AG G A             G  C A  G   C CAGGCGC  GCGC TA C   CCAAGgG GA
   CTC CGTT G  C         GGA  C      T A                  G  C   G    C  AGTC     CG   A C    CCAAGG
   C   CGT  G              TGA C      A                   T                 GG  C      C      CgGGT
   C   CG   G               TA C      A                   T                 GT C       C      CGGGT
   T   G                    C  C      A                                     GT C       C      CGGT
       G                    C          C                                    GT C               GG
       G                    C          C                                    TT                 G
       G                    C          C                                    TT                 G
   GGGGGG                   C                                               T                  G
   GGGGGTT                                                              TTTTT  T               GGGTTT
```

C

```
      +1         +10        +20        +30        +40        +50        +60        +70        +80        +89
3' GAGAGCCATC GGTTCAACCA AATTCCGCGT TCTGAAATTA AATAGTGATG CTTTAGAACT CTAGCCCGCA AGCTGAGCGG GGGCCCTCT 5'
   AGC AGGGCG CC C    A    GA C       GCACC G              AGGG       C AA GC C   GCG C C      CA CC   GA
   CGC AGGG   C        G    GA C       G GC                C  G       C CA GT C   GCG C C      A  CC   GA
   CGC CGT    C        G    GA C       G                   C  G       C CCG C C   GCG C C      C  CC   GA
       C G    C              AC                           C          C CCT G C   G   G C     GT A    A
       C      C              A                            C          C CCT G C   G   G        C       A
              C              A                            C          C CCT G C       G        C       T
                                                                     C         C                     T
                                                                     C         C                     C
                                                                                                     C
                                                                                             CCCC    C
```

Figure 25. Distribution of *SUP4*-o Base-Pair Substitutions Induced in **MGK-dp by MNNG.** For simplicity, only the region of the strand encoding the tRNA is shown above each distribution. Double base substitutions are indicated by lower-case letters. The intron extends from +40 through +53. (A) MKP-op: MNNG (30 μg/ml); (B) MGK-dp: MNNG (30 μg/ml); (C) MGK-dp: spontanoeus.

A

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

```
        A A ATT   A TT      TTA      TA              T    A     C    TT          A           A  ATAT C   T TC
        A  ATT    A         TTA      T  A            T    A           TT                      A    A  T T
        .A  C T   A         TTA      T  A                 A           TT                             A     T T
        A                              TT     T  A             A           TT                               T T
  AAAAAAA                              TT     T  A             A           TT                                   T
  AAAAAAA                              T      T  A             A           TT
  AAAAAAA                              T      T  A             A           TT
  AAAAAAA                              T      T  A             A           T
  AAAAAAA                              T      T AAA            A           T
  AAAAAAA                               TTT AAA
```

B

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

```
  AGA AGGT G  AC  G  T   C TTA CA   ACAG C          A TT      C AGGTCGG   AGC tcgCC  C A G ATA
  A  A  AGT    AC  G      C TTA C    ACA C                     C ATGTC     A C A C     C C G   C
  A  A AGT     CC         TTA C      ACA C           A          CTT        A C A C     C C G
  A  A CGT     CC         TTA C      G A             A          TT          C C  C     C C T
  A   T        C          TTA C      T A             A           T
  A   C        T          TTA C      T A             A
  A   C        T          TTA C      T               A
  A   C                   TA         T
                          TC         T
  AAAAA                   TTTTTTTC
  AAAAAA                  TTTTTTTCC
  AAACCC
```

C

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

```
  AGC AGGGCG  CC C   A    GA C      GCACC G         A    AGGG   C AA GC C   GCG   CA CC     CCAGG GA
  CGC AGGGG   C      G    GA C      G GC             A    C  G   C CA GT C   GCG   A CC      CGGG GA
  CGC CGT     C      G    GA C                      A    C  G   C CG G C    GCG   C CC      CGGG GA
      C  G    C           A C                       A    C  G   C CT G C    G     C C       G T A
      C       C           A                              C      C CT G G    G     T C        T T A
              C           A                              C      C CT G G    G     C C        T A
                                                         C                        C C        T C
                                                                                  C C        C C
                                                                                  C          C C
                                                                                  CCCC        C
```

Table 48. G·C to A·T Transitions Induced in MGK-dp by EMS or MNNG

		Frequency (x 10^{-6})						
	Sequence[b]	EMS (1%)				MNNG (30 µg/ml)		
Site[a]	5′ to 3′	MKP-op[c]		MGK-dp		MKP-op[c]		MGK-dp
3	AGA	6.5	(10)[d]	-		1.1	(1)	1.0 (4)
32	AGA	2.6	(4)	0.2	(1)	14.3	(13)	2.3 (5)
56	AGA	7.2	(11)	0.2	(1)	11.0	(10)	3.3 (7)
61	AGA	0.6	(1)	-		-		-
88	AGA	2.6	(4)	-		4.4	(4)	0.5 (1)
10	AGC	-		-		-		-
25	AGG	1.9	(3)	0.3	(2)	5.5	(5)	3.3 (7)
1	AGT	9.8	(15)	0.2	(1)	58.5	(53)	10.3 (22)
15	AGT	0.6	(1)	-		1.1	(1)	-
34	AGT	9.8	(15)	0.8	(5)	16.6	(15)	3.3 (7)
86	GGA	3.2	(5)	0.5	(3)	6.6	(6)	-
11	GGC	2.6	(4)	0.2	(1)	3.3	(3)	0.9 (2)
26	GGC	1.9	(3)	-		14.3	(13)	10.8 (23)
67	GGC	1.3	(2)	0.2	(1)	9.9	(9)	0.9 (2)
79	GGC	0.6	(1)	-		3.3	(3)	-
66	GGG	6.5	(10)	0.8	(5)	7.7	(7)	1.4 (3)
7	GGT	5.2	(8)	0.3	(2)	3.3	(3)	1.4 (3)
19	GGT	1.3	(2)	-		1.1	(1)	-
5	CGA	6.6	(10)	0.2	(1)	2.2	(2)	1.4 (3)
51	CGA	3.2	(5)	0.3	(2)	2.2	(2)	-
64	CGA	0.6	(1)	-		-		0.9 (2)
72	CGA	-		-		-		1.4 (3)
77	CGA	4.5	(7)	0.3	(2)	1.1	(1)	0.9 (2)
27	CGC	6.5	(10)	0.7	(4)	3.3	(3)	3.7 (8)
78	CGC	-		-		1.1	(1)	-
6	CGG	0.6	(5)	0.3	(2)	2.2	(2)	0.5 (1)
65	CGG	2.6	(4)	0.8	(5)	-		1.4 (3)
83	CGG	1.9	(3)	0.3	(2)	-		0.5 (1)
84	CGG	-		0.5	(3)	-		-
69	CGT	2.6	(4)	-		-		-
59	TGA	1.3	(2)	-		-		0.5 (1)
18	TGG	2.6	(4)	1.8	(11)	2.2	(2)	0.5 (1)

[a] G·C to A·T events can be detected at all sites listed (see Table 3).

[b] In each trinucleotide, the central G is the mutated base.

[c] Data for MKP-op are taken from Table 37.

[d] Numbers in parentheses are the number of mutants recovered.

91% for MNNG treatment. Although these ranges were similar, the mean reduction in the mutation frequency was greater for EMS (84%) than MNNG (62%). This was also true for the individual frequency decreases at sites +1 and +34, the transition hotspots for treatment of MKP-op with EMS (98% and 92%, respectively) or MNNG (82% and 80%, respectively). These results are consistent with the elevated dCTP level in MGK-dp having a greater effect on EMS than MNNG mutagenesis. In addition, the data indicate that the influence of dNTP imbalance likely was mediated, at least in part, by DNA sequence context.

The G·C to C·G transversions constituted the major class of substitution occurring spontaneously in MGK-dp or induced in this strain by EMS but not MNNG (Tables 46 and 47). Of the 39 sites where G·C to C·G changes can be detected in *SUP4*-o, this transversion occurred spontaneously at 29 sites and was induced by EMS or MNNG at 35 or 25 sites, respectively (Figures 24 and 25, Table 49). The spontaneous and EMS-induced transversions were found at a total of 37 sites with 27 common to the two distributions. Similarly, the spontaneous and MNNG-induced transversions were detected at 35 different sites but only 19 were identified in both distributions. The sites mutated most frequently differed for the EMS (+6, +84), MNNG (+12, +29) and spontaneous (+64, +79) distributions. With a few exceptions for EMS treatment (+11, +56, +64, +67), the transversion frequencies at the sites common to the distributions for spontaneous and EMS- and MNNG-induced substitutions were higher for the induced events. The increases ranged from 1.7- to 33-fold with a mean of 12-fold for EMS treatment and 1.8- to 78-fold with a mean of 17-fold for MNNG treatment. Taken collectively, the data indicate that the G·C to C·G transversions were distributed dissimilarly for the three

Table 49. G·C to C·G Transversions Induced in MGK-dp by EMS or MNNG

Site[a]	Sequence[b] 5′ to 3′	Frequency ($\times 10^{-7}$)					
		Spontaneous		EMS (1%)		MNNG (30 μg/ml)	
3	AGA	1.3	(5)[c]	8.3	(5)	18.7	(4)
32	AGA	1.0	(4)	1.7	(1)	4.7	(1)
56	AGA	1.6	(6)	1.7	(1)	-	
88	AGA	0.8	(3)	1.7	(1)	-	
10	AGC	0.3	(1)	6.7	(4)	4.7	(1)
25	AGG	-		3.3	(2)	-	
1	AGT	0.5	(2)	1.7	(1)	14.0	(3)
15	AGT	-		1.7	(1)	9.4	(2)
34	AGT	-		1.7	(1)	-	
86	GGA	0.8	(3)	5.0	(3)	-	
11	GGC	1.6	(6)	1.7	(1)	9.4	(2)
26	GGC	0.8	(3)	5.0	(3)	-	
67	GGC	1.6	(6)	1.7	(1)	-	
79	GGC	3.7	(14)	8.3	(5)	18.7	(4)
66	GGG	-		5.0	(3)	9.4	(2)
80	GGG	0.8	(3)	-		4.7	(1)
81	GGG	-		3.3	(2)	18.7	(4)
82	GGG	0.3	(1)	3.3	(2)	-	
85	GGG	0.8	(3)	11.7	(7)	14.0	(3)
7	GGT	0.5	(2)	1.7	(1)	4.7	(1)
19	GGT	-		-		-	
5	CGA	0.3	(1)	6.7	(4)	4.7	(1)
51	CGA	-		3.3	(2)	-	
64	CGA	2.6	(10)	1.7	(1)	4.7	(1)
72	CGA	0.8	(3)	3.3	(2)	-	
73	CGA	1.6	(6)	3.3	(2)	4.7	(1)
77	CGA	0.5	(2)	8.3	(5)	4.7	(1)
27	CGC	-		10.0	(6)	18.7	(4)
68	CGC	0.3	(1)	10.0	(6)	9.4	(2)
78	CGC	-		-		4.7	(1)
6	CGG	1.0	(4)	33.3	(20)	18.7	(4)
65	CGG	0.3	(1)	6.7	(4)	4.7	(1)
83	CGG	0.8	(3)	5.0	(3)	9.4	(2)
84	CGG	0.8	(3)	18.3	(11)	-	
69	CGT	-		1.7	(1)	4.7	(1)
59	TGA	1.0	(4)	5.0	(3)	-	
29	TGC	1.0	(4)	10.0	(6)	32.8	(7)
12	TGG	0.3	(1)	-		23.4	(5)
18	TGG	0.5	(2)	6.7	(4)	-	

[a] G·C to C·G events can be detected at all sites listed (see Table 3).
[b] In each trinucleotide, the central G is the mutated base.
[c] Numbers in parentheses are the number of mutants recovered.

conditions. In addition, they argue that the site-specificity of transversion induction by the alkylating agents was influenced by DNA sequence context.

The hypergeometric test (Skopek and Adams 1987) was used to statistically compare the differences between all pair-wise combinations of the five distributions shown in Figures 24 and 25. For all possible comparisons, the probability of random sampling error being responsible for differences in the distributions was less than 1 in 500 (the upper limit of the 90% confidence interval on each estimate of P was 0.002).

4.6.2.5 Sequence Context and Strand Specificity of EMS- or MNNG-Induced Base-Pair Substitutions in MGK-dp

It was shown in this study that G·C to A·T transitions were preferentially induced at 5'Pur-G sites in MKP-op by MNNG but not EMS (Table 38) and that there was no strand bias for the induction of these events by either agent (Table 40). To determine whether the elevated dCTP levels in MGK-dp affected these features of EMS and MNNG mutagenesis, the bases immediately flanking the transition sites and the strand specificity of mutation induction were examined in greater detail. This analysis revealed that while EMS-induced G·C to A·T transitions did not occur more frequently at 5'Pur-G than 5'Pyr-G sites, induction of these changes by MNNG occurred 2.8-fold more often at the former than the latter sites (Table 50). Although this bias was less pronounced than observed for MNNG mutagenesis in MKP-op, it was significant ($P < 0.01$). For EMS treatment the induction of G·C to A·T transitions did not show a strand preference (Table 51). Yet, the induction of these events by MNNG did show a slight bias for the transcribed strand ($P <$ at least 0.02) and it was significant

Table 50. Influence of the 5' Base on EMS- or MNNG-Induced G·C to A·T Transitions in MGK-dp

5' Base	EMS (1%)				MNNG (30 μg/ml)		
	No. of available sites	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available
A	10	5	10	1.0	7	53	5.3
G	8	5	12	1.5	5	34	4.2
Pur	18	10	22	1.2	12	87	4.8
C	12	8	21	1.7	8	23	1.9
T	2	1	11	5.5	2	2	1.0
Pyr	14	9	32	2.3	10	25	1.8

Table 51. Strand Specificity of EMS- or MNNG-Induced G·C to A·T Transitions in MGK-dp

Strand	EMS (1%)				MNNG (30 μg/ml)		
	No. of available sites	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site	No. of sites detected	No. of mutants recovered	Average no. of mutants per available site
TS[a]	12	8	17	1.4	11	61/39[b]	5.1/3.2[b]
NTS[a]	20	11	37	1.8	11	51/28[b]	2.5/1.4[b]

[a] TS: transcribed strand; NTS: non-transcribed strand.

[b] Data for MNNG hotspots in MGK-dp at positions +1 and +26 included/excluded.

whether the data for the hotspot at position +1 and +26 were included in the calculation or not (Table 51).

In the previous section it was reported that elevated dCTP levels increased the frequency of $G \cdot C$ to $C \cdot G$ transversions induced by EMS and MNNG treatment. The site and strand specificities of this effect were investigated assuming that $G \cdot C$ to $C \cdot G$ transversions resulted from misincorporation of dCTP. There was no obvious preference for the EMS-induced transversions to occur next to a particular 5' base, while there did seem to be a slight bias (2-fold) for MNNG-induced transversions to take place at 5'-TX-3' sites (Table 52). However, this preference was not significant ($P > 0.05$) when compared to the expected random distribution of mutations based on the number of sites available. Analysis of the 3' bases at EMS- or MNNG-induced $G \cdot C$ to $C \cdot G$ transversion sites did not reveal any significant preferences. EMS-induced $G \cdot C$ to $C \cdot G$ transversions did not preferentially occur on the transcribed or non-transcribed strand whether the base inserted immediately after the misinsertion site was a C or not (Table 53). On the other hand, MNNG-induced transversions at 5'-XC-3' sites exhibited a 7.6-fold preference for the transcribed strand ($P < 0.05$) but only when the MNNG transversion hotspot data (position +29) were included in the calculation. When the hotspot data are omitted from the analysis, the bias is not significant ($P > 0.2$). Similarly, the apparent, albeit slight, preference for the transcribed strand when the next base to be inserted was other than a C was not significant ($P > 0.1$).

Table 52. Influence of the 5' or 3' Base on EMS- or MNNG-Induced G·C to C·G Transversions in MGK-dp

Sequence[a] 5' to 3'	No. of available sites[b]	EMS (1%)			MNNG (30 μg/ml)		
		No. of sites detected	No. of mutants recovered	Average no. of mutants per site available	No. of sites detected	No. of mutants recovered	Average no. of mutants per site available
A X	9	9	17	1.9	9	11	1.2
C X	14	13	67	4.8	11	19	1.4
T X	4	3	13	3.3	2	12	3.0
G X	12	10	28	2.3	7	17	1.4
X A	12	12	30	2.5	6	9	0.8
X C	9	8	32	3.6	7	21	2.3
X T	6	5	5	0.8	4	7	1.2
X G	12	10	58	4.8	8	22	1.8

[a] Misinserted base: C. X: site of misinsertion.

[b] Sites where G·C to C·G transversions can be detected in SUP4-o.

Table 53. Strand Specificity of EMS- or MNNG-Induced G·C to C·G Transversions in MGK-dp

	EMS (1%)								MNNG (30 µg/ml)					
Next base inserted	No. of available sites[a]		No. of sites detected		No. of mutants recovered		Average no. of mutants per site available		No. of sites detected		No. of mutants recovered		Average no. of mutants per site available	
	TS[b]	NTS[b]	TS	NTS	TS	NTS	TS	NTS	TS	NTS	TS	NTS	TS	NTS
C	5	4	5	3	24	8	4.8	2.0	5	2	19/12[c]	2	3.8/2.4[c]	0.5
A/T/G	13	17	11	16	27	66/35[d]	2.1	4.1/2.1[d]	9	9	22	16	1.7	0.9

[a] Sites where C misinsertions can be detected in SUP4-o.

[b] TS: transcribed strand; NTS: non-transcribed strand.

[c] Data for MNNG hotspot at position +29 included/excluded.

[d] Data for EMS hotspots at positions +6 and +11 included/excluded.

DISCUSSION

5 DISCUSSION

5.1 Nucleotide Permeability and Canavanine Sensitivity

Yeast strains with defects in the thymidylate synthase gene (*TMP1*) require dTMP rather than thymine or thymidine (Little and Haynes 1979) because *S. cerevisiae* lacks the enzyme thymidine kinase (Grivell and Jackson 1968) and so is dependent on the *de novo* pathway for the synthesis of thymine nucleotides. Normally yeast is impermeable to nucleotides but mutants (*tup*) able to take up thymidylate have been isolated (Jannsen *et al*. 1973; Laskowski and Lehmann-Brauns 1973; Fäth *et al*. 1974; Wickner 1974). To date, five different complementation groups have been described and others may exist (Wickner 1974; Bisson and Thorner 1982a).

Although the precise mechanism(s) of dTMP uptake remains to be elucidated, *tup* mutations also allow uptake of other nucleotides and nucleotide analogs including AMP, IMP, araCMP, dAMP, dCMP, dGMP, dUMP, BrdUMP, FdUMP and IdUMP as well as inorganic phosphate and its analog arsenate (Kunz *et al*. 1980; Bisson and Thorner 1982a; McIntosh and Haynes 1984; Brendel 1985; McIntosh *et al*. 1986; Ross *et al*. 1987). On the other hand, entry of purine and pyrimidine bases and nucleosides is not promoted (Bisson and Thorner 1982a). Furthermore, Tup$^-$ strains are no more sensitive to the metabolic inhibitors cycloheximide, nystatin and cordecypin than are their Tup$^+$ parents (Bisson and Thorner 1982a). Taken collectively, these findings suggest that *tup* mutations specifically facilitate the transport of phosphate and certain phosphate-containing molecules. Consequently, it was rather surprising to find that recovery of canavanine-resistant (Can^R) mutants was severely reduced in a thymidylate auxotroph and that this decrease was associated with enhanced sensitivity to canavanine (Figure 16, Table 6). The effect could only be observed in

strains carrying a *tup* mutation whether they also had a mutation in the *TMP1* gene or not (Table 7). This was particularly intriguing because there was no obvious connection between dTMP permeability and the uptake of canavanine, a molecule showing no structural resemblance to compounds known to be affected by *tup* mutations.

How then might enhanced sensitivity to canavanine in *tup* strains be explained? Arginine permease transports canavanine and arginine into yeast cells and is believed to be encoded by the *CAN1* gene (Grenson *et al.* 1966; Hoffmann 1985; Ahmad and Bussey 1986). In MKP-o and its derivatives, this gene carries an ochre mutation (*can1-100*) and so is defective, although the results of the labelling experiment for MKP-o (Table 8) suggest that it is somewhat leaky. Even so, the *can1-100* allele confers resistance to 30 μg/ml canavanine on MKP-o but not on SBT-TL, SBT-T or SBT (Figure 17). In addition, it was shown that dTMP uptake was not linked to the arginine permease function (Table 9). Thus, one possible explanation for elevated canavanine uptake in the dTMP permeable strains is that the *tup* mutation(s) might somehow suppress the ochre *can1-100* allele thereby increasing the synthesis of arginine permease. This seems unlikely since neither *ade2-1* nor *lys2-1*, which are also ochre alleles, were suppressed in SBT-TL (Table 5). Furthermore, incorporation of labelled arginine was markedly increased in SBT-TLp, where *can1-100* is efficiently suppressed by *SUP4*-o, to levels greater than those attributable to suppression of *can1-100* alone (Table 8).

Uptake of arginine and canavanine in yeast can also occur via the general amino acid permease (GAP) (Grenson *et al.* 1970). The GAP system is inhibited by ammonia and ammonium ion is found in the yeast media used in this study. Conceivably, the *tup* mutation(s) might cause loss of ammonia

inhibition of GAP activity. This condition sensitizes arginine permease-deficient strains to canavanine (Grenson *et al.* 1970) but canavanine resistance can be restored by growing the cells in high concentrations (100 μg/ml) of the amino acids that inhibit the GAP system (the mechanism of inhibition by the amino acids differs from that for GAP inhibition by ammonium ions) (Grenson *et al.* 1970). The effective amino acids include several (alanine, methionine, phenylalanine and valine) that do not inhibit uptake of arginine by arginine permease. So it was reasoned that if enhanced sensitivity to canavanine in SBT-TL resulted from loss of ammonia inhibition of GAP activity, specific inhibition of the GAP system by these amino acids should restore resistance to canavanine. However, when SBT-TL was grown in media containing canavanine (30 μg/ml) and the amino acids (100 μg/ml) listed above, canavanine inhibition of cell growth was not affected (Table 10). This indicated that the *tup* mutation(s) did not render SBT-TL sensitive to canavanine by alleviating ammonia inhibition of the GAP system.

Another explanation for increased canavanine sensitivity in the *tup* strains is that the *tup* mutation(s) might enhance the activity of any arginine permease synthesized as a consequence of leakiness or suppression of the *can1-100* allele. At present, I cannot explain how deficiencies in genes that play roles in phosphate transport or metabolism could have such an effect. Perhaps membrane alterations which affect arginine permease might be involved. In any event, it should be noted that *tup1* has been found to be pleiotropic, conferring high levels of iso-2-cytochrome *c*, clumpy growth, morphological abnormalities and insensitivity to catabolite repression as well as defects in sporulation, mating and possibly UV-induced mutagenesis (Lemontt *et al.* 1980). Possibly, other *tup* mutations

also might influence processes unrelated to nucleotide uptake. In this regard, it is was recently suggested that transcriptional regulation might be altered in *tup1* strains (Thrash-Bingham and Fangman 1989) and several findings are consistent with this possibility. First, the *TUP1* gene product was implicated as a negative regulator in the phenomenon of catabolite or glucose repression, since mutations in the *TUP1* gene result in constitutive expression of several catabolite-repressible genes in yeast (Trumbly 1986). Second, the predicted *TUP1* protein sequence features polyglutamine tracts which have been detected in several yeast regulatory proteins (Williams and Trumbly 1990). Third, experimental evidence suggests that the *TUP1* gene product might be part of a protein complex that possibly can interact with transcriptionally active domains of chromosomes (Williams *et al.* 1991).

Yeast cells carrying a *tup1* mutation are dumbbell-shaped during growth and form clumps in liquid medium (Lemontt *et al.* 1980). However, SBT, as well as its *tmp1* derivatives SBT-T and SBT-TL, did not clump during growth and exhibited normal cellular morphology. This indicates that the dTMP permeability of the *tup* strains used in this study was not due to a *tup1* mutation. Nevertheless, it is conceivable that the *tup* mutation(s) in SBT-TLp might promote transcription of the apparently leaky *can1-100* allele so that sufficient arginine permease could be produced to increase cellular sensitivity to canavanine. If so, the increased transcription of *can1-100* and suppression of this allele would have to interact synergistically. The uptake of labelled arginine in SBT-TLp, where both the *tup* phenomenon and suppression of *can1-100* contribute to arginine transport, was markedly greater than the combined uptakes in SBT-TL and MKP-op where only the *tup* effect or suppression function, respectively

(Table 8). Furthermore, the finding that uptake of the chemically distinct substrates, canavanine and dTMP, which are associated with two separate uptake mechanisms (canavanine but not dTMP interfered with the uptake of labelled arginine) is affected by the same *tup* mutation(s) supports the idea that the *TUP* gene product could be involved in regulation.

In conclusion, permeability to thymidylate in a dTMP auxotroph of yeast is associated with increased uptake of canavanine. This phenomenon might also be a characteristic of other mutations that promote dTMP permeability. A search of the literature, prompted by these findings, revealed that *tup* strains isolated by other investigators also exhibit abnormally low frequencies of spontaneous mutation to canavanine resistance (Lemontt 1977; Lemontt *et al.* 1980; Brendel 1985; Bender and Brendel 1988).

5.2 Analysis of dNTP Pools

The concentrations of all four DNA precursors were measured in MKP-op, MGK-dp and SBT-TLp, in the latter case following either dTMP starvation (0 μg, 12 h) or treatment with excess dTMP (1 mg, 6 h). The dNTP levels in MKP-op were used as a reference to determine the extent of dNTP imbalance in MGK-dp and SBT-TLp. In MKP-op, dATP, dCTP and dGTP had about the same concentration (0.6 - 0.7 pmol/10^6 cells) while the dTTP levels were about two-fold higher (1.4 pmol/10^6 cells) (Table 16). These results were very similar to earlier measurements of dNTP levels in asynchronously growing yeast cells (Eckstein *et al.* 1974) consistent with the sizes of the dNTP pools in yeast being intermediate to those in bacteria (<0.2 pmol/10^6 cells) and mammalian cells (>5 pmol/10^6 cells) (Snyder 1984; Albert and Gudas 1985; Platz *et al.* 1985; Arezzo 1987; Eriksson *et al.* 1987; Myers *et*

al. 1987; Sargent and Mathews 1987; Arecco *et al.* 1988).

It can be roughly estimated that the dNTP pools in MKP-op would suffice, at best, for several minutes of chromosomal DNA synthesis. A normal haploid yeast cell has a chromosomal DNA content of 1.6 x 10^7 bp (= 53 pmol dNTPs/10^6 cells) (Mortimer and Schild 1985). Exponential phase cells of MKP-op growing in uracil omission medium have a measured generation time of 135 min and so S phase, which typically is equivalent to 25% - 50% of the generation time (Newlon 1988), would presumably occupy 34 - 68 min. Thus, 10^6 MKP-op cells would have to synthesize approximately 0.8 - 1.6 pmol dNTP/min during S phase to sustain chromosomal DNA replication. On this basis, and given that yeast chromosomal DNA has a base composition of 60% A + T (Fangman and Zakian 1981), the measured dATP, dTTP, dGTP and dCTP pools would suffice for 1.5 - 2.9 min, 3.3 - 6.6 min, 1.9 - 3.7 min and 2.0 - 3.9 min of DNA synthesis, respectively. These approximations correlate well with the estimates for the length of time that dNTP concentrations in mammalian cells could support DNA replication (Reichard 1988).

dTMP starvation of SBT-TLp for a 12 h period resulted in a dramatic drop in the intracellular dTTP concentration to the point where the pool of this DNA precursor was too low to be measured accurately (Table 16). In addition to the decrease in the dTTP concentration, increases in the dATP, dCTP and dGTP levels were found. Elevated dCTP concentrations were expected, on the basis of lack of feedback inhibition of dTTP at the ribonucleotide reductase, but the dCTP pool was smaller than anticipated (Larsson and Reichard 1966; Maybaum *et al.* 1981).

An increase in the dATP level has been observed after dTTP depletion and it was suggested that dTTP is involved in the regulation of dATP

levels (Neuhard 1966; Meuth *et al.* 1979a). Although dTTP was not identified as a negative regulator of ADP reduction in yeast *in vitro* (Lammers and Follmann 1984), it might conceivably act as one *in vivo*. Recent studies of the phage T4 ribonucleotide reductase indicate that the *in vivo* regulation of this enzyme by dTTP is different from its *in vitro* regulation (Ji *et al.* 1991). Neuhard (1966) suggested that increases in the concentrations of dCTP might be counteracted by high dATP levels, an inhibitor of the ribonucleotide reductase. If so, the small increase (3.5-fold) in the dCTP concentration found after dTMP starvation in this study, might be a result of the large increase (120-fold) in the dATP levels. The increase (8.5-fold) in the dGTP pool was somewhat surprising. Reduction of GDP via the ribonucleotide reductase is promoted by dTTP (Lammers and Follmann 1984, 1986; Reichard 1985) so that a decrease in dTTP levels would be expected to result in a reduction in the dGTP concentration. Furthermore, cells with elevated dATP levels have been reported to have low dGTP pools (Dahbo and Eriksson 1985; Yoshioka *et al.* 1987b). Other investigators have reported that dGTP levels are not changing or decreased during dTTP depletion in bacteria and mammalian cells (the observed decrease was even more pronounced than that for dTTP) (Neuhard 1966; Meuth *et al.* 1979b). At this point, the reason(s) for dTTP depletion causing an increase in dGTP levels in SBT-TLp remains to be determined.

In addition to the dNTP pools measured in this study, dTTP depletion has been associated with an accumulation of dUMP and ultimately dUTP in bacteria and mammalian cells (Goulian and Beck 1966; Tattersall and Harrap 1973; Jackson 1978; Goulian *et al.* 1980; Sedwick *et al.* 1981). The combination of an increase in UDP reduction (promoted by low dTTP levels)

and the inability to process dUMP to dTMP via thymidylate synthase is assumed to be responsible for the abnormally high dUTP levels. Although dUTP concentrations cannot be measured with the dNTP pool assay employed in this study, it is reasonable to think that dUTP levels might be increased during dTTP depletion in SBT-TLp. If so, misincorporation of dUTP in place of dTTP during DNA synthesis might play a role in the mutational specificity of dTTP depletion. However, it can be deduced that if dUTP levels were elevated in SBT-TLp under conditions of dTTP depletion, they did not interfere with the efficiency of the polymerase assay. If the DNA polymerase used in this assay would have incorporated dUTP in significant amounts, it would have resulted in the concentration of dTTP appearing to be much greater than actually measured.

Inactivation of dCMP deaminase in bacterial and mammalian cells, and growth of mammalian cells on medium containing high thymidine concentrations, alter intracellular dNTP levels (Meuth 1981b; Weinberg *et al*. 1981, 1985; Sargent and Mathews 1987; Wilkinson and McKenna 1989). On the basis of these studies and known properties of yeast ribonucleotide reductase (Lammers and Follmann 1984, 1986), it was anticipated that dCMP deaminase deficiency and dTMP treatment would lead to marked enhancements of the dCTP or dTTP and dGTP pools, respectively, in yeast. These expectations were confirmed by measuring the intracellular dNTP concentrations in appropriate strains under the requisite conditions.

Changes in the dNTP levels in the yeast *dcd1* strain (Table 25) were qualitatively similar to those noted for dCMP deaminase deficient mammalian cells (Weinberg *et al*. 1981, 1985; Bianchi *et al*. 1987; Arecco *et al*. 1988). The small decrease in the dTTP pool is consistent with previous results indicating that in yeast UDP reduction is a more

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important source of cellular dUMP, which is used for dTMP biosynthesis, than is dCMP deamination (McIntosh and Haynes 1984). Although dCMP deaminase deficiency also has been found to cause small increases in the dATP pool in mammalian cells (Weinberg *et al.* 1981; Arecco *et al.* 1988), a drop in the dGTP concentration has not been reported previously. Possibly a decrease in the pool of dTTP, which promotes GDP reduction in yeast (Lammers and Follmann 1984, 1986) was responsible for the diminished level of dGTP.

Treatment of SBT-TLp with excess dTMP enhanced the dGTP and dATP pools (Table 25). This presumably reflects the fact that dTTP and dGTP are positive effectors for reduction of GDP and ADP, respectively, by yeast ribonucleotide reductase (Lammers and Follmann 1984, 1986). Interestingly, it was noted that treatment with excess dTMP did not diminish dCTP levels. Yet, CDP reduction by class I ribonucleotide reductases is normally inhibited by dTTP (Thelander and Reichard 1979) and the yeast enzyme is considered a class I reductase (Reichard 1988). Moreover, there is evidence that dTTP is a negative effector of CDP reduction in yeast extracts (Lammers and Follmann 1984; E.M. McIntosh: personal communication). A potential explanation for the apparent lack of effect of dTMP treatment on dCTP pools involves altered regulation of ribonucleotide reductase activity in response to dTTP stress. The large subunit of the reductase is believed to carry binding sites for the allosteric effectors of reductase activity and the yeast genes *RNR1* and *RNR3* encode alternate large subunits (Elledge and Davis 1990). *RNR1* is ordinarily expressed at much higher levels than *RNR3* but transcription of both *RNR3* and *RNR2* (which encodes the small reductase subunit) is effectively induced by treatment with hydroxyurea, which blocks DNA replication by inhibiting the

reductase (Elledge and Davis 1989, 1990; Yagle and McEntee 1990). Thus, it has been proposed that one purpose for induction of *RNR3* and *RNR2* might be to produce a species of reductase having modified allosteric properties in order to restore dNTP balance and so DNA replication (Yagle and McEntee 1990). Perhaps expansion of the dTTP pool also leads to the induction of *RNR3* (and *RNR2*) and the maintenance of normal dCTP levels in SBT-TLp is a consequence of this induction. This suggestion is strengthened by the finding that growth of nucleotide-permeable yeast cells in medium containing excess dTMP increases ribonucleotide reductase activity (Lammers and Follmann 1984).

5.3 Stability of YCpMP2 Under Different Conditions

To analyze spontaneous and induced mutagenesis in yeast, mutations were selected in the yeast tRNA gene *SUP4*-o by scoring for loss of suppressor activity. As described above (section 3.1), *SUP4*-o is carried on the shuttle vector YCpMP2. This vector mimics the behaviour of yeast chromosomes. Routinely, to determine whether any of the treatment conditions used in this study might influence chromosome stability, plasmid retention was measured. This was accomplished by comparing the number of colonies that formed on medium selective for the plasmid with the number of colonies that emerged on non-selective medium. Approximately 90% of the cells retained YCpMP2 under normal growth conditions in the wild-type strain MKP-op (section 4.3). This value is comparable to those established from other studies on the stability of autonomously replicating plasmids carrying the same centromere sequence (*CEN4*) as YCpMP2 (Stinchcomb *et al*. 1982; Mann and Davis 1986).

Elevated dCTP levels in a *DCD1* deficient strain (MGK-dp) and EMS or

MNNG treatment of cells, with balanced dNTP or elevated dCTP levels, did not affect the stability of YCpMP2 (Tables 26, 34 and 43). In contrast to these findings, cells with the disrupted *TMP1* gene progressively lost YCpMP2 during periods of dTMP starvation or exposure to excess concentrations of dTMP (Tables 17 and 26). Since loss of the plasmid is monitored by the reappearance of uracil dependence, it might be argued that mutation of the plasmid-borne *URA3* gene could account for this observation. However, neither treatment increased the frequency of Can^R or *SUP4*-o mutations by more than 148-fold above the corresponding spontaneous values in MKP-op ($< 7 \times 10^{-5}$, Table 19 and Figure 20). Therefore, it seems unlikely that mutational inactivation of the *URA3* gene on YCpMP2 could be responsible for the loss of the plasmid in 65% and 48% of the cells starved for dTMP (12 h) or exposed to excess dTMP (1 mg/ml, 6 h), respectively.

In principle, loss of this centromere plasmid could be caused by effects on the replicative and/or partition functions of the *ARS* or *CEN* sequences, respectively. It might be suggested that one approach to distinguish between these possibilities would be to subject cells harbouring plasmids that lack centromeres (i.e., replicating plasmids that contain *ARS* or episomal plasmids that have the yeast 2μ plasmid replication origin) to thymidylate stress. Unfortunately, such plasmids exhibit segregation biases during meiosis and/or mitosis and normally are maintained at high copy numbers (Gunge 1983; Murray and Szostak 1983; Struhl 1983). The high copy number would make it difficult, at best, to selectively score loss of individual plasmids and the segregation biases would obscure effects on plasmid stability that otherwise might be attributable to dTMP stress. Clearly, the use of such vectors would not allow one to determine whether replicative and/or partition functions are

affected. However, it should be emphasized that the *ARS* and *CEN* sequences responsible for these properties are normal yeast chromosome components and that YCpMP2 behaves as a mini-circular chromosome. Thus, it would seem reasonable to think that if plasmid instability under conditions of thymidylate stress is a consequence of interference with the replicative or partition functions, then dTMP stress also might affect the same properties of yeast chromosomes.

5.4 DNA Sequence Analysis of Spontaneous Mutations in the *SUP4*-o Gene

Earlier studies have shown that spontaneous mutations can involve a variety of DNA alterations (deJong *et al*. 1985; Schaaper *et al*. 1986; Ashman and Davidson 1987b; Nalbantoglu *et al*. 1987). Consistent with such findings, eight different mutational classes were identified during the analysis of 334 spontaneous mutations in the *SUP4*-o gene in MKP-op. These included single and double base-pair substitutions, single and multiple base-pair deletions, single base-pair insertions, insertion of the yeast transposable element Ty, duplications and more complex changes (Table 12).

Single base-pair substitutions contributed the largest fraction (81%) of the spontaneous mutations analyzed (Table 12). All possible types of substitution were identified but there was a bias towards transversions and G·C sites (Table 13). Spontaneous base substitution mutagenesis at the chromosomal *SUP4*-o locus of *S. cerevisiae* also shows a preference for transversions and G·C sites (Kurjan *et al*. 1980), suggesting that the same mutational processes act on the plasmid and chromosomal copies of *SUP4*-o. Base-pair substitutions were recovered throughout *SUP4*-o but five locations were mutated more frequently than others (Figure 18), indicating that DNA sequence context influences the site specificity of mutagenesis.

No point mutations were detected outside the region encoding *SUP4*-o and only one position (+51) was mutated within the tRNA intron. Outside the intron, spontaneous base-pair substitutions were recovered at 63 of the 68 available sites in the gene (compare Table 3 and Figure 18). Only at one other intron position (+43) and at five exon sites (+4, +13, +70, +71 and +72), where spontaneous substitutions were not found, can changes be detected (Table 3). Thus, spontaneous base-pair substitutions were not limited to a small subset of sites or types of change. A variety of different mechanisms that may be responsible for spontaneous substitutions have been proposed. For example, it has been suggested that altered base-pairing during DNA replication due to tautomeric shifts or deamination of cytosine, replication errors made by DNA polymerase and failure to correct such errors or repair of spontaneous DNA damage may be involved (Speyer *et al*. 1966; Topal and Fresco 1976; Coulondre *et al*. 1978; Glickman *et al*. 1978; Sargentini and Smith 1981; Schaaper *et al*. 1983; Kunkel 1984).

Deletions (loss of 1 to 807 bp) constituted the second largest class (9%, including deletions in complex changes) of the spontaneous sequence alterations identified (Table 12). In 20 mutants, the deletions involved the loss of a single G·C pair from a run of 3 to 5 G·C pairs (Table 14) and may have been mediated by local misalignment of the complementary strands as proposed by Streisinger *et al*. (1966). Alternatively, given that the mutational target is a tRNA gene, it is conceivable that DNA secondary structure may have played a role in generating some of these changes (Ripley and Glickman 1983; Glickman and Ripley 1984). Processing of a DNA secondary structure, formed by pairing within a quasi-palindromic sequence, could have generated the complex change in which the sequence 5'-GATCTCA-3 at +58 to +64 was replaced by 5'-CCGGG-3' (Figure 26A ->

Figure 26. A Secondary Structure (A) that Can Potentially Direct Two Sequence Changes in the *SUP4*-o Gene. A -> B -> C details replacement of the sequence 5'-CCCGG-3' with 5'-TGAGATC-3'. A -> B' -> C' depicts replacement of the sequence 5'-GATCTCA-3' with 5'-CCGGG-3'. In each case, the appropriate segment would be removed and DNA synthesis, using the remaining single-stranded segment as a template, would effect the replacement. To simplify the diagram, only the transcribed strand is shown.

C'

A - T 50
A - T
A - T
T - A
T - A
C - G
C - G
C - G
G - C
G - C
G - C
G - C
G - C
G - C
G - C
G - C
A
A
G
C
T
92 5' A A C G 3'

B'

A - T 50
A - T
A - T
T - A
C - G
T - A
C A C T
C
C
82 G
G - C
G - C
C - G
G - C
A
A
G
C
T
G
A
T
C
86
94 5' A A C G 3'

A

A - T 50
A - T
A - T
T - A
C - G
T - A
C A C T 58
C
C
82 G
G - C 64
G - C
C - G
G - C
A
A
G
C
T
G A T
C
86
94 5' A A C G 3'

B

A - T 50
A - T
A - T
T - A
C - G
T - A
A C T 58
C
C
C
G
G - C 64
G - C
C - G
G - C
T - A
A
A
G
C
T
G A T
C
94 5' A A C G 3'

C

A - T 50
A - T
T - A
C - G
T - A
T - A
A - T
G - C
A - T
T - A
G - C
G - C
C - G
G - C
A
A
G
C
T
96 5' A A C G 3'
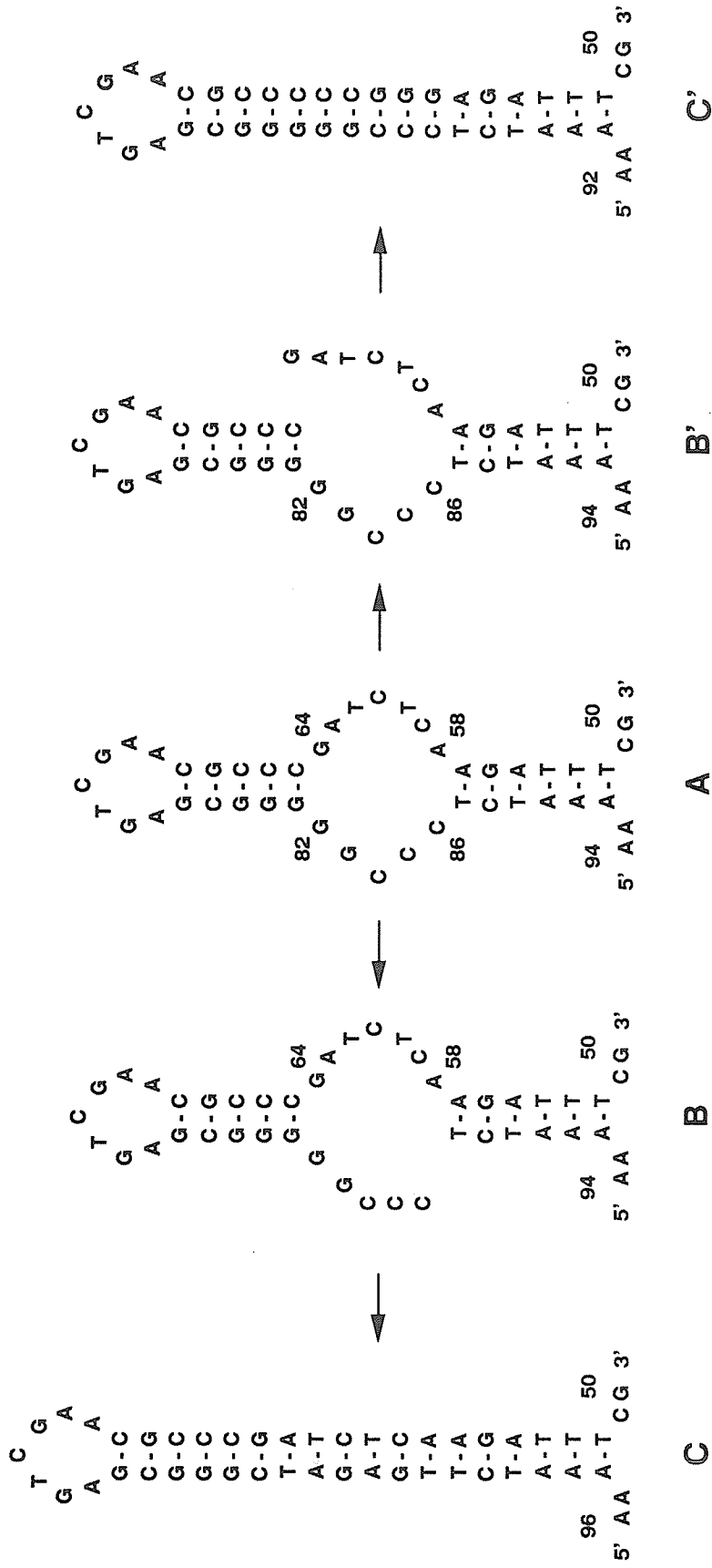
B′ -> C′). This possibility is strongly supported by the finding that the solitary complex event detected in SBT-TLp involved the other sequence replacement (5′-CCCGG-3′ at +82 to +86 converted to 5′-TGAGATC-3′) that could have been templated by the same secondary structure (Figure 26A -> B -> C).

Three mutants each carried a multiple base-pair deletion extending into the 3′ flanking sequence (Table 12). These ran either from +88 to +119, +89 to +96 or +89 to +97. The +90 to +96 region of the 3′ flanking sequence of *SUP4*-o functions as a signal for the termination of transcription (Figure 9), and deletions in this region decrease the efficiency of transcription termination *in vitro* (Allison and Hall 1985). However, among all of the spontaneous and induced *SUP4*-o mutants analyzed so far (over 5,000), those which have a deletion in the region of the termination signal have also lost at least the last base-pair (+89) of the *SUP4*-o coding sequence (B. A. Kunz, personal communication). Thus the effect of these deletions on suppression cannot be unequivocally ascribed solely to loss of the transcription termination signal. Two mutants, carrying deletions of 248 or 807 base-pairs in length, retained single copies of very short repeated sequences originally present at both deletion termini and so their generation might have involved some form of illegitimate recombination (Nalbantoglu *et al.* 1986).

In addition to base-pair substitutions and deletions, insertions were relatively common (8.1%) among the spontaneous *SUP4*-o mutants (Table 12). The majority (96%) were insertions of the yeast transposable element Ty (Table 15). Ty insertions were detected at three sites within the *SUP4*-o sequence, one being a hotspot for transposition (5′ to +38), one being located in the intron of the tRNA gene (5′ to +44) and one within the A

box of the internal promotor (5' to +18). The size of plasmids carrying a Ty insertion was either consistent with the presence of an intact Ty element or of a solo delta sequence. In the latter case, the insertion was most likely the remainder of a transposon that had excised by homologous recombination between its flanking delta sequences (Roeder and Fink 1983; Rothstein et al. 1987). Although the factors that promote transposition are not well understood, insertion generally has been found to occur in A·T-pair-rich sequences (Fink et al. 1980; Gafner and Philippsen 1980; Williamson et al. 1983). Consistent with this observation, Ty elements integrated at SUP4-o sites in regions of high A·T pair density with the site of most frequent integration (5' to +38) being in the region of greatest A·T pair density.

5.5 Mutational Specificity of dTTP Depletion

More than 96% of the mutations recovered after dTMP depletion are single base-pair events (Table 20) suggesting that DNA replication errors are the main reason for the mutagenesis associated with this treatment. The majority (71%) of the base-pair substitutions involve a replacement by an A·T pair and are consistent with misincorporation of the nucleotide in excess (dATP), while misincorporation of a dGTP (representing the dNTP pool with the second largest increase after dTTP depletion) could explain the remaining events (Table 21). However, dTTP depletion is commonly associated with a high dCTP:dTTP ratio (Neuhard and Munch-Peterson 1966; Maybaum et al. 1981) and based on this, an increase in the fraction of A·T to G·C transitions might be anticipated. Results obtained in reversion systems using bacteriophage T4 or E. coli seemed to confirm this expectation (Smith et al. 1973; Kunz and Glickman 1985). However, more

recent studies employing DNA sequence analysis indicated that A·T to G·C transitions did not constitute the majority of base-pair substitutions induced in the *E. coli lacI* gene by thymine starvation or drug treatment to inhibit thymidylate synthase (Glickman *et al*. 1988; Veigl *et al*. 1991). These findings are in agreement with results obtained during this investigation where only 5% of the base-pair substitutions induced by dTTP depletion were A·T to G·C transitions (Table 21). This, plus the predominance of substitutions involving replacement by an A·T pair, is consistent with the dATP:dTTP ratio being much higher than the dCTP:dTTP ratio in the dTMP starved cells (Table 16). However, it should be cautioned that dNTP concentrations measured in whole cell extracts might not accurately reflect the concentrations of dNTP's at the replication fork.

As mentioned above, dTTP depletion has been associated with elevated dUTP levels (Goulian and Beck 1966; Tattersall and Harrap 1973; Goulian *et al*. 1980; Sedwick *et al*. 1981). In turn, increased dUTP can result in misincorporation of dUTP in DNA in place of dTTP (Makino and Munakata 1978; Tamanoi and Okazaki 1978; Warner *et al*. 1981). Since both thymine and uracil pair with adenine and enzymes of DNA replication do not efficiently differentiate between uracil and thymine (Kornberg 1980), one might think that misincorporation of dUTP in place of dTTP should not result in mutations. However, *E. coli* mutants defective in dUTPase, which acts to limit the size of the dUTP pools, are mutators (Hochhauser and Weiss 1978; Sedwick *et al*. 1986). Uracil residues normally are rapidly excised from the DNA by the action of uracil-DNA glycosylase, leaving an AP site (Lindahl 1979; Caradonna and Cheng 1982). (Uracil-DNA glycosylase has been isolated from many different prokaryotic and eukaryotic

organisms, including yeast (Burgers and Klein 1986; Percival *et al.* 1989)). Thus, misincorporation of uracil into DNA could give rise to mutations in two ways. The first is based on the finding that purine dNTPs, in particular dATP, are preferentially inserted across from AP sites during DNA replication *in vitro* (Sagher and Strauss 1983; Randall *et al.* 1987; Takeshita *et al.* 1987). Insertion of dATP opposite an AP site resulting from excision of an uracil (misincorporated in place of thymine) would not cause a mutation, while the insertion of dGTP could lead to an A·T to G·C transition. However, in the *lacI* gene of an *E. coli* mutant (*dut*) with elevated dUTP levels due to a defect in its dUTPase, A·T to G·C transitions occurred only at one position and constituted only a minority (5/23) of the base-pair substitutions characterized (Sedwick *et al.* 1986). Similarly, only 5% of the base-pair substitutions recovered in this investigation following dTTP depletion were A·T to G·C transitions (Table 21) and some, if not all, of these could have been due to misincorporation of guanines. The second way in which misincorporation of uracil into DNA would give rise to substitutions during dTTP depletion would be if repair of the AP site was error-prone under conditions of dNTP imbalance. In this case, a variety of substitutions might be expected reflecting the particular dNTP imbalance. Consistent with this possibility several types of base-pair substitution were recovered in a *dut⁻* strain of *E. coli* (Sedwick *et al.* 1986) suggesting that mutations induced by elevated dUTP levels can be quite diverse. However, as discussed in section 5.2, the results of the dNTP assay tend to argue against dUTP misincorporation as major contributor to the induction of base-pair substitutions by dTTP depletion in yeast.

Increased dNTP levels could reduce the fidelity of replication either

by enhancing the extension of mismatched primers (Mendelman *et al.* 1990) or by inhibiting proofreading (Fersht 1979; Kunkel *et al.* 1981). Under conditions of imbalanced DNA precursor levels, this would lead to larger error rates at sites where the next nucleotide to be inserted is one present in excess. In order to probe for such effects, the bases 3' and 5' to substitution sites were analyzed, but no distinct preferences could be found possibly because the levels of dATP, dGTP and dCTP were increased by dTMP starvation of SBT-TLp (Tables 22 and 23). Nevertheless, following dTMP starvation, base-pair substitutions were not recovered at all sites where such changes can be detected in *SUP4*-o (compare Table 3 and Figure 19). In addition, individual frequencies at the mutated sites varied substantially. These findings argue that DNA sequence context of the mutation sites might play a role in the specificity of mutagenesis induced by dTTP depletion.

Single base-pair deletions constituted the next major class of mutation due to dTMP starvation (Table 20). It was suggested that incisions due to uracil removal could lead to deletions or DNA strand breaks during dTTP starvation (Breitman *et al.* 1972) and there is evidence that uracil incorporation during thymine deprivation can result in DNA strand breaks (Makino and Munakata 1978; Tamanoi and Okazaki 1978; Tye *et al.* 1978; Warner *et al.* 1981). However, all of the single base-pair deletions detected involved the loss of a G·C pair. This argues against a major role for incision resulting from the replacement of a thymine by uracil in the formation of single base-pair deletions. All of these deletions occurred at runs of 3 or 5 G·C pairs (positions +65 to +67 or +79 to +83) so that they could have been due to slipped mispairing of the template and nascent strands during replication through the runs

(Streisinger et al. 1966). However, it has been demonstrated that base-pair deletions can be a consequence of dNTP misincorporation when the misinserted dNTP is complementary to the next nucleotide in the template (Bebenek and Kunkel 1990). Misalignment of the template strand to loop out the nucleotide opposite which misinsertion occurred would efficiently place the misincorporated nucleotide ahead by one position. Correct base-pairing could stabilize this intermediate and extension of the primer would result in the loss of a single base-pair. Insertions could occur in a similar fashion when the misinserted nucleotide is complementary to the preceding template nucleotide. In this case, the nascent strand could be realigned so that the misinserted nucleotide would be moved back by one position and then extension of the primer would lead to the gain of one base-pair. Analysis of the DNA sequences at deletion or insertion sites revealed that for SBT-TLp the misinsertion of dGTP at positions +65, +78 or +84 on the transcribed strand and position +83 on the non-transcribed strand could have given rise to all of the single base-pair deletions and insertions recovered after dTTP depletion. Therefore, these types of change might have been caused by the misincorporation of one of the dNTPs in excess.

In addition to single base-pair deletions, the frequency of larger deletions was increased by dTTP depletion (Table 20). One of these deletions involved the loss of five base-pairs within the sequence +35 to +43. Due to the sequence in this region, the loss of five different sequences (+35 to +39, +36 to +40, +37 to +41, +38 to +42 or +39 to +43) could have accounted for the deletion in question. However, within this sequence (+35 to +43) there is a four base-pair repeat at positions +35 to +38 and +40 to +43 (Figure 27A). A strand misalignment during DNA

Figure 27. Model for a 5 Base-Pair Deletion Involving Strand Misalignment.
A: wild-type *SUP4*-o sequence, the four base repeats are
enclosed in boxes. B and B': DNA synthesis reaches the end of
the 5' repeat on the transcribed (B) or non-transcribed strand
(B'). C and C': slippage of the template strand allows the 5'
repeat to pair with the repeat on the nascent strand resulting
in the 5 intervening nucleotides on the template strand (C: +39
to +43; C': +35 to +39) forming a loop. D and D': DNA synthesis
then continues on the shortened template, the loop may or may
not be excised. E: mutant duplex is formed immediately, if the
loop was excised, or following the next round of replication if
the loop was not excised.

A

35   43

5'-A-G-A-C-T-T-A-A-T-T-T-A-T-C-A-C-3'
. . . .
3'-T-C-T-G[A-A-A-T][A-A-A-T]A-G-T-G-5'

B

5'-A-G-A-C[T-T-T-A][A]T-C-A-C-3'
. . . .
3'-A-A-A-T-A-G-T-G-5'

C

T   T
T   A
5'-A-G-A-C[T-T-T-A]T-C-A-C-3'
. . . . . .
3'-A-A-A-T-A-G-T-G-5'

D

T   A
T   A
5'-A-G-A-C[T-T-T-A]T-C-A-C-3'
. . . . . .
3'-T-C-T-G-A-A-A-T-A-G-T-G-5'

B'

5'-A-G-A-C-T-T-A-3'
. . . .
3'-T-C-T-G[A-A-A-T][A-A-A-T]A-G-T-G-5'

C'

5'-A-G-A-C-T-T-A-3'
. . . .
3'-T-C-T-G[A-A-A-T]A-G-T-G-5'
A   T
A   T
A

D'

5'-A-G-A-C-T-T-A-T-C-A-C-3'
. . . . . .
3'-T-C-T-G[A-A-A-T]A-G-T-G-5'
A   T
A   T
A

E

5'-A-G-A-C-T-T-T-A-T-C-A-C-3'
. . . .
3'-T-C-T-G[A-A-A-T]A-G-T-G-5'

synthesis (Drake *et al.* 1983) through this region might have been responsible for the deletion as diagrammed in Figure 27. During synthesis of the transcribed strand, strand misalignment could result in the formation of an intermediate structure in which the five bases at +39 to +43 on the template strand form a loop (+35 to +39) and the bases from the repeat at +35 to +38 pair with the nascent strand at positions +40 to +43 (Figure 27B, C and D). This intermediate can be resolved by deleting the loop and continuing replication or, if the loop is not deleted, by a second round of DNA replication. In either case, the result will be the deletion detected (Figure 27E). In a similar fashion, misalignment during the synthesis of the non-transcribed strand could result in the loss of the bases at position +35 to +39 (Figure 27B′, C′, D′ and E). Loop-formation could also be mediated by the misalignment of repeats consisting only of two or three base-pairs within +35 to +43 (e.g. +37 to +38 with +42 to +43 or +36 to +38 with +41 to +43). Such misalignments could lead to the loss of the five base-pairs at +36 to +40, +37 to +41 and +38 to +42. However, because these misalignments would involve shorter repeats, they probably would result in less stable intermediates. It is interesting to note that the next two bases to be incorporated after the first four-base repeat during synthesis of the transcribed strand (Figure 27 B) are thymines. Considering the low dTTP levels in dTMP starved cells, it is conceivable that DNA replication would slow at this point. This might encourage the formation of the misaligned structure during dTTP depletion and favour the loss of the bases extending from +35 to +39.

None of the three multiple base-pair deletions detected after dTTP depletion had repeated sequences at its endpoints. Thus, it is possible that they resulted from some type of illegitimate recombination. In this

regard, it is worth pointing out that all three deletions occurred within, or were flanked on one or both sides by at least five A·T pairs. This leaves open the possibility that DNA incisions at the sites of misincorporated uracils could have been involved in the formation of multiple base-pair deletions. Such a possibility is supported by the finding that repair of closely opposed uracil residues on opposite DNA strands can generate double-strand breaks (Dianov et al. 1991) and such breaks can induce recombination (Kunz and Haynes 1982).

5.6 Mutational Specificity of dCMP Deaminase Deficiency and Excess dTMP

The mutagenesis associated with the dNTP perturbations observed in the dCMP deaminase deficient strain and after treatment with excess dTMP appeared to be due primarily to DNA replication errors. More than 98% of the total mutations were single base-pair events (Table 28). The majority (77% or 100% for MKP-op and SBT-TLp treated with excess dTMP, respectively) of the base-pair substitutions were attributable to misinsertion of the dNTP(s) in excess (Table 29). As outlined in section 5.5 single base-pair deletions and insertions can also be a consequence of misincorporation of a dNTP present in excess. Inspection of Figure 21 reveals that for MGK-dp, misinsertion of dCTP at position +79 on the transcribed strand of SUP4-o and at position +84 on the non-transcribed strand, followed by the appropriate misalignment, could have generated 18 of the 19 deletions and the single insertion detected in this strain. For SBT-TLp, misincorporation of dTTP at positions +7 and +89 on the non-transcribed strand and of dGTP at positions +68 and +83 on the non-transcribed strand and position +78 on the transcribed strand, could have given rise to all of the deletions and insertions recovered in this

strain. Of the various substitutions required to generate the deletions and insertions in each strain, only misinsertion of dGTP at position +68 on the non-transcribed strand in SBT-TLp was not detected (Figure 21). Clearly, the majority (40/42) of single base-pair deletions and insertions that occurred under conditions of dNTP imbalance could have resulted from misincorporation of the dNTP(s) in excess. It should be cautioned here, however, that it is not certain how accurately the dNTP pool measurements reflect dNTP concentrations at replication forks in growing yeast. Examination of the distributions of the base substitutions revealed that the mutations occurring under conditions of dNTP imbalance were unlikely to result solely from dNTP misinsertion. Putative replacements by T or C were found at only one-half or two-thirds, respectively, of the sites where such changes can be detected in *SUP4*-o. Furthermore, T to C transitions, and possibly A to C transversions, in MGK-dp exhibited a bias for sites where dCTP was the next correct nucleotide to be inserted (Table 31). Similarly, C to T and A to T substitutions recovered after dTMP treatment occurred preferentially at sites where the next correct nucleotide to be incorporated was dTTP or dGTP (Table 31).

These site preferences were consistent with two mechanisms whereby increased levels of the next correct nucleotide could reduce the fidelity of replication: enhanced extension of mismatched primer termini (Mendelman *et al.* 1990) and inhibition of proofreading (Fersht 1979; Kunkel *et al.* 1981). The effect of dNTP concentration on extension of mismatched primer termini is thought to apply only to situations where extension occurs in the absence of proofreading (Mendelman *et al.* 1990). Yeast DNA polymerases (pol) α, ϵ and δ (formerly termed pol I, II and III (Burgers *et al.* 1990)) are essential for DNA replication *in vivo* (Johnson *et al.* 1985; Boulet *et*

al. 1989; Sitney *et al.* 1989; Morrison *et al.* 1990) and 3' to 5' exonuclease activities, which may function in proofreading, have been detected for all three polymerases (Bauer *et al.* 1988; Kunkel *et al.* 1987; Hamatake *et al.* 1990; Morrison *et al.* 1990; Brooke *et al.* 1991). Recently, it has been suggested that pol α initiates DNA synthesis at replication origins and primes lagging strand synthesis while replication of the leading and lagging strands is primarily the responsibility of pol ϵ and δ, respectively (Morrison *et al.* 1990). According to this view, the normally high fidelity of DNA synthesis in yeast would be due, in part, to proofreading during replication of both the leading and lagging strands. Thus, it seems reasonable to think that the next-nucleotide-like effects observed in this study were a consequence of inhibiting proofreading rather than enhancing extension of mismatched primer termini. If so, the finding that there was no strand preference for presumptive nucleotide misinsertion events would support the possibility that proofreading occurs in yeast during replication of each strand in a duplex.

Although these data suggested that both dNTP misinsertion and next-nucleotide effects contribute to the mutagenicity of dNTP imbalances in yeast, it seems likely that other factors, which may influence these processes, also are involved. The dTTP:dCTP ratio in dTMP-treated cells of SBT-TLp (excess dTMP) and the dCTP:dTTP ratio in MGK-dp were both increased by about the same magnitude (120-fold) over the corresponding ratios in the parental strain MKP-op (Table 25). Nevertheless, the *SUP4*-o mutation rate was enhanced to a much greater extent in SBT-TLp (excess dTMP) than in MGK-dp. With regard to nearest-neighbour influences, there was no next-nucleotide effect for G·C to C·G transversions in MGK-dp, despite the fact that these events constituted the majority of base-pair

substitutions in this strain, or for G·C to T·A transversions in SBT-TLp (excess dTMP). In addition, the specificity of base-pair substitutions in both strains exhibited features that were not anticipated on the basis of the measured dNTP pool perturbations and previous results for pyrimidine dNTP imbalances in Chinese hamster ovary cells (Phear and Meuth 1989a,b). In those studies, elevated dTTP or dCTP levels were associated with notable increases in the relative fractions of G·C to A·T or A·T to G·C transitions, respectively. Among transversions, the only increase was in the proportion of A·T to C·G events when the dCTP pool was enhanced. In this study, the proportion of A·T to G·C transitions in MGK-dp remained the same as for MKP-op but the fractions of G·C to C·G and A·T to C·G transversions increased. For dTMP treatment of SBT-TLp, the proportion of G·C to A·T transitions did not change relative to MKP-op whereas there was a large increase in the fraction of A·T to T·A transversions and the fraction of G·C to T·A transversions decreased by 50%.

Here factors, other than obvious differences in experimental systems, that might account for the results of this study are considered. The much greater increase in the *SUP4*-o mutation rate in dTMP-treated SBT-TLp vs. MGK-dp might have been partially due to the larger final size of the dTTP pool in SBT-TLp than of the dCTP pool in MGK-dp. Conceivably, exceeding a certain dTTP level might have caused a dramatic increase in the rate of dTTP misinsertion. Alternatively, unknown aspects of the *SUP4*-o DNA sequence may have favoured misinsertion of dTTP in SBT-TLp over dCTP in MGK-dp under conditions of dNTP imbalance. Certainly, the presence of hotspots and coldspots for substitution mutagenesis in both strains suggests that DNA sequence context can influence nucleotide misinsertion.

Another possibility is that error correction by proofreading might

have been diminished to a much greater extent in SBT-TLp (excess dTMP). This might have occurred in three non-mutually exclusive ways. First, the very large dTTP pool generated by dTMP treatment might have more effectively inhibited proofreading via a next-nucleotide effect than the smaller dCTP pool in MGK-dp. Second, the marked disturbances in the dTTP levels might have offset the control of proofreading activity. A defect in the *mutD* (= *dnaQ*) gene, which encodes the *E. coli* DNA polymerase III subunit (ϵ) with 3' to 5' proofreading activity (Echols *et al.* 1983; DiFrancesco *et al.* 1984), confers a mutator phenotype (Degnen and Cox 1974). Growing cells in minimal medium supplemented with thymidine enhances this effect (Degnen and Cox 1974) and a thymine nucleotide, was implicated as the mutagenic effector (Erlich and Cox 1980). These findings, together with identification of a binding site specific for dTTP on the ϵ subunit (Biswas and Kornberg 1984) led to the suggestion that binding of dTTP to ϵ is involved in the control of proofreading (Biswas and Kornberg 1984; Cox and Horner 1986). If only dTTP plays a similar role during DNA synthesis in yeast, the increased dTTP levels in SBT-TLp treated with dTMP might have promoted mutagenesis by altering the regulation of proofreading activity *in vivo*. Third, the elevated levels of dTMP also expected to be present in these cells might have contributed to the inhibition of proofreading. This suggestion is based on the findings that treatment of a yeast dTMP auxotroph with 0.8 g/l thymidylate for 6 h increased the intracellular dTMP concentration by 30-fold (Toper *et al.* 1981) and the 3' to 5' exonuclease activities of *E. coli* DNA polymerase I and rabbit DNA polymerase δ were inhibited by dTMP, although the effect was less pronounced for the mammalian polymerase (Byrnes *et al.* 1977; Que *et al.* 1978).

Neither G·C to C·G transversions in MGK-dp, nor G·C to T·A trans-versions in SBT-TLp (excess dTMP) were associated with a next-nucleotide effect (Table 31). *In vitro* evidence indicates that 3' to 5' exonucleo-lytic proofreading may excise particular mispairs much less efficiently than others during DNA replication (Reyland *et al*. 1988). Thus, one simple explanation for the absence of a next-nucleotide effect would be that mispairing of dCTP or dTTP with template C is not proofread efficiently in yeast. If so, one might expect that other means to reduce such replication errors have evolved. Although the specificity of proofreading by yeast DNA polymerases has yet to be addressed, it is interesting to note that dCTP-C and dTTP-C mispairs are among those formed at the lowest rates *in vitro* by purified yeast DNA polymerase α-primase, a complex that lacks 3' to 5' exonuclease activity (Kunkel *et al*. 1987).

The production of a base-pair substitution by dNTP imbalance not only requires misinsertion of an incorrect nucleotide and failure to proofread the error, but also that the mispair not be restored to the correct base-pair prior to the next round of DNA replication. Thus, it might be expected that the specificity of base substitution mutagenesis induced by perturbation of dNTP levels would be influenced by the relative efficiencies of correcting different mismatches. In yeast, C-C and T-T mispairs are among the least efficiently corrected base mismatches (Bishop *et al*. 1989; Kramer *et al*. 1989). Consequently, the increase in G·C to C·G transversions in MGK-dp and A·T to T·A transversions in SBT-TLp treated with dTMP might partially reflect the inefficiency of correcting C-C and T-T mismatches, respectively (it should be noted that the enhanced fraction of G·C to C·G transversions in MGK-dp was also consistent with the very large increase (184-fold) in the dCTP:dGTP ratio in this strain

compared to MKP-op). Similarly, the lack of change in the proportions of A·T to G·C and G·C to A·T transitions in MGK-dp and SBT-TLp (excess dTMP), respectively, and the small increase in the fraction of A·T to C·G transversions in MGK-dp, might be explained in part by the finding that A-C and G-T mismatches are among those repaired more efficiently in yeast whereas T-C mismatches are somewhat less efficiently corrected. This line of reasoning is attractive but the role of mismatch correction in the specificity of dNTP-induced base substitution should be interpreted cautiously. DNA sequence context effects and variations in genetic background may blur distinctions in correction efficiencies (Bishop et al. 1989; Kramer et al. 1989). This might be one reason why G·C to C·G and A·T to T·A transversions were not recovered to any significant extent among aprt mutations induced by elevated dCTP or dTTP levels in Chinese hamster ovary cells (Phear and Meuth 1989a,b) even though C-C and T-T mismatches are corrected with low efficiency in monkey and human cells (Brown and Jiricny 1988; Holmes et al. 1990). Furthermore, it remains to be determined whether dNTP imbalances can modulate the specificity and/or efficiency of mismatch correction.

5.7 Elevated dCTP Levels Alter the Specificity of EMS and MNNG Mutagenesis
5.7.1 Effect of Alkylation Treatment on Intracellular dNTP Levels

The sizes of the four dNTP pools were reduced in mock-treated cells of MKP-op and MGK-dp (the treatment procedure was performed with sterile water in place of an alkylating agent) compared to untreated cells (Tables 33 and 41). The ratio of the four dNTPs was the same in both cases indicating that all four dNTP pools were affected equally by the treatment conditions. The simplest explanation for the general reduction in pool

sizes is that experimental conditions prevented the cells from replicating and therefore fewer cells were in S phase producing dNTPs for DNA synthesis.

dNTP levels in EMS- or MNNG-treated cells of MKP-op and MGK-dp were in general higher than the corresponding levels in mock-treated cells (2- to 6-fold in MKP-op; 1.8- to 2.5-fold in MGK-dp). The only exception was the elevated dCTP level in MGK-dp where approximately the same concentrations were evident for mock-, EMS- and MNNG-treated cells (Table 41). Furthermore, the dNTP levels in MNNG-treated cells of MKP-op and MGK-dp seemed to be increased on average more than the dNTP levels for cells treated with EMS. DNA degradation, excision of nucleotides during repair or decreased incorporation of dNTPs into DNA during DNA replication have been suggested to cause elevated dNTP levels following treatment with mutagens (Slaby *et al.* 1971; Eckstein *et al.*. 1974; Meuth 1989). However, these possibilities cannot explain the effect of the mutagens on dNTP pools in this study. Only dNTP levels can be determined with the enzymatic assay employed here and the degradation of DNA would release dNMPs while repair enzymes (alkyl-DNA glycosylase, alkyl-DNA transferase) that correct alkylation damage would excise bases or remove alkyl groups. In addition, during alkylation treatment the cells do not multiply and the mock-treated cells have decreased dNTP levels.

Another alternative is that dNTP levels might be increased after mutagen treatment to supply dNTPs required for DNA repair synthesis of the induced damage. This possibility is supported by findings that in yeast some genes encoding enzymes (dTMP kinase, subunits of the ribonucleotide reductase) involved in DNA precursor synthesis are induced by DNA damaging agents (Elledge and Davis 1987, 1990). Near matches of two consensus

sequences, which have been associated with regulating the damage inducibility of genes encoding the ribonucleotide reductase subunits, can also be seen to be present upstream of the coding sequences for the dCMP deaminase, dihydrofolate reductase, dUTPase and thymidylate synthase in yeast (Birkenmayer et al. 1984; McIntosh and Haynes 1986; Lagosky et al. 1987; Taylor et al. 1987; Sebastian et al. 1990; Yagle and McEntee 1990). Although the damage inducibility of the latter four genes has yet to be investigated, these findings provide grounds for thinking that induction by DNA damage of genes required for DNA precursor synthesis might account for the increased dNTP levels observed after treatment with EMS or MNNG.

5.7.2 Role of Neighbouring Bases and Assessment of Strand Specificity in EMS and MNNG Mutagenesis

DNA sequence analysis of 152 EMS-induced and 166 MNNG-induced SUP4-o mutations revealed that, in both cases, over 96% were G·C to A·T transitions (Table 36). These results are in agreement with numerous observations (Prakash and Sherman 1973; Coulondre and Miller 1977; Dodson et al. 1982; Loechler et al. 1984; Bhanot and Ray 1986; Burns et al. 1986, 1987; Lebkowski et al. 1986; Lucchesi et al. 1986; Ashman and Davidson 1987a; Reed and Hutchinson 1987; Richardson et al. 1987a) which suggest that O^6-alkylguanine is the major premutational lesion produced by EMS and MNNG. A few transversions at G·C sites also were identified after EMS and MNNG treatment. For EMS, two of the three transversions recovered were each part of double mutants where the transversions were separated by 1 or 3 base pairs from G·C to A·T transitions (Figure 23). Presumptive double mutations have been detected in the E. coli lacI gene after EMS treatment (Coulondre and Miller 1977). These findings are consistent with the

suggestion (Reed and Hutchinson 1987) that, occasionally, an alkylating agent-induced G·C to A·T transition may trigger a second, nearby substitution event. The remaining EMS-induced transversion (G·C to C·G at +80) and three of the MNNG-induced transversions (G·C to T·A at +73 and +80; G·C to C·G at +5) also have been found among a collection of 334 spontaneous *SUP4*-o mutations (Figure 18) and so may have originated spontaneously. However, one MNNG-induced transversion (G·C to C·G at +81) was recovered at a site where such an event has not yet been found to arise spontaneously, suggesting that it may have been induced. Alternatively, the G·C to T·A substitution could have been the result of alkylation at the *N*-7 or *N*-3 positions of guanine (Singer and Kusmierek 1982; Saffhill *et al.* 1985). Methylation or ethylation at these positions induces AP sites via destabilization of the ß-*N*-glycosyl bonds (Loeb and Preston 1986) and preferential incorporation of adenine across from the AP sites (Sagher and Strauss 1983) would produce G·C to T·A transversions. A similar explanation might account for the occurrence of the G·C to C·G substitution since guanine can be inserted opposite AP sites *in vitro* (Sagher and Strauss 1983).

In addition to events at G·C sites, one A·T to G·C transition after EMS treatment and two after MNNG treatment were also identified. It seems reasonable to suggest that these substitutions may have been due to the production of O^4-alkylthymine. This lesion has been detected after alkylation of DNA *in vitro* (Singer and Kusmierek 1982; Saffhill *et al.* 1985; Singer 1986) and can mispair with guanine to give rise to A·T to G·C transitions (Saffhill and Hall 1985; Preston *et al.* 1986, 1987; Singer *et al.* 1986). Furthermore, it is produced to a much lesser extent than O^6-alkylguanine (Singer and Kusmierek 1982). It is unlikely that the A·T

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to G·C transitions were a consequence of alkylation at the O^6 position of guanines in deoxyribonucleotide pools (Topal and Baker 1982) followed by insertion of O^6-alkylguanines opposite template thymines. Although it was shown that modified dNTP's can be incorporated into the DNA, most of these experiments were done under non-physiological conditions (Dodson et al. 1982; Hall and Saffhill 1983; Toorchen and Topal 1983; Eadie et al. 1984; Snow et al. 1984b). Furthermore, O^6-methylated precursors (including bases, nucleosides and nucleotides) accounted for >0.1% of the unmodified intracellular purines (Topal and Baker 1982). Their conversion to triphosphates is often prevented due to a lack of enzyme recognition of the modified precursors (Pegg and Swan 1979; Ball et al. 1983; Hsie et al. 1986; Snow and Mitra 1987). In addition, modified precursors are often highly susceptible to degradation (Hsie et al. 1986; Snow and Mitra 1987).

An analysis of the influence of neighbouring base sequence on mutation induction showed that MNNG, but not EMS, mutagenesis occurred more frequently at 5'Pur-G sites than at 5'Pyr-G sites (Table 38). These results are in general agreement with previous findings in prokaryotic systems (Burns et al. 1986, 1987; Reed and Hutchinson 1987; Richardson et al. 1987a). To account for this preference, it has been suggested (Burns et al. 1987; Richardson et al. 1987a) that flanking bases may : 1. influence the reactivity of the O^6 position of guanine and so the distribution of DNA damage; 2. affect the accessibility of O^6 positions to methylating species by influencing local DNA helix geometry; 3. modulate the efficiency of mismatch correction or O^6-methylguanine repair by methyltransferase; or 4. influence the fidelity of DNA replication. In the prokaryotic studies, 5'G-G sites were found to be preferred over 5'A-G sites whereas results in this study seem to indicate that the converse is true for yeast. This

interpretation must be viewed cautiously, however. If the data for the MNNG hotspot at position +1 in *SUP4*-o are excluded from the analysis, then although 5'Pur-G sites are still preferred, there is no difference between 5'A-G and 5'G-G sites. Yet, the preference for 5'A-G sites might be genuine since the *SUP4*-o region from +79 to +86, which consists of a run of 5 guanines on the transcribed strand followed by a tract of 3 guanines on the non-transcribed strand, was a relatively poor target for MNNG mutagenesis (Figure 23). Nevertheless, it is clear from these findings that 5'G-G sites are not preferred over 5'A-G sites in *SUP4*-o. Thus, these results indicate that subtle differences may distinguish the site specificity of MNNG mutagenesis in prokaryotes and yeast.

Neither EMS nor MNNG mutagenesis in *SUP4*-o showed a preference for G·C sites having the guanine on the non-transcribed strand (Table 40), a feature that has been observed for MNNG mutagenesis in some prokaryotic systems (Reed and Hutchinson 1987; Richardson *et al.* 1987a). It must be stressed that such a preference was not found for EMS or MNNG mutagenesis of the *E. coli lacI* gene carried on an F' episome or for MNNG mutagenesis of the phage P22 *mnt* gene inserted into the bacterial plasmid pBR322 (Burns *et al.* 1987; Lucchesi *et al.* 1987). However, it has been pointed out (Reed and Hutchinson 1987) that in these two cases, the target genes are extrachromosomal in nature. Although the *SUP4*-o gene also is located on a plasmid, the vector in question mimics the behaviour of yeast chromosomes (Pierce *et al.* 1987; Giroux *et al.* 1988). Furthermore, it was found that observed features of spontaneous mutagenesis at *SUP4*-o on the plasmid and at its chromosomal locus are comparable (section 5.4) suggesting that the same mutational mechanisms act on the plasmid and chromosomal copies of the gene. Consequently, it seems reasonable to think

that the extrachromosomal location of *SUP4*-o is not responsible for the lack of strand preference in EMS or MNNG mutagenesis.

To account for the strand preference, it has been proposed that alkyl-transferase may act less efficiently on the non-transcribed strand since it is single-stranded during transcription and the enzyme is known to repair double-stranded DNA more efficiently (Reed and Hutchinson 1987). However, MNNG mutagenesis still exhibited a preference for guanines on the non-transcribed strand in an unadapted *E. coli* mutant that has a 3,000-fold less active alkyltransferase than wild-type (Richardson *et al.* 1987a). Furthermore, yeast has an alkyltransferase (Yarosh 1985; Pegg 1990; Sassanfar and Samson 1990) and shows no strand bias for alkylation mutagenesis. Therefore, an explanation based on a strand-specific action of an alkyltransferase seems unsatisfactory.

More recently, it was suggested that rather than differences in the metabolic make-up of organisms, non-random distribution of mutational target sites could be the cause for the observed strand bias of MNNG-induced mutations in some mutational studies (Gordon and Glickman 1988; Gordon *et al.* 1988; Horsfall *et al.* 1990). Most mutational targets are genes that encode proteins. MNNG predominantly alkylates guanines and in *E. coli* guanines preceded 5' by a G are hit most frequently (Burns *et al.* 1987; Reed and Hutchinson 1987; Richardson *et al.* 1987a; Glickman *et al.* 1987; Gordon *et al.* 1988). It was therefore argued, that amino acids encoded by triplets containing two neighbouring guanines (mutational target site is on the non-transcribed strand) or two consecutive cytosines (mutational target site is on the transcribed strand) are potential hotspots for MNNG mutagenesis. G·C to A·T transitions at most of these codons (UCC, ACC, GCC, CGG, AGG) cause rather conservative amino acid

replacements and mutations at these sites are therefore likely to be silent. However three possibilities are worth discussing in more detail. Glycine (GGX; X being any base) can be a flexible component of hinges and turns and can be an intrinsic part of the protein structure. Mutations at these codons could result in a mutant phenotype. In addition, G·C to A·T transitions at the tryptophan codon (UGG) leads to the formation of an amber or ochre stop-codon. Proline (CCX; X being any base) is known to cause bends in protein structures and is therefore of structural importance. However, G·C to A·T transitions at the first C residue results in replacement by a serine which, similarly to proline has a high potential for the formation of ß-turns (Chou and Fasman 1978). Due to these properties of the amino acids and their respective codons, a large number of mutable glycines and tryptophan residues could result in a higher frequency of MNNG-induced mutations on the non-transcribed strand, while an over-representation of mutable prolines would shift the bias to the transcribed strand. It was shown (Gordon and Glickman 1988) that in the target gene used by Reed and Hutchinson (1987) the majority of mutations examined (14/18) occurred at glycine residues and these residues accounted for 5 of the 8 mutation sites detected. Furthermore, analysis of the MNNG-induced G·C to A·T transitions reported by Richardson *et al.* (1987a) indicated that 50% of the mutations (18/37) occurred at sequences coding either for tryptophan or glycine. This suggests that the bias observed (Reed and Hutchinson 1987; Richardson *et al.* 1987a) for the non-transcribed strand could be due to a non-random distribution of target sites. Since *SUP4*-o is a tRNA gene, studies of mutational specificity in *SUP4*-o are not influenced by codon usage.

Finally, this portion of the study demonstrates that EMS and MNNG

mutagenesis of the *SUP4*-o gene have certain features in common. There is considerable overlap in the sites that were mutated and those that were missed by each agent (Figure 23, Table 37). Similarly, EMS- and MNNG-induced G·C to A·T transitions were found to occur at many of the same sites in the *lacI* gene of *E. coli* (Coulondre *et al.* 1977; Burns *et al.* 1986, 1987). Furthermore, several sites in *SUP4*-o were mutated frequently by both EMS and MNNG (Table 37), a feature also seen for the *lacI* gene (Coulondre *et al.* 1977; Burns *et al.* 1986, 1987). Where there are differences in the frequencies of induced *SUP4*-o and *lacI* mutations at sites mutated by EMS and MNNG, they often appear to be attributable to the lack of preference of EMS for 5'Pur-G sites (Table 38; Burns *et al.* 1986, 1987). However, dissimilarities in the reactivities of EMS and MNNG with DNA, steric accessibility of the agents to reactive sites in DNA and recognition and repair of methylated or ethylated DNA adducts also may contribute (Singer and Kusmierek 1982; Saffhill *et al.* 1985; Cooper and Waters 1987). Even so, the results of this study and the *lacI* data argue that while the site-specific frequencies of MNNG mutagenesis are influenced by the 5' flanking base, there must be additional factors that are able to direct the distribution of both EMS- and MNNG-induced mutations.

5.7.3 Elevated dCTP Levels and Alkylation Mutagenesis

A number of studies have provided evidence for links between dNTP levels and induced mutagenesis (Kunz 1988). EMS and MNNG mutagenesis in particular were found to be affected by high and low dTTP:dCTP ratios, high ratios increasing and low ratios decreasing the frequency of EMS- or MNNG-induced mutations (Meuth 1981a, 1983; Peterson *et al.* 1985a). In

agreement with these expectations, EMS- and MNNG-induced *SUP4*-o mutation frequencies were reduced by 60% and 40%, respectively, in a dCMP deaminase deficient strain (MGK-dp) having elevated dCTP levels compared to results obtained under conditions of normal dNTP balance (MKP-op) (Table 42). DNA sequence analysis of 203 EMS- and 202 MNNG-induced *SUP4*-o mutations confirmed that the frequency of G·C to A·T transitions was substantially reduced (by 90% and 70%, respectively; Tables 46 and 47). This finding is consistent with the prediction that the frequency of EMS- and MNNG-induced O^6-alkylG·T mispairs would be reduced by the presence of elevated dCTP concentrations. Surprisingly, however, these reductions in the G·C to A·T transition frequencies were larger than the decreases observed in the overall *SUP4*-o mutation frequency. An increase in the frequency of EMS- and MNNG-induced G·C to C·G transversions (35- and 12-fold, respectively) was mainly responsible for this discrepancy.

Even though the increase of EMS- and MNNG-induced G·C to C·G transversions in MGK-dp was unexpected there are several potential explanations for it. First, G·C to C·G transversions constitute the major class (58%) of base-pair substitutions occurring spontaneously in MGK-dp (Table 29). So the increase in G·C to C·G events following alkylation treatment might simply reflect the mutational specificity of the *dcd1* mutator. However, this change was detected at least 7.5-fold more frequently after EMS or MNNG treatment. Therefore, the majority of G·C to C·G transversions were induced by the alkylation treatment and were not part of the spontaneous background in MGK-dp.

Second, the increase in G·C to C·G transversions might be due to dCTP acting as a target for alkylation mutagenesis (Topal and Baker 1982). The O^2 and *N*-3 positions of cytosine are potential targets for alkylation by

EMS and MNNG (Table 1) and the addition of an alkyl group at these positions could possibly interfere with hydrogen bonding by cytosine. Indeed, a codon-anticodon study revealed that O^2-ethylcytidine acts almost entirely as if it is uracil (Singer et al. 1979). However, if alkylated dCTP is involved in a mechanism which leads to the observed increase in G·C to C·G transversions, then the formation of an alkylC·C intermediate would be required. To date, no evidence for the role of such a mispair in alkylation mutagenesis has been reported.

Third, elevated dCTP levels might modify the accuracy of repair processes. The N-7 position of guanine is the major target in DNA for alkylation by EMS and MNNG (Table 1). Removal of N-7-alkylguanine by alkyl-DNA glycosylase or destabilization of its β-N-glycosyl bond can create an AP site. Repair of such sites involves the action of AP endonuclease, exonuclease, DNA polymerase and DNA ligase (Sancar and Sancar 1988). Under balanced dNTP levels this type of repair is virtually error-free. Yet, it is possible that in cells having elevated dCTP levels, dCTP might be misincorporated during repair of apurinic sites induced at G·C pairs. If left unrepaired, the resulting C-C mismatch would lead to a G·C to C·G transversion during the next round of DNA replication. Should this mechanism be responsible for the induction of G·C to C·G transversions, it would suggest that only the AP site is removed during repair. Otherwise, a specific increase in G·C to C·G events would not be expected.

Frequencies of A·T to G·C transitions and G·C to T·A and A·T to T·A transversions were also enhanced relative to the spontaneous frequencies in MGK-dp by alkylation treatment (Tables 46 and 47). EMS- and MNNG-induced A·T to G·C transitions occurred more frequently (2.5- and 4-fold, respectively) in MGK-dp than in MKP-op (Tables 46 and 47). The observed

increase could have been the result of C misincorporation during DNA replication past AP sites created by the removal of N-3-alkyladenine, a lesion produced by EMS and MNNG treatment (Table 1). This would link the induction of at least a fraction of these base-pair substitutions to the elevated dCTP levels in MGK-dp. EMS- and MNNG-induced G·C to T·A transversions were detected at about the same frequencies in MKP-op and MGK-dp and it is therefore unlikely that their induction was influenced by the elevated dCTP levels in MGK-dp. Although A·T to T·A transversion were not detected among the EMS- or MNNG-induced changes in MKP-op, recovery of just one or two of these events would have given frequencies similar to those for MGK-dp (Tables 46 and 47). The G·C to T·A and A·T to T·A transversions in MGK-dp could have resulted from the preferential incorporation of an adenine across from an AP site (Sagher and Strauss 1983) created by the removal of N-7- or N-3-alkylguanine or by the removal of O^4-alkylthymine, respectively (Singer and Kusmierek 1982; Saffhill et al. 1985).

MNNG- but not EMS-induced G·C to A·T transitions preferentially occurred at 5'Pur-G sites in MKP-op. Neither of the two agents showed a preference for inducing mutations at sites having guanine on the transcribed or non-transcribed strand in this strain. Elevated dCTP levels did not affect these features of EMS and MNNG mutagenesis (Tables 50 and 51). This suggests that although the recovery of EMS- and MNNG-induced G·C to A·T transitions was reduced by elevated dCTP levels, the overall mechanism of G·C to A·T induction was not affected.

5.8 Future Research

This work presented in this thesis points to several lines of

investigation. In addition to imbalances in the supply of pyrimidine dNTPs, alterations in the levels of purine DNA precursors are of interest, in particular because enzyme deficiencies in purine metabolism and purine dNTP imbalances have been associated with human immunodeficiency diseases. *Tup* strains are capable of taking up dNMPs other than dTMP and can be used to induce purine dNTP imbalances in yeast.

Given the results observed for alkylation mutagenesis in MGK-dp, it would be informative to investigate the influence of excess dTTP and dTTP depletion on EMS and MNNG mutagenesis. Based on the dCTP:dTTP ratios observed in this study, excess dTTP should favour the formation of O^6alkylG·T mispairs and lead to an increase in EMS- or MNNG-induced G·C to A·T transitions compared to MKP-op, while dTTP depletion should, as seen for alkylation mutagenesis in MGK-dp, favour the incorporation of cytosine opposite O^6-alkylguanine. However, after dTTP depletion dCTP levels are much lower than the ones observed in MGK-dp and so it is likely that the effect of dTTP depletion on the induction of G·C to A·T transitions would be less pronounced. If the G·C to C·G transversions observed following treatment of MGK-dp with EMS or MNNG were the result of dCTP misincorporation during the repair of AP sites, it is possible that elevated dTTP levels during alkylation mutagenesis would lead to G·C to T·A transversions.

Analysis of EMS- and MNNG-induced mutations in MKP-op and MGK-dp suggested that the repair of alkylation damage under conditions of dNTP imbalance might play a role in alkylation mutagenesis. Characterization of alkylation-induced mutations in strains deficient for enzymes responsible for the repair of alkylation damage and for enzymes involved in DNA precursor synthesis could help to probe the mechanisms involved. For

example, cells lacking the alkyl-DNA glycosylase are unable to remove N-7-alkylguanine and will not produce AP sites via this repair mechanism. Consequently, if the induction of $G \cdot C$ to $C \cdot G$ transversions in alkyl-DNA glycosylase deficient cells having elevated dCTP levels were decreased, it would support the hypothesis that misincorporation of dCTP during the repair of AP sites gives rise to these transversions.

Such avenues of research could provide a better understanding of the links between dNTP levels and cellular responses to mutagenesis.

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