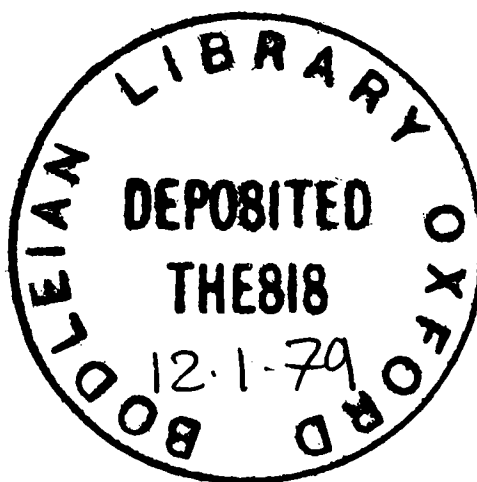


Genetics of Nonsense Suppressors
in Yeast

A thesis presented by
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for the degree of Doctor of Philosophy
at the University of Oxford.

Long Vacation 1978.



Acknowledgements

I am greatly indebted to my supervisor Dr. Brian Cox who was a continual source of encouragement, advice and constructive criticism. I wish to thank Professor F.R. Whatley for allowing me to use the facilities at the Botany School. Oxford. Special thanks are due to Dorothy Hoy, Wendy Blakey and my wife, Janice, for typing this thesis. I would also like to acknowledge the help and valuable advice of Drs. Clegg Waldron, Shirley McCready and Laura diCaprio during the preparation of this thesis.

This work was carried out with the aid of a Science Research Council Studentship (1975-1978).

ABSTRACT

This thesis is a genetic study of the cytoplasmically-inherited determinant $[\bar{\text{psi}}]$ of Saccharomyces cerevisiae.

$[\bar{\text{psi}}]$ is a potentiator of ochre suppression. The molecular basis of $[\bar{\text{psi}}]$ was investigated using mutagenesis as a probe. The psi^+ phenotype (efficient suppression) can be mutated to psi^- phenotype (loss of suppression) by ultra-violet light (UV) and nitrosoguanidine (NTG). The UV-induced mutation was a single-hit event and the pre-mutational lesion was partly photoreactivable. Repair or expression of UV-induced mutation to the $[\bar{\text{psi}}]$ determinant was under the same genetic control as for nuclear mutation. It was concluded that $[\bar{\text{psi}}]$ has a DNA genome. The 'extrachromosomal mutagens' thymidylate starvation, 5-fluorouracil, manganese chloride and cycloheximide failed to induce psi^- mutants whilst guanidine hydrochloride, dimethyl sulphoxide and potassium chloride were shown to induce this mutation at frequencies up to 100%. Several other physical and chemical agents caused a high frequency of loss of the psi^+ phenotype.

A new class of recessive nuclear mutation (pnm) was shown to cause a loss of the psi^+ phenotype. A simple complementation test was devised to distinguish them from cytoplasmic psi^- mutants. The dominant PNM^- mutation was shown not to cause a physical loss of the $[\bar{\text{psi}}]$ genome. Two mutants with

a modified PNM⁻ phenotype were analysed. Attempts to demonstrate genetically the involvement of /psi/ with the 80S ribosome were unsuccessful. The psi⁺ phenotype was conclusively demonstrated to be inherited independantly of the nucleus using a 'heterokaryon test'. Two models for the /psi/ phenomena were proposed; one postulating the presence of a DNA 'plasmid' and one postulating the involvement of a stable, self-perpuating metabolic state.

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Abbreviations Used In This Thesis

J/m ²	Joules per square metre
mg	milligram
ug	microgram
ml	millilitre
ul	microlitre
sec	second
min	minute
hr	hour
<	less than
>	greater than

Those for media are explained in Materials and Methods.

All other abbreviations will be explained as they arise in the text.

CHAPTER 1

INTRODUCTION

Heritable differences that obey the laws of Mendelian genetics do so because their determinants, the genes, are located on chromosomes within the nucleus and these laws are a direct consequence of the behaviour of chromosomes at mitotic and meiotic cell divisions. Within a decade of the re-discovery of Mendel's Laws of Inheritance in 1902 exceptions were being reported (Correns 1909; Baur 1909). Such non-Mendelian inheritance was attributed to the existence of cytoplasmic genes.

The phenomenon of cytoplasmic inheritance of characters was, over the next 50 years, uncovered in a number of diverse organisms e.g. Chlamydomonas (Sager 1954), Drosophila (L'Heritier and Teissier 1937), Zea mays (Rhoades 1933) and Saccharomyces cerevisiae (Ephrussi et al., 1949). From the earliest investigations of cytoplasmic inheritance two classes of cytoplasmic organelle, chloroplasts and mitochondria, were put forward as bearers of cytoplasmic genes. It was not until the early 1960's, with the discovery of DNA's in chloroplasts (Ris and Plaut 1962; Sager and Ishida 1964) and mitochondria (Nass and Nass 1963; Luck and Reich 1964) that this supposition was directly confirmed.

Beyond the recognition of many phenotypes under non-Mendelian genetic control no formal genetic analysis of cytoplasmic systems was undertaken until Sager and her co-workers isolated a large number of mutations, in the unicellular alga Chlamydomonas, exhibiting non-Mendelian inheritance (see Sager 1972 for a review). Their work led to the discovery of a single cytoplasmic linkage group or "chromosome" which is now known to be located on chloroplast DNA (Sager and Ramanis 1976). Vegetative cells of Chlamydomonas are genetically diploid for this linkage group and genetical analysis with multiply marked crosses revealed regular distribution

at cell division, extensive changes between homologs leading to recombination, and circularity.

In parallel with Sager's work other research groups started to look at cytoplasmic inheritance in other micro-organisms which were amenable to rapid genetic analysis. The most intensively studied was the petite mutation in Saccharomyces cerevisiae by Ephrussi, Slonimski and their colleagues. They demonstrated that it was a cytoplasmically inherited change responsible for loss of mitochondrial function and that petite mutants lacked respiratory enzymes, notably cytochrome C oxidase and cytochrome a and b (Ephrussi 1953). Not until 1966, when some petite mutants were shown to contain altered mitochondrial DNA as judged by buoyant density (Mounolou et al., 1966), was the mitochondrial location of the petite mutation substantially established. The isolation of non-Mendelian mutations resistant to the antibacterial antibiotic erythromycin on non-fermentable medium (Linnane et al., 1968; Thomas and Wilkie 1968b) enabled formal genetic analysis of the mitochondrial genome to be undertaken (see Wilkie 1975; Nagley et al., 1977 for review). A large number of mitochondrial genes have now been mapped using deletion mapping procedures with petite mutants (Molloy et al., 1975). Recombination between mitochondrial genes has been demonstrated both genetically (Thomas and Wilkie 1968a) and physically by restriction mapping (Fonty et al., 1978). Transmission of mitochondrial genes is influenced by both nuclear and mitochondrial factors (see Wilkie 1975 and Nagley et al., 1977 for recent review).

The chloroplast and mitochondrion therefore represent extra-nuclear entities capable of some form of continuity from one cell

generation to the next. These organelles display a certain degree of genetic autonomy but nuclear genes are known to influence organelle formation and it seems likely that each genetic system codes for a particular set of organelle proteins.

All cytoplasmically inherited phenotypes do not however result from the expression of alterations to organelle DNA and thence to organelle structure and function. There are at least two other classes of extrachromosomally located genetic elements:-

(1) plasmids: these are small accessory double-stranded (ds) DNA circles found in some bacteria separate from the bacterial chromosome and capable of autonomous replication. Some plasmids show reversible insertion into the bacterial chromosome and, in the integrated state, are termed "episomes". Plasmids carry a number of genes coding for example antibiotic resistance and colicin production (for recent review see Lewin 1977). The finding of small circular pieces of dsDNA in the cytoplasm of Saccharomyces cerevisiae (Guerineau et al., 1971) that are cytoplasmically inherited (Livingston 1977) suggests that plasmids may not be restricted to prokaryotes. No copy of the yeast plasmid has yet been found stably integrated into a site on nuclear chromosomal DNA (Cameron et al., 1977; Guerineau 1977; Tabak 1977).

(2) Viruses and other endosymbionts: some types of cytoplasmic inheritance are caused by the presence of endosymbionts in the cytoplasm and in particular by the presence of viruses or virus-like particles (VLP's). Several examples of apparent cytoplasmic inheritance in Drosophila are known in which viral infection is involved e.g. carbon dioxide sensitivity is due to sigma,

a virus that resembles vesicular stomatitis virus (L'Heritier 1970). In fungi dsRNA viruses have been associated with cytoplasmically inherited characters e.g. the 'killer' character of yeast (Herring and Bevan 1974) and of Ustilago maydis (Wood and Bozarth 1973; Koltin and Day 1976). A miscellaneous assortment of other endosymbionts acting as agents of extranuclear inheritance have been described including algae, bacteria (some containing viruses) and rickettsiae (for recent reviews: Preer 1971; Beale and Knowles 1978).

The study of cytoplasmic inheritance in yeast has uncovered a number of genetic elements not apparently associated with mitochondria. The three main criteria for mitochondrial inheritance of a particular trait in yeast are:-

- (a) mitotic segregation of alleles after zygote formation,
- (b) no segregation of alleles on meiosis of a homoplasmic diploid (4:0 or 0:4 tetrads) and,
- (c) loss of the trait associated with the extranuclear petite mutation (ρ^0 or ρ^-).

All of the cytoplasmically inherited phenotypes listed in Table 1.1 fail mainly on the last of these criteria. In some instances the non-mitochondrial location of a phenotype has been confirmed by the failure of the phenotype to segregate with known mitochondrial genes e.g. ψ_7 , (Young and Cox 1972), glucosamine resistance (Kunz and Ball 1977).

Apart from mitochondrial DNA 4 other persistent, cytoplasmically-inherited, nucleic acid species have been found in the yeast cytoplasm. They are the two dsRNA species (possibly three, see Wickner 1976) which are encapsidated separately, but

in identical protein coats, to give isometric virus-like particles of mean diameter 39nm (Bevan et al., 1973; Herring and Bevan 1974). These virus-like particles show no infective cycle (Mitchell et al., 1976b; P. Chambers and B. Cox personal communication). Cells carrying both species of dsRNA release a toxin that is lethal to sensitive cells lacking the P2 or both P1 and P2 dsRNA species (see Wickner 1976 for a recent review). The P1 dsRNA has been transcribed in vitro and shown to code for the largest major polypeptide found in the virus-like particles (Hopper et al., 1977).

A 20S single stranded RNA species has recently been identified which is synthesised only during sporulation and is associated with 18-20 identical protein subunit in a 32S ribonucleoprotein particle (Wejksnora and Haber 1978). The ability of a cell to synthesise 20S RNA depends upon an unidentified dominant genetic element that is distinct from other previously identified cytoplasmic genetic elements (see Table 1.1) (Garvik and Haber 1978; C. Waldron personal communication). Accumulation of the 20S RNA is insensitive to antinomycin D suggesting that it is transcribed from a cytoplasmic RNA which does not appear to be either of the /killer/-associated dsRNAs P1 and P2 (Garvik and Haber 1978).

The /2μ/ DNA plasmid originally discovered by Sinclair et al., (1967) and characterised by Guerineau et al., (1971), is a dsDNA circle 2μ long and is present as an homogenous population of between 50 and 100 copies per cell which are believed to be compartmentalised within the cell (Livingston 1977) /2μ/. DNA replicates in vivo (Peters and Williamson 1975) and certain

TABLE 1.1 Cytoplasmically inherited genetic elements and phenotypes in yeast NOT associated with mitochondrial DNA.

GENETIC* ELEMENT	NUCLEIC ACID	PHENOTYPE	REF*
[KILLER]	P2 ds RNA ⁶ (MW:1.4x10 ⁶)	Killing (K ⁺), resistance (R ⁺)	12
[L]	P1 ds RNA ⁶ (MW:2.5x10 ⁶)	virion Proteins (?)	2
[psi]	?	Potentiatio <u>n</u> of ochre and frameshift suppression	4
[URE3]	?	Uptake of ureidosuccinic acid and its conversion to uracil	1
[2μ]	2μ DNA ⁶ (MW:4x10 ⁶)	Pleitropic drug resistance (?)	6
[20SRNA]	RNA?	Accumulation of A 32S ribonucleoprotein particle during sporulation (20S ⁺)	5
?	?	Resistance to rhodamine 6G, venturicidin, triethyltin and cycloheximide	3
?	?	Resistance to oligomycin, chloramphenicol and cycloheximide	7
?	?	Glucosamine resistance	11
?	?	Resistance to janus green, oligomycin, ethidium bromide and chloramphenicol	10
?	?	Tetracycline resistance	8
?	?	Modification of nuclear-coded nystatin resistance	9

* [] indicates cytoplasmically located.

* REFERENCES	-	1	Aigle & Lacroute (1975)	7	Guerineau et al (1974)
		2	Bevan et al (1973)	8	Hughes & Wilkie (1972)
		3	Carignani et al (1977)	9	Karunakaran & Johnston (1977)
		4	Cox (1965)	10	Kruszewska & Szczesniak (1978)
		5	Garvik and Haber (1978)	11	Kunz and Ball (1977)
		6	Guerineau et al (1971)	12	Somers and Bevan (1968)

enzymes, required for nuclear DNA replication, are also required for $\sim 2 \mu$ DNA replication (Livingston and Kupfer 1977). There is some preliminary evidence that it is transcribed in vivo (Gubbins et al., 1977; Guerineau 1977). In a coupled transcription-translation system in E. coli mini cells $\sim 2 \mu$ DNA has been shown to direct the synthesis of a few discrete protein products (Hollenberg et al., 1977; Gubbins et al., 1977). Genetical evidence has implicated $\sim 2 \mu$ DNA in antibiotic resistance via an informational role in the biogenesis and/or function of cellular membranes (Guerineau et al., 1974) but a direct function has not yet been established. The $\sim 2 \mu$ DNA therefore has characteristics similar to bacterial plasmids (see Lewin 1977).

In prokaryotes some DNA sections flanked by a duplication-inversion sequence can be transferred from one genome to another (Kleckner 1977). $\sim 2 \mu$ DNA has a long duplication-inversion comprising 20% of the molecule (Guerineau et al., 1976; Hollenberg et al., 1976) but $\sim 2 \mu$ DNA cannot be detected in nuclear DNA in an integrated form (episome) (Cameron et al., 1977), even when the cell lacks all detectable cytoplasmic $\sim 2 \mu$ DNA (Guerineau 1977; Tabak 1977). It is unclear whether nuclear DNA copies of dsRNA exist. Vodkin (1977a) has reported homology between dsRNA and nuclear DNA with approximately 30 copies per haploid genome and DNA complementary to P1 dsRNA. The hybridisation data of Wickner and Leibowitz (1977) however suggests less than 0.5 copies per haploid genome of P1 dsRNA sequences and less than 0.1 copies per haploid genome of P2 dsRNA sequences occur. Preliminary evidence also suggests that the 20S RNA species fails to hybridise to nuclear DNA (Wejksnora and Haber 1978). There is therefore no firm evidence for integration of

cytoplasmic "genomes" into nuclear DNA in yeast.

Several, apparently different, mutants resistant to a number of inhibitors of mitochondrial function, have been described (Hughes and Wilkie 1972; Guerineau et al., 1974; Carignani et al., 1977; Kruszewska and Szczesniak 1978) which show characteristics of both cytoplasmic and nuclear inheritance, but are not associated with mitochondrial DNA. It has been tentatively suggested that these may represent a novel class of yeast hereditary factor resembling bacterial plasmids or episomes. A one to one correlation between the presence of $\phi 2\mu$ DNA and one of the genotypes has been demonstrated by Guerineau et al., (1974) and they hypothesise that $\phi 2\mu$ DNA can mutate giving drug resistance, and become integrated in a nuclear chromosome, where it behaves like a nuclear gene.

Since its discovery in 1965 (Cox 1965) the nature of the cytoplasmically-inherited ϕpsi factor of yeast has remained something of an enigma. Its phenotypic effect on nonsense suppression, namely modifying the efficiency of certain nonsense suppressors, suggests that it plays a role, directly or indirectly, in controlling the fidelity of translation.

Since the pioneering work of Hawthorne and Mortimer (1963) numerous nonsense suppressors have been isolated in Saccharomyces cerevisiae. Suppressors are genes which suppress the expression of mutant phenotypes and are themselves mutant genes. Nonsense suppressors (sometimes called 'informational suppressors') act upon only certain alleles at any given locus with any single suppressor acting upon a wide variety of mutant phenotypes i.e. they are "allele-specific".

Nonsense suppression was first discovered in Escherichia coli and the bacteriophage T₄ where it was demonstrated that the alleles acted upon by these informational suppressors were nonsense mutations. Nonsense mutations are mutations arising from the presence of one of the three polypeptide chain terminating "nonsense" codons UAA (ochre) UAG (amber) or UGA at an intralocus position. Nonsense mutations consequently give rise to prematurely terminated gene products. The gene product of the informational suppressor (= nonsense suppressor) must intervene at some step in the translation of this mutant mRNA so as to lead to the synthesis of a complete functional gene product. Evidence that E. coli nonsense suppressor genes code for a mutant tRNA has been reviewed by Garen (1968) and Gorini (1970).

tRNA molecules can gain activity in suppression of nonsense codons by;

(a) a base substitution directly in the anticodon of the tRNA such that the tRNA recognises a certain nonsense codon instead of - or in addition to - the codons which it usually recognises. This results in the aminoacid, with which the tRNA is charged, being inserted at the site of the nonsense codon, or,

(b) a base substitution outside the anticodon e.g. a trp.tRNA in E. coli, showing a G to A base change in the D-stem of the molecule, can recognise a UGA codon (Hirsh 1971). This results in a conformational change in the tRNA such that only the first two bases of the codon (UG) are recognised by the trp.tRNA anticodon CCA. (Buckingham and Kurland 1977)

Initially yeast nonsense suppressors (also termed 'super-suppressors') were classified mainly by their characteristic

suppression of particular nutritional markers. Eleven different classes were uncovered (Class I - Class XI) representing 4 major types of nonsense suppressor;

- a) amber-specific suppressors
- b) ochre-specific suppressors
- c) UGA-specific suppressors

and d) omnipotent suppressors acting on all three nonsense codons. A detailed review of the isolation and classification of these nonsense suppressors in yeast is given by Hawthorne and Leupold 1974 and will not be discussed here.

It was not until UAA and UAG mutants of the iso-1-cytochrome C structural gene (CYC1⁺) had been identified in yeast (Sherman and Stewart 1974) by amino acid sequence analysis of the iso-1-cytochrome C, that the means for determining the suppressible codons and for determining the amino acid inserted by nonsense suppressors, became available. In addition these cyc1 mutants have been used to estimate the efficiencies of suppression by determining the amount of iso-1-cytochrome C in strains, carrying a suppressor and a cyc1 nonsense mutant, by spectrophotometric measurements after chromatographic separation (Sherman et al., 1973).

Table 1.2 lists the major groups of ochre and amber suppressors examined using the cyc1 system. The high efficiency ochre and amber suppressors map at only 8 independent loci on 6 different chromosomes (Gilmore et al., 1971; Liebman et al., 1976). All insert the amino acid tyrosine at the site of the nonsense codon.

Although yeast nonsense suppressors showed many characteristics of E. coli nonsense suppressors it was not until very recently that the suppressing activity of, at least some, yeast nonsense

TABLE 1.2 The major groups of UAA and UAG suppressors examined with the cycl system.

CODON	EFFICIENCY OF SUPPRESSION	LOC1	NO. OF LOC1	AMINO ACID INSERTED	REFERENCE
UAA (ochre)	HIGH	<u>SUP2 - SUP8</u> <u>SUP11</u>	8	tyrosine	Gilmore et al, 1971
	LOW	<u>SUP5(=SUP16)</u> <u>SUP17</u>	2	serine	Liebman et al, 1975a Ono et al, 1978
	VERY LOW	<u>SUP26 - SUP29</u> <u>SUP32 - SUP33</u>	6	leucine	Ono et al (in prep)
	RECESSIVE LETHAL	NONE	NONE	-	
UAG (amber)	HIGH	<u>SUP2 - SUP8</u> <u>SUP 11</u>	8	tryosine	Liebman et al, 1976
	LOW	<u>SUP52*</u>	?	leucine	Liebman et al, 1977
	VERY LOW	NONE	NONE	-	
	RECESSIVE LETHAL	<u>RLI(=SUP16)*</u>	?	serine	Branpriss et al, 1976

* others have been found but not characterised (F. Sherman, pers. comm.)

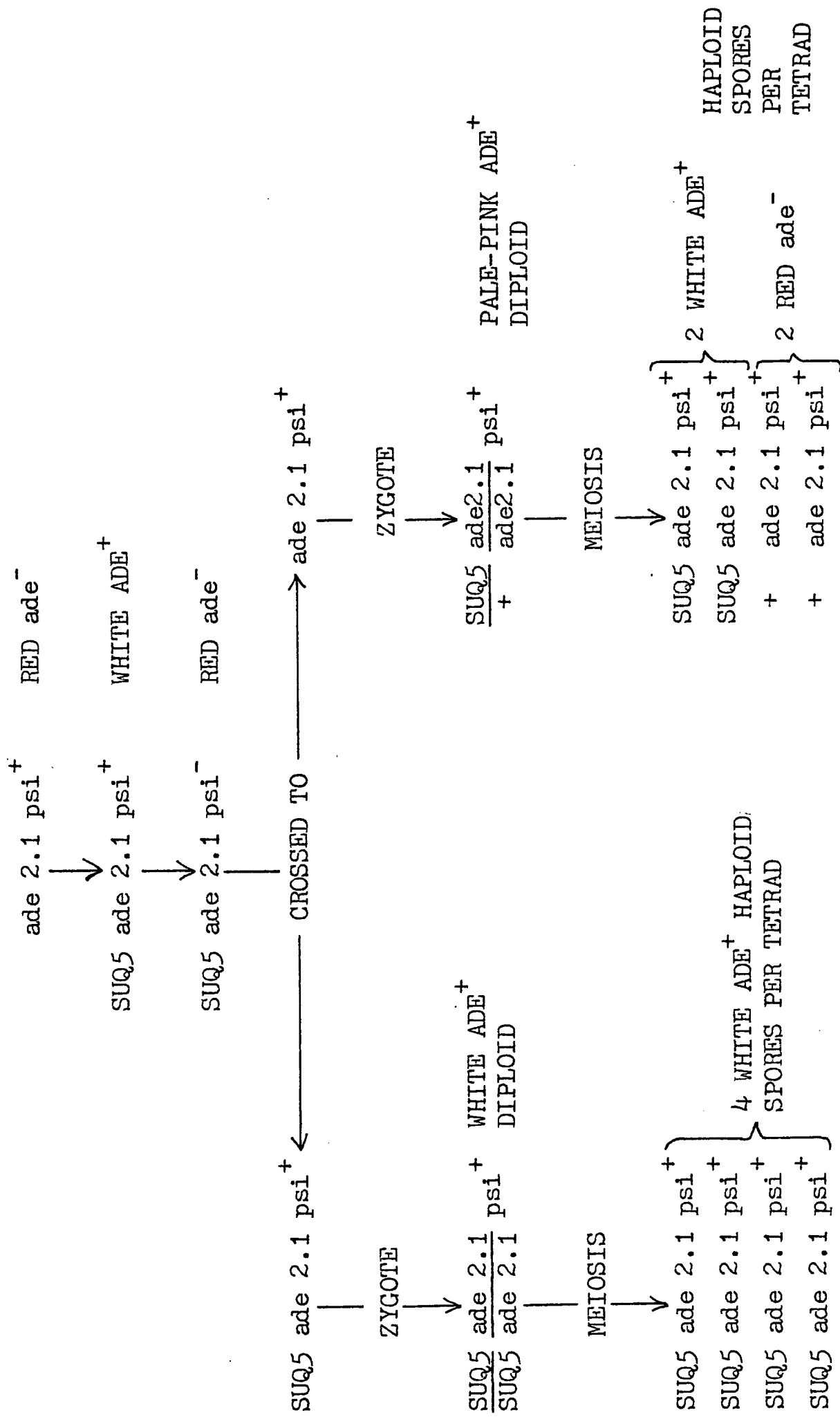
suppressors was conclusively demonstrated to be due to altered tRNAs. Four approaches have been used to demonstrate that yeast suppressors code for mutant tRNAs:-

- (1) reversed phase column chromatography of tRNA species (Bruenn and Jacobson 1972),
 - (2) 'in vitro' translation of mRNA containing nonsense codons (Capeocchi et al., 1975; Gesteland et al., 1976),
 - (3) direct sequencing of the tRNA molecule (Piper et al., 1976; Piper 1978),
- and (4) direct nucleotide sequencing of the suppressor gene (Goodman et al., 1977).

Using these techniques the tyrosine-inserting ochre suppressors SUP2-SUP8 and SUP11, their amber-suppressing alleles, the serine-inserting ochre suppressor SUQ5, the recessive lethal amber suppressor SUPRL1 and one UGA suppressor have all been shown to code for mutant tRNA molecules (M. Capeocchi personal communication). In the case of SUPRL1, SUP5 (amber) and SUP4 (ochre) the predicted base change in the anticodon loop has been demonstrated (Piper et al., 1976; Goodman et al., 1977; Piper 1978).

All yeast nonsense suppressors so far mentioned are dominant. However there also exist a class of nonsense suppressors that are recessive. These are the omnipotent suppressors sup35 and sup45 (Hawthorne and Leupold 1974) / also known as supP and supQ (Gerlach 1975) and sup1 and sup2 (Inge-Vechtomov 1965) which suppress UAA, UAG and UGA codons in vivo. From mutational studies (Hawthorne and Leupold 1974; Gerlach 1976) it was suggested that they code for mutant ribosomal proteins rather than tRNAs. This follows the precedent of the ribosomal

FIGURE 1.1 Summary of backcrosses reported by Cox (1965) demonstrating the existence of a putative extrachromosomal element $[\psi^-]$.



* diagram after Young (1969).

ambiguity (ram) mutants of E. coli which code for altered ribosomal proteins and are able to suppress all 3 nonsense codons (Gorini 1974). The recent report that the sup2 mutant of Inge-Vechtsov (1965) has different amounts of a protein of the 60S ribosomal subunit from the sup2⁺ parent (Smirnov et al., 1978) would appear to add weight to this supposition.

The effect of the /psi factor on yeast nonsense suppression was first demonstrated by Cox (1965) during a study of the ochre suppressor SUQ5. SUQ5 was shown to suppress the ade2.1 mutation (an adenine-requiring, ochre mutation which results in accumulation of a red pigment) to give white prototrophic colonies. Upon investigation of a rare red/white sectorized spore colony arising from a SUQ5/+ ade2.1/ade2.c diploid, it was demonstrated, by a series of backcrosses to stock SUQ5 ade2.1 and SUQ5⁺ ade2.1 strains, that the red sector still carried the SUQ5 mutation but in an inactive state. Suppressor activity was restored when cells from the red sector were crossed to the SUQ5⁺ ade2.1 strain the resulting diploid being ADE⁺ and segregated 2 white and 2 red spores in each tetrad. These backcrosses are illustrated in Figure 1.1.

To explain the failure of SUQ5 to suppress ade2.1 in the red sector it was postulated that a cytoplasmic component psi⁺, which was dominant in diploids, was required for the expression of SUQ5 and that the rare red sector was the result of a spontaneous psi⁺ to psi⁻ mutation. Two other spontaneous red sectorized colonies examined by Cox (1965) gave identical results. Backcrosses and intercrossoes between cells from the 3 different red sectors gave only red diploids. All three were psi⁻.

Since its discovery several different characteristics of the effect of the psi⁺ phenotype on nonsense suppression have been uncovered:-

(a) the 8 tyrosine-inserting ochre suppressors SUP2-SUP8 and SUP11 are only expressed in a psi⁻ background. In a psi⁺ background they are lethal to the cell (Cox 1971).

(b) the SUQ5 ochre suppressor inserts serine at the UAA site of the ochre mutation cyc1-72 efficiently only in a psi⁺ background (Liebman et al., 1975a). A second serine-inserting ochre suppressor SUP17 also requires psi⁺ (Ono et al., 1978). These two psi⁺-dependent suppressors are unlinked; SUQ5 is on chromosome XVI (Liebman et al., 1975a); SUP17 is on chromosome IX (Ono et al., 1978).

(c) amber-suppressing alleles of the 8 tyrosine-inserting suppressors are not affected by psi⁺. They have been shown to act with equal efficiency, in both psi⁺ and psi⁻ backgrounds, on the amber mutant cyc1-179 (Liebman et al., 1975b). No lethality was seen when the amber-specific allele of SUP11 was crossed into a psi⁺ background.

(d) psi⁺ increases the efficiency of ochre suppression. Table 1.3 demonstrates the effect the psi⁺ phenotype has on the efficiency of suppression of several ochre suppressors using the cyc1 system. The psi⁺-induced lethality in cells carrying any one of the tyrosine-inserting ochre suppressors has been suggested to result from an enhancement of the efficiency of suppression to a level lethal to the cell by suppression of normal termination codons. This would give rise to protein readthrough products (Cox 1971).

(e) using the cyc1 system, to measure quantitatively the effect of the psi⁺ phenotype on efficiency of ochre suppression, it was demonstrated that psi⁺ is apparently a weak ochre suppressor since it elevates the level of iso-1-cytochrome C from a non-detectable level in a psi⁻ cyc1-72 strain to approximately

TABLE 1.3 Effect of $\overline{[\psi]}$ on the efficiency of nonsense suppression.*1

SUPPRESSORS*2	cyc1 allele*3	Approximate % of iso-1-cytochrome C compared to wild type	
		ψ^+	ψ^-
NONE	1-72	1-2	0.06
NONE	1-2	0.3	NOT TESTED
SUQ5 (UAA)	1-2	15	1
	1-9	1.2-1	NOT TESTED
	1-179	0.1	NOT TESTED
SUP16 (UAA)	1-72	25	3
SUP7 (UAA)	1-2	LETHAL	12
SUP7 (UAG)	1-179	40	40
<u>SUQ5</u> (UAA) +	1-2	7-11	NOT TESTED

*1 data from Liebman et al (1975a, 1975b), Ono et al (1978)
and F. Sherman, personal communication.

*2 SUP16 is allelic to SUQ5 (Ono et al, 1978).

*3 cyc1-2 cyc1-9 cyc1-72 are och re alleles, cyc1-179 is an amber allele.

2% of the wild type level in a psi⁺ cyc1-72 strain (Liebman et al., 1975b, see Table 1.3). Psi⁺ also suppresses the ochre allele trp5.48 (B. Cox personal communication).

(f) Genetic and biochemical studies have shown that psi⁺ increases the efficiency of three frameshift suppressors SUF1, SUF4 and SUF6 whilst having no effect on four other genetically distinct frameshift suppressors SUF2, SUF3, SUF5 and SUF7 (Culbertson et al., 1977). The three /psi-dependent frameshift suppressors show reductions in levels of gly3tRNA unlike the other four SUF mutants. The finding that psi⁺ affects frameshift suppression, that is suppression of mutations which add or delete bases from DNA, suggests that /psi does not affect termination factors. Psi⁺ is unable to suppress the frameshift allele his4.38 in the absence of a frameshift suppressor (Culbertson et al., 1977).

/Psi is therefore a modifier of nonsense suppression enhancing the efficiency of ochre and frameshift suppression. Psi⁺ has no other apparent phenotypic effect on the cell - it has a non-essential function.

Two classes of nuclear mutation also directly modify the efficiency of nonsense suppression in yeast;

(1) Antisuppressors; recessive antisuppressors mapping at 8 different nuclear loci (asu1-asu8) have been found. They abolish the ability of the SUP2, SUP11 and SUQ5 ochre suppressors to suppress ade2.1 and diminish overall suppressor efficiency (McCready and Cox 1973, 1976). They prevent lethal phenotype of SUP11 in a psi⁺ background (McCready and Cox 1973) but have no affect on the ability of the amber-suppressing allele of SUP11

to suppress the trp1.1 amber mutation (McCready and Cox 1976). Antisuppressors for amber suppressors (Hawthorne 1967; Hawthorne and Leupold 1974) and for informational missense suppressors (Gorman and Gorman 1971) have been reported.

The molecular basis of antisuppression in Saccharomyces cerevisiae is unknown but recently sin1, one of the 14 recessive antisuppressors mutants (sin1-sin14) isolated in Schizosaccharomyces pombe (Thuriaux et al., 1975), has been shown to be defective in an enzyme required for the modification of a base adjacent to the 3' end of the anticodon loop of the suppressor tRNA (F. Janner personal communication). In E. coli this base has an essential role in the binding of tyrosine tRNA to ribosomes (Geftter and Russell 1969). Many antisuppressor mutants of E. coli have been shown to be defective in tRNA biosynthesis, in particular in modification enzymes (see Altman 1975 for a recent review).

(2) Allosuppressors; are recessive nuclear mutations mapping at 5 distinct loci (sal1-sal5), that result in an increase in the level of ochre suppression (Young and Cox 1975; Cox 1977b). They allow SUQ5 to suppress efficiently in a psi⁻ background and are lethal when coupled with the high-efficiency, tyrosine-inserting suppressors SUP2 SUP3 and SUP11 in a psi⁻ background (Cox 1977b). They appear to be chromosomal mimics of the psi⁺ phenotype, but show no ochre-suppressor activity by themselves in a psi⁺ or psi⁻ background.

The sal mutants described by Hawthorne (Hawthorne 1967; Hawthorne and Leupold 1974) abolish the antisuppressor phenotype of SIN2 (a dominant mutation which prevents the full expression of the amber-suppressing allele of SUP5) and enhances the efficiency of ochre suppressors. They appear to be different from Cox's sal mutants in that they are weak suppressors by themselves.

A mutation (ups) that increases the activity of nonsense suppressors in E. coli has been recently isolated (Davidoff-Abelson and Mindich 1978) and is believed to code for a soluble protein involved on termination of protein synthesis. Presumably the ups mutant (and possibly the yeast sal mutants) affects competition between suppressor tRNA and the termination system for the nonsense codon site such that suppressor tRNA is favoured.

By isolating red non-suppressed mutants from a white SUQ5 ade2.1 psi⁺ suppressed strain, following UV-irradiation mutagenesis Young and Cox (1971) isolated a mutant designated "U16". It had the following properties: it gave a red, non-suppressed diploid segregating only red, non-suppressed spores when crossed to a standard red SUQ5 ade2.1 psi⁻ strain; crossed to a white SUQ5 ade2.1 psi⁺ it yielded pink, non-suppressed diploids which segregated an excess of red, non-suppressed spores. By back-crossing the four red non-suppressed spores from a 0:4 ascus from the latter cross, to standard SUQ5 ade2.1 psi⁺ strains, it was demonstrated that a dominant nuclear gene mutation was responsible for the psi⁻ phenotype; two spores yielded white suppressed diploids segregating only suppressed (psi⁺) haploid progeny and 2 gave red non-suppressed diploids segregating an excess of red non-suppressed (psi⁻) haploid progeny. The nuclear mutation, now designated PNM1 (formerly R), was shown to cause a progressive loss of the psi⁺ phenotype in the vegetative descendants from a SUQ5 ade2.1 psi⁺ X SUQ5 ade2.1 PNM1 psi⁻ zygote (Young and Cox 1971). A second, unlinked PNM mutation (PNM2) has also been isolated (B. Cox personal communication). Thus at least 2 nuclear genes are known to be involved in maintenance of the

psi⁺ phenotype and possibly function in the replication or transmission of the /p̄si/ factor.

The study by Young and Cox (1971) also demonstrated that the non-suppressed phenotype could arise by a reversion of the SUQ5 mutation (SUQ5 → SUQ5⁺) or by mutation from psi⁺ → psi⁻.

Studies on the basis of the /p̄si/ factor have, so far, proved unfruitful. Young (1969) reported that both ultra-violet and ethyl methane sulphonate mutated psi⁺ to psi⁻ this being consistent with /p̄si/ having a DNA genome. Linkage studies between psi⁺ and two mitochondrial genome markers rho⁻ and ery^r (resistance to erythromycin) revealed that the psi⁺ phenotype segregated independently of rho⁻ and ery^r (Young and Cox 1972). Psi⁺ is not therefore linked to mitochondrial DNA. Psi⁺ rho⁺ strains can also be converted to rho⁰ (lacking all detectable mitochondrial DNA) by treatment with ethidium bromide whilst retaining their psi⁺ phenotype (B. Cox personal communication).

Both killer psi⁺ and killer psi⁻ strains carry the P1 and P2 species of dsRNA (B. Cox personal communication, C. Waldron personal communication) and both psi⁺ and psi⁻ diploids are able to synthesise the 20S RNA species during sporulation (C. Waldron personal communication). There is no apparent difference in length or in length distribution of /2μ/ DNA molecules between psi⁺ and psi⁻ strains (McCready and McLaughlin 1977) although this does not rule out the possibility that psi⁻ may be a point mutation on the /2μ/ "genome". The fact that psi⁻ can revert to psi⁺ (Young and Cox 1975) adds weight to this possibility; no reversion would be seen if genetic information

was physically lost.

An extensive search, using electron microscopy, caesium chloride equilibrium density gradients and 1D polyacrylamide gel analysis, has failed to reveal an extrachromosomal nucleic acid species associated with the psi⁺ (or psi⁻) phenotype (S. McCready personal communication, C. Waldron personal communication).

The /psi factor represents a unique phenomenon; an apparently cytoplasmically inherited genome which directly, or indirectly, affects the translation process in yeast and in particular the fidelity of that process. The psi⁺ phenotype shows no apparent association with any previously identified yeast cytoplasmic genetic element and the /psi factor may therefore represent a new, but as yet unidentified, plasmid of yeast.

This thesis is concerned with attempts to:

- (1) clarify the molecular nature and location of the yeast /psi factor using mutagenesis as a probe .
- (2) demonstrate conclusively that the psi⁺ phenotype is not associated with either mitochondrial DNA or the two dsRNA species.
- (3) determine the types of mutation, nuclear or cytoplasmic, that affect maintenance of the psi⁺ phenotype and to study in particular the role of the nuclear genome in maintaining this phenotype.
- (4) determine, using genetic studies, whether the psi⁺ or psi⁻ phenotype affects one of the components of the yeast translational apparatus namely the ribosome.

CHAPTER 2

MATERIALS AND METHODS

- 2.1 Media
- 2.2 General Techniques
- 2.3 Yeast Strains
- 2.4 Genetic Analysis
- 2.5 Determination of Genotypes and Phenotypes
- 2.6 Mutagenesis
- 2.7 Statistical Procedures

MATERIALS AND METHODS

2.1 Media

a) Complete Medium (YC)

This is a rich growth medium used by Cox and Bevan (1962). A stock solution of X20 the required final concentration was prepared as follows:

(i)	Difco peptone	60g
(ii)	Difco yeast extract	100g
(iii)	Difco casein hydrolysate	10g
(iv)	DL tryptophan	0.2g
(v)	Vitamin solution	50ml
(vi)	Distilled water	950ml

(i) - (iv) were added to (v) and (vi) and the mixture heated until dissolved. The solution was then boiled for 10 minutes, cooled and stored in 500ml medical flats at 4°C over chloroform.

The ingredients of the vitamin solution ((iv) above) were:

Pyridoxine	0.04g
i-Inositol	2.50g
Thiamin	0.50g
Nicotinic acid	0.50g
Calcium pantothenate	0.50g
para-amino benzoic acid	0.25g
Riboflavin	0.50g

These were dissolved, in the order given above, in a D-Biotin solution of concentration $2.5 \times 10^{-5}\%$ (W/V). Liquid YC medium was made up as follows:

Stock solution	50 ml
Glucose	40 g
Distilled water	950ml

For solid YC medium 15g/l of Oxoid agar No. 3 was added to the above prior to sterilisation. YC medium was used in preference to the more conventional 1% yeast extract, 1% peptone, 1.25% agar, 2% glucose (YEPD) medium because strains carrying the ade2.1 mutation will accumulate the characteristic red pigment on YC even when these strains are respiratory-deficient (petites), whereas petites carrying the ade2.1 mutation appear as white colonies on YEPD. It was found, however, that by either reducing the yeast extract in YEPD to 0.1% or increasing the glucose concentration to 4% or 8% one obtained identical results to YC medium.

b) Minimal Medium (MM)

The minimal medium was made up as follows:

Difco yeast nitrogen base without amino acids	6.7g
Glucose	10g
Distilled water	1000ml

The medium was solidified by adding 12.5g/l of Oxoid ion agar No. 2, or Oxoid purified agar, prior to sterilisation. The Difco yeast nitrogen base without amino acids is as described in the Difco manual. 1ml, per 100ml of MM, of the following supplement stock solutions was added after sterilisation.

<u>Supplement</u>	<u>Stock Concentration</u> (W/V)
adenine	0.2%
DL histidine	0.2%
DL lysine	0.1%
DL tryptophan	0.2%
uracil	0.1%
DL methionine	0.2%
L tyrosine	0.1%
DL threonine	0.1%

L arginine	0.2%
DL leucine	0.5%

This medium (OM) was used to score auxotrophic requirements by leaving out the required supplement. To score resistance to canavanine, arginine had to be left out of the medium and canavanine sulphate added, to a final concentration of 30 µg/ml, after sterilisation of the medium.

c) Sporulation Medium (FMA)

The sporulation medium used was based on that described by Hurst and Fogel (1964) except that adenine was added to a final concentration of 20 µg/ml. This additional supplement was necessary because many of the diploids used in this study were homozygous for adenine requirement. The medium contained:

Potassium acetate	10.0g
Difco yeast extract	2.5g
Glucose	0.5g
Oxoid agar No. 3	15.0g
Distilled water	1000ml

d) Glycerol Medium (YEPG)

This medium was used to test for petite colonies and contained the following:

Yeast extract	10g
Bacto peptone	10g
Glycerol	20g
Oxoid agar No. 3	12.5g
Distilled water	1000ml

e) Killer Assay Medium (KM)

The medium used to assay for the killer phenotype was that of Somers and Bevan (1968) and contained:

Yeast extract	10g
Bacto peptone	10g
Glucose	40g
Oxoid agar No. 3	12.5g
Buffer (pH 4.8)	1000ml

The buffer was made by adding 25.3ml of 0.1M citric acid to 24.7ml of 0.2M Na_2HPO_4 and diluting to 100ml with distilled water. Methylene blue was added, after autoclaving the medium, to a final concentration of 0.003M. The medium was cooled to 48°C and seeded with 0.1ml of a stationary phase culture of a killer sensitive (K^-) strain, KL4359D (supplied by Dr P. Slonimski, Gif-sur-Yvette, France).

f) Sterilisation and Plate Preparation

All media and heat-stable supplement solutions were sterilised for 20 minutes at 151b in $^{-2}$ pressure in a 'British Steriliser Autoclave'. Heat-labile solutions were filter-sterilised.

Media was allowed to cool for up to 1 hour at room temperature before pouring into plastic, single-vent, petri dishes (approximately 20ml of medium per plate). This operation was carried out in a sterile cabinet and the plates were allowed to dry for 1 hour with their lids removed. All plates, with the exception of KM plates, were stored at room temperature in sealed, sterile plastic bags. KM plates were stored at 4°C to prevent growth of the sensitive lawn.

2.2 General Techniques

Standard microbiological techniques were used in plating and culturing strains.

a) Incubation was carried out generally at 28°C, either on agar plates in a Gallenkamp oven, or by shaking liquid suspensions of cells on a Gallenkamp shaking water bath. Temperature-sensitive (ts) mutants were grown at 23°C.

b) Suspensions. Cell suspensions and dilution series were made in sterile saline (0.85% W/V sodium chloride).

c) Plating of Suspensions was carried out by spreading 100 or 200ul of the suspension evenly over the surface of the agar plate with a glass "spreader" previously sterilised by dipping into 95% ethanol and flaming. Even distribution of cells was facilitated by the use of a specially constructed, hand-revolved, 'plating wheel'.

d) Replica Plating. The technique described by Woods (1963) was used. A brass ring was used to hold sterile filter paper (Whatman No. 1, 11cm diameter) in position on a 9cm diameter brass block. A velvet pad was placed under the filter paper. Prior to replica plating the filter paper was briefly flamed. A freshly inoculated YC master plate was used to ensure that only a thin film of cells was transferred to each replica plate. Up to 8 replicas could be made from one print of the YC master plate on the sterile filter.

e) Storage. Strains were stored for periods up to twelve months on YC plates, sealed with $\frac{1}{2}$ inch insulating tape, at 4°C. For long term storage (up to 3 years) strains were stored at -80°C in liquid YEPG (with 20% W/V rather than 2% W/V glycerol).

f) Estimating Cell Numbers. An "Improved Neubauer" haemocytometer slide was used to estimate cell number. Cells were

generally suspended in saline and counted at a concentration of approximately 5×10^6 cells/ml. Clumped cells were separated, prior to counting, by subjecting a 10ml saline suspension of the cells to 25 seconds sonication, at 1.25 amps, in an "MSE Ultrasonic Power Unit". The cell suspension was held in a McCartney bottle which was surrounded by ice. This treatment had no effect on cell viability.

2.3 Yeast Strains

"Yeast" throughout this thesis refers to the budding yeast Saccharomyces cerevisiae.

a) Nomenclature: each strain constructed for this study was given a unique number:

A / BC e.g. MT182 / 8d

where A = the number of the diploid,

B = the number of the tetrad and

C = refers to the ascospore number in the tetrad B.

The nomenclature for the mutant alleles used in this study are those proposed by Plischke et al., (1974).

The symbols used for the two mating types are a and α. A wild type phenotype is designated with a "+" symbol e.g. ADE^+ and the corresponding mutant phenotype is given a "-" symbol e.g. ade^- , except in the case of drug resistance where the wild type phenotype is designated "s" e.g. CYH^s and the mutant phenotype is designated "r" e.g. cyh^r , for "sensitive" and "resistant" respectively. An extrachromosomal element is indicated by square brackets e.g. $[\bar{psi}]$.

b) Gene Markers used: A list of all the gene markers used in this study is given in Table 2.1.

TABLE 2.1 Genetic markers in this thesis.
(taken from Plischke et al 1974)

GENE MARKER	MAP ^{*1} POSITION	PHENOTYPE	NATURE OF ALLELE
1 <u>AUXOTROPHIC MARKERS</u>			
ade 2.1	15R	requires adenine;red	ochre
his 5.2	9L	requires histidine	ochre
lys 1.1	9R	requires lysine	ochre
trp 5.48	7L	requires tryptophan	ochre
arg 4.17	8R	requires arganine	ochre
ura 3.1	5L	requires uracil	missense
leu 1	7L	requires leucine	missense
ade 1	1R	requires adenine; red	missense
ade 2.40	15R	requires adenine; red	missense
his 4	3L	requires histidine	missense
arg 9	5R	requires arganine	missense
2 <u>RADIATION SENSITIVE</u>			
rad 1.1	16L	UV sensitive; defective in excision repair	missense
rad 3.12	-	UV-sensitive; defective in excision repair	missense
rad 6.1 rad 6.3	7L	UV and x-ray sensitive; defective in error-prone repair	?
rev 3.1	-	moderately UV-sensitive; defective in error-prone repair	missense
umr 1.1	-	slightly UV-sensitive; defective in error-prone repair	missense

TABLE 2.1 cont'd

GENE MARKER	MAP* 1 POSITION	PHENOTYPE	NATURE OF ALLELE
3 <u>CELL DIVISION CYCLE MUTANTS</u> (temperature sensitive; restrictive 36°C, permissive 23°C)			
cdc 4	6L	cell cycle arrest at plaque separation	missense
cdc 7	4L	cell cycle arrest at initiation of DNA synthesis	missense
cdc 8	10R	cell cycle arrest at DNA elongation	missense
cdc 9	4L	cell cycle arrest at nuclear division	missense
cdc 21≡tmp 1	15R	cell cycle arrest at DNA elongation	missense
4 <u>DRUG RESISTANCE</u>			
can 1.100	5L	resistance to canavanine	ochre
cyh 2	7L	resistance to cycloheximide	missense
cry 1	3R	resistance to cryptopleurine	missense
cry 3	-	resistance to cryptopleurine	missense
tcm 1	15R	resistance to trichodermin	missense
5 <u>NONSENSE SUPPRESSORS AND THEIR MODIFIERS</u> *2			
SUQ5	16R	(psi)-dependant serine-inserting ochre suppressor	missense
SUP 2	4R	high efficiency, tyrosine-inserting ochre suppressor	missense
SUP 3	15L	high efficiency tyrosine-inserting ochre suppressor	missense
asu1-asu8	-	anti suppressors of ochre suppressors	missense
sal 1-sal 5	-	allosuppressors; enhancing efficiency of ochre suppressors	missense
PNM 1-PNM 2	-	elimination of <u>psi</u> ⁺ phenotype	missense

TABLE 2.1 cont'd

GENE MARKER	MAP ^{*1} POSITION	PHENOTYPE	NATURE OF ALLELE
6 MISCELLANEOUS			
a, α	3R	mating type	missense
kar 1.1	-	blocked in karyogamy	missense
mak 10	5L	unable to maintain (<u>killer</u>) plasmid	missense

*1 chromosome number and arm (R = right L = left) are given;
- indicates not known.

*2 an amber-suppressing allele of SUQ5 or SUPX mutants are
designated SUQ5-a or SUPX-a. Lack of a suffix implies the
ochre-suppressing allele.

c) Derivation of strains will be given in the relevant chapters.

2.4 Genetic Analysis

a) Isolation of Zygotes. For the construction of all diploid strains the two haploid parents, of opposite mating type, were grown overnight on a YC plate at room temperature (18°C - 20°C) and then mixed on a fresh YC plate and incubated at 28°C for 3-5 hours to allow zygotes to form. These were picked from the mating mix spread into a thin film on the agar using a Singer micromanipulator (Barer and Saunders-Singer 1948) and a fine glass loop (5-10 μ in diameter) under a Watson binocular microscope at a magnification of 200X. The glass loop was made on a deFonbrunne micro-forge (de Fonbrunne 1949). Up to 5 zygotes were isolated from each cross and allowed to grow up into single colonies by incubating for 2-3 days at 28°C .

b) Sporulation. Diploid clones were induced to sporulate by subculturing onto FMA and incubating at 28°C for 3-7 days.

c) Tetrad Dissection. After microscopic examination, to confirm sporulation had occurred, a loopful of the sporulating diploid culture was added to 100 μ l of a 1% W/V, filter-sterilised solution of Helicase (Industrie Biologique Francaise S.A., Gennevilliers, France) and left at room temperature for 1 hour. Alternatively 100 μ l of a 5% W/V solution of Gluculase (Endo laboratories, New York) was used, with the incubation period reduced to 15 minutes. These enzymes dissolve the ascus wall, leaving the ascus membrane intact, and holding the 4 spores together. After the incubation period a drop of the ascal suspension was spread onto one half of a YC plate and 10-12 complete asci separated into their 4 ascospore components, at

marked positions on the YC plate, using the micro-manipulation procedure described in a) above. After 3 days incubation at 28°C the spore clones were either subcultured and tested for auxotrophic requirements by replica plating or replica plated directly onto various omission media.

Generally only complete tetrads (containing 4 viable spores) were genotyped. Spore viability

$$\frac{(\text{number of viable spores})}{(\text{number of spores dissected})} \times \frac{100}{1}$$

was scored in all crosses undertaken.

2.5 Determination of Genotypes and Phenotypes

a) Auxotrophic requirements. These were scored by replica plating strains onto OM lacking one or other of the supplements mentioned in Section 2.1 b). Growth or non-growth was generally scored after an incubation period of 3-4 days at 28°C or 5-7 days at 23°C unless otherwise stated in the results.

b) Drug resistance. This was determined by either:

i. replica plating onto complete medium containing the drug under test. The drug was added to the medium just prior to pouring. Results were scored after an incubation period of 7-10 days. (Table 2.2 lists the drugs, solvents and media used).

ii. a more sensitive "well test" was used. Plates containing exactly 20ml of YC were used with Oxoid Purified Agar added, instead of Oxoid Agar No. 3, to facilitate drug diffusion. 200µl of a saline suspension of the strain under test (c. 5×10^6 cells/ml) was spread evenly over the agar and allowed to dry. A well, of diameter 1.2cm or 1.4cm, was bored in the middle of the plate using a flame sterilised "well borer" and 200µl of the drug solution added to the well. Plates were incubated at 23°C and the diameter of the zone of inhibition scored after

TABLE 2.2 Drugs Used in this Study.

DRUG	ABBREVIATION	SOLVENT* ¹	STOCK CONC.	MEDIUM USED	SOURCE
cycloheximide	cyh	water	2mg/ml	YC	'Sigma'
canavanine sulphate	can	water	3mg/ml	OM-arg	'Calbiochem'
cryptopleurine	cry	DMSO	500µg/ml	YC	C. McLaughlin
trichodermin	tri	DMSO	10mg/ml	YC	C. McLaughlin
anisomycin	ani	DMSO	500µg/ml	YC	C. McLaughlin
erythromycin	ery	MeOH	10mg/ml	YEPC	'Sigma'
nystatin	nys	DMSO	5mg/ml	YC	'Sigma'

*¹ DMSO - dimethyl sulphoxide , MeOH - methanol.

All stock solutions were stored at -20°C.

7 days incubation.

c) Petite Phenotype. The respiratory capacity of strains was tested in either of two ways:

- i. Tetrazolium Overlay (Ogur, St. John and Nagai 1957).
0.1g of tetrazolium salt (BDH) was dissolved in 100ml of phosphate buffer (pH 7.0), 1.25g of Oxoid Agar No. 3 added, and the solution boiled to dissolve the agar. This was allowed to cool to approximately 48°C and then 10-15ml was poured over 2-3 day old colonies growing on a YC plate. The plate was then incubated for up to 12 hours at 23°C. Respiration proficient (grande) colonies turn red, whilst respiration deficient (petite) colonies remain uncoloured.
- ii. YEPG. Subcultures of the strains under test, or YC plates with 100-200 individual colonies of the strain under test, were replica plated onto a YEPG plate. Excess transfer of cells was removed by pressing the YEPG plate onto a fresh sterile filter paper. Plates were incubated for 2-3 days at 28°C, or 5-7 days at 23°C, before the phenotype was scored. Petite strains are unable to utilise a non-fermentable carbon source such as glycerol and thus fail to grow on YEPG. Grande strains grow normally.

The latter method was generally used.

Colonies that showed petite sectors were classified as grande with only those colonies showing a 100% petite phenotype being classified as petite. The petite phenotype of strains which were red (i.e. carrying either the ade1 or ade2 mutation) could be distinguished visually because petite colonies appeared a dis-

tinct brown colour on YC against the normal red colour of grandes. On YEPD medium petites appeared white against the grande colonies red colour. These observations were confirmed by replica plating such red and brown (or white) colonies onto YEPG.

d) Killer Phenotype. This was determined using the killer assay medium (KM) previously described. Subcultures of strains under test, or YC plates containing 50-100 individual colonies of a particular strain, were replica plated onto KM and the KM plate incubated at room temperature ($18^{\circ}\text{C} \pm 1^{\circ}\text{C}$) for 7 days. The killer phenotype was ascertained by the degree of inhibition of growth of the sensitive strain seeded in the medium. A strain with normal killing ability (designated K^{+}) gave rise to a clear zone around itself, this zone being bordered by a ring of dead cells, which become stained dark blue due to an uptake of the methylene blue in the plate. A strain was said to show weak killing ability (designated K^{W}) if this zone of inhibition was not present, but the colony was surrounded by a ring of dead, methylene blue-stained cells. No killing ability (designated K^{-}) was deduced when neither the zone of inhibition, nor the ring of dead, stained cells, was present. The killer phenotype designation is that suggested by Wickner (1976). The resistance phenotype (R) of the strains was not determined.

e) UV-sensitivity. Mutants with a very UV-sensitive phenotype (e.g. rad1) were determined by replica plating subcultures of strains under test onto YC medium and UV-irradiating these plates with 20 or 50 J / m². Resistance and sensitivity were

scored after 3 days incubation at 28°C in the dark. For mutants which gave only a moderate UV-sensitive phenotype (e.g. rad3, rev3) a more sensitive "UV spot test" was used. Saline suspensions of cells to be tested were made up to c. 5×10^6 /ml. 10ul drops were spotted onto YC plates and allowed to dry. These plates were then irradiated at UV doses up to 80 J / m^2 and growth scored after 3 days incubation in the dark.

Strains carrying the umr1 gene mutation could not be successfully monitored by UV-sensitivity alone. Thus, because umr1 strains are blocked in UV-induced forward mutation to canavanine resistance (Lemontt 1977), this phenotype was also scored. 10ul drops of a saline cell suspension (c. 5×10^6 /ml) were inoculated onto OM+ can. plates and allowed to dry. The plates were then UV-irradiated at 20 or 40 J / m^2 . After an incubation period of 10 days the number of can^r colonies was determined. Table 2.3 gives results obtained with two tetrads derived from a diploid heterozygous for umr1 (i.e. UMR⁺/umr1).

f) Classification of non-suppressed mutants

Where possible a strain of the following general phenotype was used in mutagenesis experiments:-

SUQ5 ade2.1 his5.2 can1.00 ura3.1 (or leu1) psi⁺

The phenotype of this strain is ADE⁺ HIS⁺ CAN^Sura⁻ (leu⁻) and is white on YC medium. A non-suppressed mutant of this strain is red because of the expression of the ade2.1 ochre mutation which, being unable to convert 5-aminoimidazole ribonucleotide (AIR) into further intermediates of the adenine biosynthetic

TABLE 2.3 Determination of the umr1 phenotype.

STRAIN ^a	YC(J/m ²) ^b		OM + can. (J/m ²) ^c			GENOTYPE
	20	40	0	20	40	
1a	+	+	3	13,3	13,3	UMR+
1b	+	+	0	0,0	0,0	umr1
1c	+	+	3	0,0	0,1	umr1
1d	+	+	0	10,15	13,12	UMR ⁺
2a	+	+	2	0,0	0,0	umr1
2b	+	+	3	0,2	0,2	umr1
2c	+	+	0	10,13	10,2	UMR ⁺
2d	+	+	0	4,1	0,1	UMR ⁺

- a, the diploid number was MT195/-.
- b, '+' indicates no UV-sensitivity.
- c, the number of can^r colonies arising in two independent experiments are given.

pathway, (Silver and Eaton 1969) converts AIR into a red pigment. Such a non-suppressed mutant would also be histidine-requiring and canavanine resistant because the ochre-suppressible his5.2 and can1.100 mutations would also be expressed. Ura3.1 and leu1 are non-suppressible markers.

After treatment with a mutagen, e.g. ultraviolet light (UV), the cells were plated out onto solid YC plates to give 200-800 colonies per plate. After 4 days incubation at 28°C (7 days at 23°C) the plates were stored for 1-5 days at 4°C. This enabled one to distinguish with greater confidence true, non-suppressed, red mutants arising on the plate, from suppressed colonies, by increasing the strength of the red colour of the mutant. The total number of colonies on each plate were counted using a "Gallenkamp Colony Counter". The red mutant colonies were screened for using a Zeiss stereo microscope at 10x magnification and were scored as whole (100% mutant) or sectorised (less than 100% mutant) colonies. Each mutant was recloned by streaking onto YC to obtain a pure mutant clone for further analysis.

There are at least 8 different classes of mutant which give rise to the non-suppressed red mutant phenotype and these can be distinguished by determining the genotype of the mutant and by complementation tests using 4 defined tester strains which are also adenine-requiring. Table 2.4 gives the complementation results expected for each different class of mutant. The theory of the complementation test is given in Chapter 3. The tester strains used for the complementation analysis are given in Table 2.5.

TABLE 2.4 Mutations giving rise to a non-suppressed (red) phenotype; their genotypes and complementation patterns.

GENOTYPE ^a	GROWTH ON OM			COMPLEMENTATION ^b				
	-ade	-his	+can	xade1	xSUQ5 psi	xSQ5 ⁺ psi ⁺	xSQ5 ⁺ psi ⁺	kar
SUQ5 ade2.1 his5.2 psi ⁺	+	+	-					
SUQ5 ade2.1 his5.2 <u>psi⁻</u>	-	-	+	W	R	W		SW
SUQ5 ade 2.1 his5.2 psi ⁺ <u>ASU</u>	-	-	+	W	R	R		R
SUQ5 ade2.1 his5.2 psi ⁺ <u>asu</u>	-	-	+	W	W	W		WW
<u>SUQ5⁺</u> ade2.1 his5.2 psi ⁺	-	-	+	W	W	R		R
SUQ5 ade2.1 his5.2 psi ⁻ <u>PNM</u>	-	-	+	W	R	R		R
SUQ5 ade2.1 his5.2 psi ⁻ <u>pnm</u>	-	-	+	W	R	W		WW
SUQ5 <u>ade2.1.x</u> his5.2 psi ⁺	-	+	-	W	W	W		WW
SUQ5 ade2.1 his5.2 psi ⁺ <u>ade1</u>	-	+	-	R	W	W		WW

b SW denotes strong complementation, WW denotes complementation bare detectable for the SUQ5⁺ psi⁺ kar tester. For all other testers R implies no complementation, W strong complementation.

a The mutation giving the non-suppressed phenotype is underlined.

TABLE 2.5 Tester strains used for complementation analysis of
non-suppressed (red) mutants.

TESTER	STRAIN NO. ^{*1}	GENOTYPE
ade 1	461/1b	α ade 1
	461/4b	a ade 1 his 2
SUQ5 psi ⁻	468/1a	a SUQ5 ade2.1 lys1.1 can1.100 ura3.1 psi ⁻
	483/1d	a SUQ5 ade2.1 lys1.1 can1.100 psi ⁻
	468/1d	α SUQ5 ade2.1 his5.2 ura3.1 psi ⁻
	483/2d	α SUQ5 ade2.1 lys1.1 can1.100 psi ⁻
SUQ5 ⁺ psi ⁺	465/2a	a ade2.1 his5.2 can1.100 ura3.1 psi ⁺
	465/4d	α ade2.1 lys1.1 can1.100 ura3.1 psi ⁺
SUQ5 ⁺ psi ⁺	MT152/3c	a ade2.1 his5.2 can1.100 leu1 kar1 psi ⁺
kar	MT152/1d	α ade2.1 his5.2 leu1 kar1 psi ⁺

*1 all strains, except MT152/3c and MT152/1d,were obtained from
Dr. B. Cox.

Complementation tests were carried out on YEPD plates with a final glucose concentration of 8% being used to give maximum expression of the red pigment. Such medium also apparently prevented growth of many contaminating fungi and bacteria. Subcultures of mutants under test, and tester strains, were grown overnight at 28°C on YC plates. The tester strains were streaked vertically on the agar surface using a tungsten wire loop. Then inocula of each mutant (which was of opposite mating type to the tester strain) was carefully streaked horizontally across each tester strain, ensuring that a fresh inoculum was taken for each cross streak. This technique (illustrated on Figure 2.1) ensured that the mutant and the tester were thoroughly mixed on the right hand side of the vertical streak. If complementation occurred, white diploids would be present, in clear contrast to the red haploid strains, on the distal side of the vertical streak.

Two SUQ5 psi⁻ testers were always used because these strains show a very high frequency reversion to prototrophy (Young and Cox 1975) and a large number of prototrophs in the SUQ5 psi⁻ tester streak, as was often found, obscured any complementation that might be occurring. Only very rarely were both SUQ5 psi⁻ testers "lost".

The theoretical basis for the use of the SUQ5⁺ psi⁺ kar testers, to distinguish psi⁻ mutants from mutants carrying a pnm mutation, will be given in Chapter 7.

To distinguish SUQ5 ade2.1 psi⁺ strains from mutants of the genotype SUQ5⁺ ADE⁺ psi⁺ or SUQ5 SUPX ade2.1 psi⁺ a complementation test using an SUQ5 ade2.1 sal3.5 PNM2 psi⁻ tester strain

was used. Only SUQ5 ade2.1 psi⁺ strains give red diploids with this tester, the diploid being homozygous for SUQ5 and ade2.1, but heterozygous for PNM2. The psi⁺ phenotype is lost in the presence of the PNM2 mutation (Young and Cox 1971). Loss of the psi⁺ phenotype, when the other two ADE⁺ HIS⁺ strains are crossed to the tester, does not affect the suppressed phenotype of the diploid because, ^{for} the SUQ5⁺ ADE⁺ psi⁺ mutant, the diploid would be heterozygous for ade2.1 whilst for the SUQ5 SUPX ade2.1 psi⁺ mutant the diploid would be heterozygous for a /psi⁻ independent, dominant, ochre suppressor. Figure 2.1 illustrates such a complementation test with 16 white, undefined ADE⁺ HIS⁺ mutants. Numbers 3, 5 and 14 fail to complement either α or a tester.

The two tester strains used were obtained from Dr B. Cox and had the following genotypes:

584/2b α SUQ5 ade2.1 his5.2 lys1.1 sal3.5 PNM2 psi⁻
 584/1d a SUQ5 ade2.1 his5.2 lys1.1 sal3.5 PNM2 psi⁻

2.6 Mutagenesis

To obtain a stationary phase culture of the strain to be mutagenised, 10ml of liquid YC was inoculated with a loopful of the strain and the culture shaken at 28°C for 3-4 days in a thermostatically controlled, Gallenkamp shaking water bath. The cell number per ml., and proportion of budded cells present, were determined with a haemocytometer, and the cells pelleted by centrifugation (10 minutes at 3000rpm in an MSE benchtop centrifuge). The pellet was resuspended in an equal volume of either saline or appropriate buffer. The proportion of budded cells was always between 2% and 10% of the population.

FIGURE 2.1 A complementation test using an α and a SUQ5 ade2.1 sal3.5 PNM2 psi⁻ tester strain to identify SUQ5 ade2.1 psi⁺ mutants from SUPX ade2.1 and SUQ5 ADE⁺ mutants.



NOTE: The vertical streaks are the α and a tester strains 584/2b and 584/1d respectively. The 16 horizontal cross-streaks are undefined ADE⁺HIS⁺ mutants.

To obtain a log phase culture of the strain a YC plate was inoculated with several loopfuls of the strain spread into a thin film over the plate. The plate was incubated at room temperature overnight and the cells then suspended in saline or appropriate buffer. The proportion of budded cells was generally greater than 60% of the population.

a) Ultra-violet (UV) irradiation

A 10ml saline suspension, containing between 3×10^6 and 2×10^7 cells/ml, was irradiated in an open, sterile petri dish at a dose rate of either 0.5, 1 or 2 J / m²/sec. depending on the sensitivity of the strain used. The petri dish was gently agitated throughout irradiation. The irradiated cell suspension was then diluted using saline, in sterile McCartney or Bijou bottles, and plated onto YC plates.

UV light was obtained using a Hanovia IIa mercury discharge tube which emitted light mainly at 254nm. The intensity was controlled by varying the distance between the tube and the cell suspension. Intensity was measured with a galvanometer attached to a photosensitive head.

All operations described above were carried out under yellow, non-photoreactivating light. YC plates were incubated in the dark.

b) N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG)

A stock solution of NTG (Koch-Light) was prepared immediately prior to use by adding 20mg of NTG to 2ml acetone. Appropriate aliquots were added immediately to 10ml of a sterile 0.2M phosphate buffer (pH7.0) in a 50ml flask covered in silver paper, and containing cells at a titre of 10^5 - 10^6 /ml. After shaking for 45

minutes at 28°C, 200µl of the suspension was added to 9.8ml of a 6% (W/V) sterile sodium thiosulphate solution and incubated at 28°C for 10 minutes to inactivate the NTG. The cell suspension was then diluted in sterile saline and spread onto YC plates. A control was always run in parallel without NTG.

c) Ethyl Methanesulphonate (EMS)

The method used was that of Lindegren et al., (1965). 300µl of EMS (Koch-Light) was added to 9.2ml 0.2M sterile phosphate buffer (pH 8.0) and 0.5ml 20% (W/V) sterile glucose solution. Cells were suspended at a titre of approximately 10^6 /ml in the buffer. After incubating at 28°C for 70 minutes, with constant agitation, 200µl of the suspension was added to 9.8ml sterile 6% (W/V) sodium thiosulphate, incubated for 10 minutes at 28°C and, after appropriate dilutions in sterile saline, plated onto YC medium. A control flask with 300µl sterile water instead of EMS was always run in parallel.

2.7 Statistical procedures

Standard statistical procedures, as described in Bishop (1971), were used to calculate 'chi-squared' (χ^2). In cases where the degree of freedom was one (df=1) 'Yates' Correction' was used:

$$\begin{aligned} \text{df} > 1 ; \quad \chi^2 &= \frac{(O-E)^2}{E} \\ \text{df} = 1 ; \quad \chi^2 &= \frac{(|O-E| - \frac{1}{2})^2}{E} \end{aligned}$$

O = observed, E = expected

CHAPTER 3

/PSI⁻/MUTAGENESIS : INDUCTION OF PSI⁻ MUTANTS WITH NUCLEAR MUTAGENS

- 3.1 Introduction
- 3.2 Methods for isolating and characterising psi⁻ mutants
- 3.3 Ultraviolet light mutagenesis
- 3.4 Nitroguanidine mutagenesis
- 3.5 Preliminary analysis of other nuclear mutagens
- 3.6 Discussion

3.1 Introduction

Many physical and chemical agents are known to cause heritable phenotypic changes in both prokaryotic and eukaryotic micro-organisms (see Auerbach 1976 for a recent review). The majority act in a direct chemical fashion on chromosomal (and cytoplasmic) DNA causing stable lesions in the DNA molecule which, if left unrepaired, can lead to mutation which is expressed as an alteration in phenotype or cell death. Many of these agents are known to be mutagenic in yeast (Mortimer and Manney 1971, and Kilbey 1975). If such mutagens can be shown to cause mutation in the $\underline{\bar{\psi}}$ factor (i.e. $\underline{\psi}^+$ to $\underline{\psi}^-$) this would be circumstantial evidence for the molecular basis of $\underline{\bar{\psi}}$ being a nucleic acid, probably DNA.

To date both electron microscopy (McCready and McLaughlin, 1977; McCready personal communication) and genetic studies (Young and Cox, 1972; Cox personal communication) have failed to identify an extrachromosomal nucleic acid species associated with the $\underline{\bar{\psi}}$ factor. In the absence of such direct evidence I have used mutagenesis as an indirect probe of the chemical nature and location of the $\underline{\bar{\psi}}$ factor. Evidence to date points to the $\underline{\bar{\psi}}$ factor being a cytoplasmically located DNA molecule present as more than one copy per haploid cell (Young 1969; McCready, Cox and McLaughlin 1977). This will be used as the "null hypothesis" for these experiments on $\underline{\bar{\psi}}$ mutagenesis.

Young (1969) was the first to demonstrate that one could mutate $\underline{\psi}^+$ to $\underline{\psi}^-$ using UV light and the alkylating agent ethyl methane sulphonate (EMS). Using this observation I have set out, in this chapter, to answer the following questions:

(1) what are the kinetics of the UV-induced mutation from psi⁺ to psi⁻ and how do they compare with the kinetics of UV-induced forward and reverse nuclear gene mutation and of the cytoplasmic petite mutation?

and,

(2) can mutagenic agents, other than UV or EMS, mutate psi⁺ to psi⁻, and if so, how does the mutation frequency compare with that for nuclear and cytoplasmic mutation?

3.2 Methods for isolating and characterising psi⁻ mutants

A mutation from psi⁺ to psi⁻ in a strain with the genotype SUQ5 ade2.1 his5.2 can1.100 psi⁺ would give rise to a mutant strain unable to suppress the three nonsense markers. The mutant clone would be red on YC, unable to grow on medium lacking adenine and histidine, but be able to grow on medium containing canavanine, because of the loss of the SUQ5 suppressing function. In a study of /psi mutagenesis two practical problems arise:

(1) how does one qualify the frequency at which mutant (non-suppressed) clones arise, from an initially non-mutant (suppressed) population, after mutagenic treatment?
and,

(2) how does one distinguish a cytoplasmic psi⁺ → psi⁻ mutation from mutations in nuclear loci which also give rise to the mutant phenotype?

3.2. (i) Quantification of induced mutation frequency

A mutant clone is distinguished primarily by its altered phenotype which, in this case, is associated with a visible change in the mutant clone, the simultaneous uncovering of at least two auxotrophic requirements, and a change, from sensitivity to

resistance to the toxic effects of the arginine analogue canavanine (can). Several methods were examined for feasibility as ways of accurately quantifying the spontaneous and induced frequency of mutation from suppressed to non-suppressed.

(a) Visible screening

One can visually detect mutant clones by plating mutagenised cultures onto complete growth medium (YC) as described in 'Materials and Methods'. The only constraint placed upon the mutant clone is that it is able to grow on YC. All classes of mutant giving a non-suppressed phenotype would be obtained and would enable one to score both pure mutant and sectorised mutant clones. The major drawback of this method is that large numbers have to be screened to obtain a statistically significant number of mutants. Attempts were therefore made to use other systems, for scoring mutation frequency, which enabled one to select for non-suppressed mutants from a population of mutagenised cells.

(b) Canavanine resistance

Spontaneous or induced, suppressor-inactive, revertants can be selected from strains of the general genotype SUPX ade2.1 can1.100 by plating cells at a high density (10^4 - 10^7 cells) onto fully supplemented minimal medium containing canavanine, but lacking arginine, and picking red can^r colonies that grow (McCready and Cox 1973). An attempt to determine whether such a system could be used as a quantitative measure of the induced frequency of mutation from suppressed to non-suppressed was therefore made. The strain used for these experiments, 507-4/2b, had the genotype SUQ5 ade2.1 his5.2 can1.100

ura3 psi⁺.

The selection medium used was basically MM+ade+his+ura+tyr+lys+can, but the ade, his, can and glucose concentrations were varied. The variations used are given in Table 3.1. The ade, his and glucose concentration were varied in an attempt to obtain maximum expression of the red pigment which failed to develop on OM+2C.

Log phase cells were UV-irradiated at 75 J / m^2 and plated, at 2 or 3 different cell densities (10^5 , 10^4 and 10^3 cells per plate), onto the selection medium to select for red, can^r colonies. An unirradiated control culture was plated similarly. Colonies were scored after 10 days growth at 28°C; further incubation resulted in the growth of many background micro-colonies which, when retested, were not can^r. Such colonies arose either because the canavanine had decomposed (McCready 1972) or as a result of canavanine-sensitive cells taking up arginine released from dead cells. Non-toxic arginine is taken up by cells in preference to its toxic analogue canavanine.

Five independent experiments using 10 different selection media were carried out. In each case unirradiated and irradiated cells were also plated onto YC to monitor survival and the number of potentially viable mutants present in the population. For each experiment approximately 1000 colonies were scored the frequency of mutant colonies being between 1.7 and 5.4 per 10^3 survivors (average 4.1 per 10^3 survivors). A total of 4348 unirradiated colonies were screened from the five experiments with only 1 non-suppressed mutant (a psi⁻ mutant) being found. Survival, after UV-irradiation, on YC varied between 13% and 41% (average 26%).

TABLE 3.1 Concentrations of adenine, histidine, canavanine and glucose in medium used for selection of can^r mutants.

MEDIUM	FINAL CONCENTRATIONS*1			
	adenine ($\mu\text{g/ml}$)	histidine ($\mu\text{g/ml}$)	canavanine ($\mu\text{g/ml}$)	glucose (% w/v)
OM+2C	20	20	60	1
1	5	20	60	1
2	5	5	60	1
3	5	20	100	1
4	2	20	60	1
5	20	20	60	2
6	20	20	60	4
7	5	20	60	4
8	10	20	180	3
9	10	20	60	3

*1 lysine, tyrosine and uracil were present in all media each at a final concentration of $10\mu\text{g/ml}$.

An experiment was carried out to check survival before and after UV-irradiation (75 J / m^2) on the selection media without canavanine using 5 or 20 $\mu\text{g/ml}$ ade. For 20 $\mu\text{g/ml}$ ade little effect on survival, compared to YC, was seen; 96.9% unirradiated, 96.1% UV-irradiated. For 5 $\mu\text{g/ml}$ survival, compared to YC, was 96.7% unirradiated and 89.4% after UV-irradiation. Overall survival on selection media without canavaine was therefore only very slightly reduced to that obtained on YC. An average of 500 colonies were scored for each experiment.

The results presented in Table 3.2 show that the majority of can^r colonies that arose in these five experiments showed no red pigmentation on the selection medium. Forward mutations to can^r occur at a single gene CAN1⁺ (Grenson et al., 1966) and are induced at a high frequency by UV-irradiation (Lawrence et al., 1974). To check whether the white can^r mutants arising on the selective media were due to second-site mutations at the can1 locus, or had just failed to produce the red pigment, individual colonies were picked and inoculated onto YC plates to check for development of the red pigment (Table 3.3). The proportion of colonies showing any red pigment on YC was between 0 and 35%. It did seem, however, that all non-suppressed can^r colonies produced the red pigment on medium numbers 2, 5, 6, 7, 8 and 9, but not on numbers .1, 3 and 4, where only the ade concentration was reduced. Increased glucosae concentrations (2-4%) gave the best pigmentation of mutant colonies. However, in all cases (with the exception of No. 4) the frequency of can^r colonies (both suppressed and non-suppressed)

TABLE 3.2 Frequencies of canavanine-resistant colonies after UV-irradiation (75J/m²) using different selection media.

E X P T	MEDIUM*1	CONTROL			UV			RED COLONIES /10 ³ SURVIVORS (YC)	% SURVIVAL (YC)
		NUMBER VIABLE CELLS PLATED (X10 ⁵)	CAN ^r COLONIES *3	CAN ^r PER 10 ⁵ PLATED	NUMBER VIABLE CELLS PLATED (X10 ⁵)	CAN ^r COLONIES *3	CAN ^r PER 10 ⁵ PLATED		
1	CM+2C	30.86	8(0)	0.26	1.95	0	-	4.10	12.56
	1	3.06	301(5)	98.37	1.95	678(0)	347.69		
	2	1.76	18(0)	4.55	4.75	179(1)	183.75		
2	3	1.76	28(0)	15.94	4.75	607(0)	127.79	4.54	27.08
	4	1.76	418(1)	238.04	0.47	686(5)	1456.47		
	5	12.32	66(9)	5.36	3.35	645(5)	192.54	4.76	27.45
3	6	12.32	68(14)	5.52	3.35	908(11)	271.04		
	7	11.88	89(0)	7.49	4.86	100(0)	20.58	5.41	40.92
	8	11.46	7(1)	0.61	2.59	5(0)	8.20	1.69	22.58
4	9	11.46	67(2)	5.85	2.59	89(0)	34.36		

*1 For details of medium see TABLE 3.1.

*3 Figures in brackets are the number of red can^r colonies on the selection plates.

TABLE 3.3 Phenotypes on YC of white can^r colonies isolated on various selection media.

MEDIUM	CONTROL						UV							
	NUMBER COLONIES TESTED			COLONY PHENOTYPE ON YC			NUMBER COLONIES TESTED			COLONY PHENOTYPE ON YC				
				RED	RED/WHITE	WHITE				DEAD	RED	RED/WHITE	WHITE	DEAD
				%*1 RED						%*1 RED				
1	66	1	7	57	1	12.31	124	2	30	83	9	27.83		
2	8	0	0	8	0	0.00	77	0	2	69	6	2.82		
3	24	0	6	17	1	26.09	83	0	12	62	9	16.22		
4	50	2	14	34	0	32.00	86	4	23	54	5	33.33		
5	35	0	0	33	2	0.00	81	1	0	80	0	1.23		
6	45	0	0	44	1	0.00	74	1	1	71	1	2.74		
7	81	3	1	77	0	4.94	76	3	1	70	2	5.40		
8	7	0	1	6	0	14.28	5	0	0	5	0	0.00		
9	67	0	2	65	0	2.98	82	0	0	82	0	0.00		

*1 Including red/white colonies.

was substantially less than the frequency of non-suppressed mutants picked up on YC in the same experiment. For medium No. 4 the frequency of non-suppressed can^r mutants after UV, calculated from the data in Table 3.2 and 3.3, was 4.96 per 10^3 survivors compared to the frequency of 4.54 per 10^3 survivors on YC. The frequency for the unirradiated control (0.77 per 10^3 survivors) was higher than predicted from the control on YC plates in this and other experiments. (see Section 3.3).

The frequency of can^r mutants, both spontaneous and UV-induced, varied considerably depending on the medium composition. Similarly the UV-induced increase in mutation frequency (i.e. UV-induced frequency/control frequency) varied from x2.75 to x49.10 depending on the medium used.

To ascertain whether a particular class of non-suppressed mutant was being selected for, on any one particular medium, a total of 98 red can^r mutants from 5 different selection media were cloned on YC, their genotypes determined by complementation and compared to the classes of mutant picked up on YC at the same UV-fluence (Table 3.4). All classes were obtained although 16/23 (69.6%) mutants isolated from medium 6 were asu compared to 7/27 (25.9%) isolated from YC.

It was therefore concluded that no particular class of non-suppressed mutant of 507-4/2b was selected for using this selection system, but that it was not a suitable method for quantitative estimation of the frequency of non-suppressed mutants because;

- (a) the frequency of red can^r mutants was substantially less than predicted by the visible screening method and

TABLE 3.4 Characterisation of red can^r colonies isolated, before and after UV (75J/m²), on different selection media .

CLASS OF MUTATION	YC		1		3		MEDIUM		4		5		6	
	CON	UV	CON	UV	CON	UV	CON	UV	CON	UV	CON	UV	CON	UV
TOTAL	1	26	16	19	0	7	7	7	15	8	3	14	9	9
ade 1	0	2	0	1	-	0	0	0	0	0	0	0	0	0
ade2.x	0	1	0	0	-	0	0	0	0	0	0	0	0	0
psi ⁻	1	5	3	4	-	1	3	2	2	1	2	3	0	0
prum	0	3	2	1	-	1	0	2	2	4	0	2	0	0
PNM/ASU	0	3	0	3	-	0	1	2	2	0	1	1	0	0
asu	0	7	3	2	-	4	2	6	6	1	0	7	9	9
SUQ5 ⁺	0	6	8	8	-	1	1	3	3	2	0	1	0	0
rho ⁻	0	4	0	2	-	0	0	0	0	0	0	0	0	0
ts(37°C)	0	1	0	0	-	0	0	1	1	0	0	0	0	0

NUMBERS OF MUTANTS IN EACH CLASS

(b) on most of the selection media used some non-suppressed can^r mutants failed to accumulate the red pigment.

(c) Selection on hypertonic medium

It was recently reported that, with the exception of SUQ5 and an undefined amber suppressor, both ochre and amber nonsense suppressor-bearing strains are unable to grow on YEPD containing 1.5M potassium chloride (KCl). Suppressor-inactive revertants however were able to grow on this hypertonic medium. (Singh 1977). I therefore determined whether strains carrying SUQ5 were sensitive to KCl at a molarity higher than 1.5M, but which did not inhibit SUP⁺ strains, and whether I could use this medium to isolate non-suppressed revertants of 507-4/2b.

A preliminary screen of a series of stock ochre and amber suppressor-bearing strains failed to confirm Singh's observations (Singh 1977) (Table 3.5). Saline suspensions of the strains were spotted onto YC plates containing KCl at 3 different molarities and growth scored at 4 days. In particular the SUP5-a and SUP52-a strains showed no osmotic-sensitive growth. 4 revertants of SUP3 that grew on 1.5M KCl were non-suppressed. The strain carrying the frameshift suppressor SUF2 showed sensitivity to 1.5M KCl, but the SUF1 and SUF5 bearing strains did not. It should be noted that YC was used for these experiments whereas Singh used YEPD.

The sensitivity of 507-4/2b to KCl was investigated in detail by plating different dilutions of an irradiated and an unirradiated log phase culture onto YC plates containing 1.5M to 3.5M KCl. $75 \text{ J} / \text{m}^2$ of UV-irradiation was given resulting

TABLE 3.5 Growth on YC+KC1 medium of strains carrying various nonsense and frameshift suppressors.

SUPPRESSOR	STRAIN*1	GROWTH RESPONSE*2		
		1.5MKC1	1.75MKC1	2.00MKC1
SUQ5 psi ⁺	507-4/2b	+++	+++	++
SUQ5 psi ⁻	483/1d	+++	+++	++
SUQ5-a psi	455	++	+	+
SUP2 psi ⁻	x2314-8C	-	-	-
SUP3 psi ⁻	x2315-14C	-	-	-
SUP4-a psi ⁻	FM11	+++	++	-
SUP5-a psi ⁻	635	+++	+++	+++
SUP6-a psi ⁻	D600	-	-	-
SUP52-a psi ⁻	381	+++	++	-
SUF1 psi ⁺	5571-6B	+++	+++	+
SUF2 psi ⁻	MC16	-	-	-
SUF5 psi ⁻	405	++	+++	-
SUP ⁺ psi ⁺	465/2a	+++	+	-
SUP ⁺ psi ⁺	465/4d	+++	+++	++

*2 +++ represents growth on YC controls plate, - no growth.

*1 strains D600, 455, 635 and 381 were obtained from Dr. F. Sherman; x2314-8C and x2315-14C from Dr. R.K. Mortimer; FM11, 5571-6B, MC16 and 405 from Dr. G.R. Fink. All others obtained from Dr. B. Cox.

in 20.65% survival and 5 non-suppressed mutants, out of 1021 colonies screened (4.89 per 10^3 survivors), on YC. The results are given in Table 3.6. No red colonies arose, but when individual colonies were picked and inoculated onto a YC plate, 40 out of 272 (14.70%) such colonies, taken from 4 different plates (1.5, 1.8, 1.9 and 2.0M), showed red mutant clones, but none were pure mutant colonies. It therefore appeared that the red pigment is not expressed on KCl medium although the possibility that it was due to phenotypic suppression was not investigated. It has however been recently demonstrated that 0.5M KCl phenotypically suppress the his5.2 and lys1.1 ochre mutations (C. Waldron personal communication). Large numbers of suppressed colonies also grew on KCl medium.

These observations, taken with the observation that growth on YC plates and in liquid YC supplemented with KCl "cures" 507-4 2b of its psi⁺ phenotype (for details see Chapter 6) rule out this approach for quantitative estimation of mutation frequency.

(d) Selection for cold resistance

Following a recent unpublished observation in our laboratory that 507-4/2b, and other strains of the genotype SUQ5 psi⁺, are unable to grow at 12°C (i.e. cold sensitive, CS) and that revertants that can grow at 12°C (i.e. CS⁺) are generally non-suppressed (C. Mundy personal communication), the possibility of using this phenomenon as a method for determining mutation frequency was investigated.

A saline suspension of log phase cells of 507-4/2b was irradiated with UV (75 J / m²). Various dilutions of both the

TABLE 3.6 Survival of 507-4/2b on YC + KC1 plates after UV-irradiation (75 J/m²).

KC1(M)	CONTROL			UV		
	NUMBER OF*1 CELLS PLATED	NUMBER OF COLONIES GROWING	SURVIVAL %	NUMBER OF*1 CELLS PLATE	NUMBER OF COLONIES GROWING	SURVIVAL %
0	572	572	100	1021	1021	100
1.5	1907	1241	65.09	638	137	21.47
1.6	1907	1269	66.56	638	131	20.53
1.7	1907	1257	65.93	638	112	17.55
1.8	1907	1050	55.07	638	74	11.60
1.9	1907	888	46.58	638	38	5.96
2.0	1907	122	6.39	638	21	3.29
2.2	1.91x10 ⁶	0*2	-	6.38x10 ⁴	0	-
2.5	1.91x10 ⁶	1	5x10 ⁻⁵	6.38x10 ⁴	0	-
3.0	1.91x10 ⁶	0	-	6.38x10 ⁴	0	-
3.5	1.91x10 ⁶	0	-	6.38x10 ⁴	0	-

*1 Estimated from the number of colonies surviving on the YC control plates.

*2 Weak background growth was seen but no discrete colonies formed after 14 days incubation.

irradiated and a control, unirradiated, cell suspension were plated onto YC. These plates were incubated in the dark, at $12^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 21 days, the temperature being continuously monitored. The number of CS^{+} revertants were scored (Table 3.7). Unirradiated and irradiated cells from this experiment were also plated onto YC and incubated at 28°C to determine the level of survival (27%) and frequency of non-suppressed viable revertants in the population (0 out of 1335 colonies for the control and 6 out of 1283 colonies after UV, a frequency of 5.14 per 10^3 survivors). Some white (suppressed?) CS^{+} revertants were found, but not scored.

An almost 10-fold increase in the frequency of non-suppressed CS^{+} revertants, per 10^3 viable cells plated, was seen after UV-irradiation. Many of the CS^{+} revertants were cloned and classified by complementation analysis (Table 3.8). From this data it was calculated that the frequency of psi⁻ (including pnm) mutants was 0.11 per 10^3 viable cells plated for the control culture and 0.59 per 10^3 viable cells plated after UV. These values are lower than those calculated by visible screening i.e. 0.18 and 1.28 respectively (see Table 3.14). The frequency of non-suppressed CS^{+} revertants was 1.15 per 10^3 viable cells plated after UV compared to 4.26 non-suppressed mutants per 10^3 survivors calculated by visible screening (see Table 3.12). The proportion of psi⁻ mutants (including pnm) among the CS^{+} revertants was much higher (50-95%) than found using the visible screening method ($< 20\%$) at 28°C .

There is a possibility that growth at 12°C may not only select for, but induce, psi⁻ mutants (C. Mundry personal

TABLE 3.7 Frequency of non-suppressed, cold-resistant revertants of 507-4/2b before and after UV-irradiation (75J/m²).

TREATMENT	DILUTION	TOTAL VIABLE*1 CELLS PLATED	TOTAL*2 CS ⁺	PIGMENTATION OF CS ⁺			FREQUENCY /10 ³
				WHOLE RED	MOSAIC RED	PINK	
0	1x	1.76x10 ⁶ (3)	CF*3	-	-	-	
	10 ⁻²	2.34x10 ⁴ (4)	3	3	0	0	0.12
	10 ⁻³	1.17x10 ³ (2)	0	0	0	0	
UV	1x	9.63x10 ⁵ (3)	CF*3	-	-	-	
	10 ⁻¹	9.63x10 ⁴ (3)	104	54	4	46	1.15
	10 ⁻²	1.13x10 ⁴ (4)	21	9	2	10	

*1 Calculated from the number of colonies growing at 28°C on YC. The figures in brackets are the number of plates used.

*2 Only non-suppressed colonies scored.

*3 At high cell densities (ie. > 1 x 10⁵ per plate) a large background growth of suppressed colonies was observed but with numerous distinct red papillae.

TABLE 3.8 Classification of non-suppressed, cold-resistant revertants of 507-4/2b.

TREATMENT	MUTANT	PHENOTYPE	NUMBER*1 ANALYSED	MUTANT CLASSIFICATION					
				psi-*3	asu	PNM/ASU	SQ5+	ade1	ade2x
0	RED	ade ⁻ his ⁻ can ^{r*2}	23(12)	23	0	0	0	0	0
	RED	ade ⁻ his ⁻ can ^r	3(0)	1	2	0	0	0	0
	TOTAL		26(12)	24	2	0	0	0	0
UV (75J/m ²)	RED	ade ⁻ his ⁻ can ^{r*2}	22(15)	18	3	1	0	0	0
	RED	ade ⁻ his ⁻ can ^r	46(24)	23	7	3	12	0	1
	PINK	ade ⁻ his ⁺ can ^r	12(7)	0	12	0	0	0	0
	TOTAL		80(46)	41	22	4	12	0	1

*1 the figure in brackets is the number which were petite.

*2 these mutants were isolated as fast growing red papillae from plates with high initial cell densities (see TABLE 3.7).

*3 cytoplasmic psi⁻ mutants were not distinguished from pnm mutants.

communication) although the data presented here shows that the frequency of CS^+ psi⁻ mutants (including pnm) was less than would be predicted from the control experiments at 28°C.

Two interesting observations were also made:

- i. there appeared to be two distinct classes of CS^+ asu mutants; those which formed pink colonies at 12°C and showing marked suppression of the his5.2 marker, and those which formed red colonies at 12°C with the his5.2 marker completely unsuppressed
- ii. a high proportion of CS^+ revertants were petite; after UV 46/80 (57.50%) CS^+ revertants were petite, against an expected 6.9% from 28°C experiments (see Table 3.15).
- iii. on the control plates virtually no nuclear mutations were found. This could reflect a higher spontaneous mutation rate for the psi⁺ → psi⁻ mutation than for forward nuclear mutation.

This method was found unsuitable for use as an estimate of mutation frequency because of the doubts about induction of psi⁻ mutants at 12°C, the finding that some SUQ5 psi⁺ strains were not CS (e.g. MT72/2b, MT182/8d) and that the estimated mutation frequencies for the different classes of mutant were substantially less than calculated by the visible screening method.

(e) General conclusion

- The visible screening procedure was found to be the most satisfactory estimate of mutation frequency for two major reasons:
- i. the only constraint made upon the mutant is that it is able to grow on complete medium, and
 - ii. it enables one to distinguish pure and sectorized mutant clones.

This method was used in all experiments on /psi mutagenesis.

3.2.a (ii) Identification of mutations giving rise to the mutant non-suppressed phenotype

(a) Classes of Mutation

One can postulate that at least 6 different classes of mutation could give rise to the mutant, non-suppressed phenotype, only one of which would be the cytoplasmic psi⁻ with which this thesis is concerned. The other 5 classes would be:

- (1) ade1; a recessive mutation in the ADE1⁺ gene, non-suppressible by SUQ5,
- (2) ade2.1.x; a second, recessive mutation, within the ade2 gene which was non-suppressible by SUQ5,
- (3) antisuppressors; dominant (ASU) and recessive (asu) nuclear mutations which reduce or eliminate suppressor function by SUQ5. Recessive asu mutants have already been characterised (McCready and Cox 1973),
- (4) /psi⁻ maintenance; dominant (PNM) and recessive (pnm) mutations in nuclear genes necessary for maintenance of the psi⁺ phenotype. At least 2 unlinked PNM loci have been found (Young and Cox 1971; Cox personal communication), which eliminate the psi⁺ phenotype.
- (5) SUQ5⁺; loss of suppressor activity due to a second-site mutation within the SUQ5 gene or reversion of the original site of mutation. Second-site mutations inactivating suppressor tRNA are known to occur in both E. coli (Smith 1972) and yeast (Di Caprio and Hastings 1976; Rothstein 1977).

(b) Complementation Test

To determine which class of mutation is responsible for the mutant phenotype a complementation test has been devised, the

practicalities of which have been given in Chapter 2 (Section 2.5f). Tables 3.9a and 3.9b set out the predicted complementation patterns given in Table 2.4 using SUQ5 psi⁻ and SUQ5⁺ psi⁺ tester strains. It should be mentioned that SUQ5 is dominant, as is psi⁺, in heterozygous crosses (Cox 1965). All classes of mutant, with the exception of the ade1 mutant, would complement a strain of genotype ade1 psi⁺ (or psi⁻), ade1 being recessive.

(c) Experimental Confirmation of the Complementary Results

To confirm the predicted segregation patterns in tetrads from diploids resulting from crossing a non-suppressed mutant to the SUQ5⁺ psi⁺ strain (in this case 465/2a) (Table 3.9b), a number of UV-induced non-suppressed mutants of 507-4/2b, giving one or other of the complementation patterns listed in Table 2.4, were crossed to 465/2a. Zygotes, isolated by micromanipulation, were grown up on YC, their phenotype determined and then induced to sporulate. 8-12 tetrads were dissected from each diploid and, after 5 days at 28°C, scored for segregation of red and white spores (Table 3.10).

The most interesting observation from this analysis was that only one mutant, of those classified as psi⁻, gave the predicted spore segregation pattern i.e. mutant number 4. The other 5 mutants gave overall segregation patterns of 3 red: 1 white spores indicating that the strains probably carried a recessive nuclear gene mutation which results in elimination of the psi⁺ phenotype. Each mutant was psi⁻ because of their failure to complement with an SUQ5 ade2.1 psi⁻ strain and the mutation must be recessive because they complemented the SUQ5⁺ ade2.1 psi⁺ strain. The mutation carried by these "nuclear

TABLE 3.9a) Theoretical basis of complementation tests for non-suppressed revertants of strains of the genotype SUQ5 ade2.1 his5.2 can1.100 psi⁺. I. crosses to SUQ5 ade2.1 his5.2 can1.100 psi⁻ tester.

MUTANT CLASS	RESULTING GENOTYPE	TYPE OF MUTATION	DIPLOID ^{*1} GENOTYPE	PREDICTED DIPLOID PHENOTYPE	PREDICTED SEGREGATION IN TETRAIDS (WHITE: RED)
NON MUTANT PARENT	SUQ5 psi ⁺	dominant cytoplasmic	$\frac{\text{SUQ5}}{\text{SUQ5}}$ psi ⁺	WHITE	4:0
psi ⁻	SUQ5 psi ⁻	recessive cytoplasmic	$\frac{\text{SUQ5}}{\text{SUQ5}}$ psi ⁻	RED	0:4
ASU	SUQ5 ASU psi ⁺	dominant nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{ASU}}{+}$ psi ⁺	RED	2:2
asu	SUQ5 asu psi ⁺	recessive nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{asu}}{+}$ psi ⁺	WHITE	2:2
PNM	SUQ5 PNM psi ⁻	dominant nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{PNM}}{+}$ psi ⁻	RED	0:4
pnm	SUQ5 pnm psi ⁻	recessive nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{pnm}}{+}$ psi ⁻	RED	0:4
SUQ5 ⁺	SUQ5 ⁺ psi ⁺	recessive nuclear	$\frac{\text{SUQ5}}{+}$ psi ⁺	WHITE	2:2
ade1	SUQ5 ade1psi ⁺	recessive nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{ade1}}{1}$ psi ⁺	WHITE	2:2
ade2.x	SUQ5 ade2.1.x psi ⁺	recessive nuclear	$\frac{\text{SUQ5}}{\text{SUQ5}}$ $\frac{\text{ade2.1.x}}{\text{ade2.1}}$ psi ⁺	WHITE	2:2

^{*1} All diploids would be homozygous for ade2.1 his5.2 and can1.100.

TABLE 3.9b) Theoretical basis of complementation tests for non-suppressed revertants of strains of the genotype SUQ5 ade2.1 his5.2 can1.100 psi⁺. II. crosses to ade2.1 his5.2 can1.100 psi⁺ tester.

MUTANT CLASS	RESULTING GENOTYPE	TYPE OF MUTATION	DIPLOID*1 GENOTYPE	PREDICTED DIPLOID PHENOTYPE	PREDICTED SEGREGATION IN TETRADS(WHITE:RED)
NON MUTANT PARENT	SUQ5 psi ⁺	dominant cytoplasmic	$\frac{+}{\text{SUQ5}}$ psi ⁺	WHITE	2:2
psi ⁻ *2	SUQ5 psi ⁻	recessive cytoplasmic	$\frac{+}{\text{SUQ5}}$ psi ⁺	WHITE	2:2
ASU	SUQ5 ASU psi ⁺	dominant nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{ASU}}{+}$ psi ⁺	RED	2:2, 1:3, 0:4
asu	SUQ5 asu psi ⁺	recessive nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{asu}}{+}$ psi ⁺	WHITE	2:2, 1:3, 0:4
PNM	SUQ5 PNM psi ⁻	dominant nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{PNM}}{+}$ psi ⁻	RED	0:4*3
pnm	SUQ5 pnm psi ⁻	recessive nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{pnm}}{+}$ psi ⁺	WHITE	2:2, 1:3, 0:4
SUQ5 ⁺	SUQ5 ⁺ psi ⁺	recessive nuclear	$\frac{+}{+}$ psi ⁺	RED	0:4
ade1	SUQ5 ade1 psi ⁺	recessive nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{ade1}}{+}$ psi ⁺	WHITE	2:2, 1:3, 0:4
ade2.x	SUQ5 ade2.1.x psi ⁺	recessive nuclear	$\frac{+}{\text{SUQ5}}$ $\frac{\text{ade2.1.x}}{\text{ade2.1}}$ psi ⁺	WHITE	2:2, 1:3, 0:4

*3 a few suppressed white spores would be expected (see Young and Cox, 1971).

*1 all diploids would be homozygous for ade2.1 his5.2 can1.100.

*2 a 'suppressive'_{psi⁻} would give a red diploid which would give a tetrad segregation of 4R:0W.

TABLE 3.10 Genetic analysis of diploids derived from crossing classified non-suppressed mutants of 507-4/2b with 465/2a (SUQ5⁺ ade2.1 psi⁺) tester.

PUTATIVE MUTATION	MUTANT NO.	DIPLOID*3 PHENOTYPE	TETRAD ANALYSIS				S: NS IN COMPLETE TETRADS			OBSERVED SPORE *4 TOTALS S: NS	PREDICTED *1 S: NS
			NO. DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS	S: NS IN COMPLETE TETRADS					
						4:0	3:1	2:2			
psi ⁻	1	S	12	85.4	6	2	2	2	32:9	2:2	
	2	S	12	97.9	11	2	8	1	37:10		
	3	S	12	81.2	7	2	4	1	32:7		
	4	S	12	79.2	6	0	0	6	21:17		
	5	S	20	62.5	8	1	3	4	36:15		
	6	S	10	92.5	7	1	5	1	27:10		
SUQ5 ⁺	1	NS	12	91.7	8	8	0	0	44:0	4:0	
	2	NS	11	86.4	8	8	0	0	38:0		
	3	NS	12	95.8	10	10	0	0	46:0		
	4	NS	10	100	10	10	0	0	40:0		
	5	NS	12	97.9	11	2	7	2	35:12		
PNM/ ASU*2	1	NS	12	95.8	10	9	1	0	45:1	4:0	
	2	NS	12	97.9	11	8	3	0	44:3		
	3	NS	12	93.8	10	7	3	0	41:4		
	4	NS	12	100	12	12	0	0	48:0		
	5	NS	12	93.8	9	4	5	0	39:6		
	6	NS	10	95.0	8	7	1	0	37:1		
asu	1	S	12	89.5	9	1	5	3	30:13	3:1	
	2	S	12	95.8	10	1	5	4	31:15		
	3	S	10	100	10	1	7	2	29:11		
	4	S	12	95.8	10	2	7	1	35:11		
	5	S	11	95.4	10	2	6	2	32:10		
	6	S	8	96.9	7	1	0	6	18:13		

TABLE 3.10 cont'd

PUTATIVE MUTATION	MUTANT NO.	DIPLOID*3 PHENOTYPE	TETRAD ANALYSIS			S:NS IN COMPLETE TETRAIDS			OBSERVED		PREDICTED *1 S:NS
			NO. DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRAIDS	4:0	3:1	2:2	SPORE*4 TOTALS S:NS		
ade2.1.x	1	S	12	100	12	3	7	2	37:11		
	2	S	12	75.0	2	0	1	1	22:14		
	3	S	12	41.7	0	0	0	0	12:8		3:1
	4	S	12	43.8	1	0	1	0	13:9		
	5	S	12	85.4	5	0	2	3	25:16		
	6	S	12	91.7	9	4	4	1	36:8		
ade1	1	S	12	77.1	6	1	2	3	26:11		3:1
	2	S	4	100	4	0	3	1	11:5		
	3	S	12	87.5	8	2	6	0	34:8		

*1 See TABLE 3.9(a) A 3:1 segregation implies 2:2, 3:1 and 4:0 tetrads expected but overall should be 3:1

*2 PNM and ASU mutants cannot be distinguished on the basis of their complementation patterns with the two standard tester strains.

*3 S = suppressed, NS = non-suppressed.

*4 Includes data from incomplete tetrads.

psi⁻ strains was designated pnm and a genetic analysis of these and other pnm mutants isolated can be found in Section 7.4 (Chapter 7).

A complementation test using a tester strain of genotype SUQ5⁺ ade2.1 kar1.1 psi⁺ was developed. This strain is unable to complete karyogamy producing transient heterokaryons (Conde and Fink 1976). SUQ5 ade2.1 psi⁻ mutants show complementation with this strain but SUQ5 ade2.1 pnm psi⁻ strains show very weak complementation. The construction of this tester strain and the theoretical basis for the "kar complementation test" are given in Chapter 7. Suffice to state here that a pnm mutant can be distinguished from a true cytoplasmic psi⁻ mutant using this simple complementation test.

Overall, the predicted segregations were found for the other classes of mutant analysed. An exception was the putative SUQ5⁺ mutant number 5 which apparently still carried a functional SUQ5 in a psi⁺ background but also carried a nuclear mutation which eliminated suppressor activity (an antisuppressor?). The SUQ5 therefore behaved in a recessive fashion in crosses to the SUQ5⁺ ade2.1 psi⁺ tester, non-suppressed diploids being obtained. SUQ5 is known to behave as a recessive mutation in certain genetic crosses (see Appendix 3, F. Sherman personal communication to B.S. Cox). Alternatively mutant number 5 carried an inactive partially dominant antisuppressor mutation.

It is interesting to note that all 6 mutants of the PNM/ASU class were PNM although ASU mutants were found in mutation studies with diploid psi⁺ strains homozygous for SUQ5 (see Section 3.4).

3.3 UV-Light Mutagenesis

The principal biological effects of UV-light (254nm radiation), namely cell death, recombination and mutation, are a result of damage produced in cellular DNA. The dimerisation of adjacent pyrimidines in the DNA to produce pyrimidine dimers ($\hat{P}P$) is the principle photoproduct responsible for these events. Other lesions in DNA are also produced by UV-light but their role, if any, in these events is not yet established.

In yeast UV-light is known to produce $\hat{P}P$ in both nuclear and cytoplasmic mitochondrial DNA (Unrau et al., 1971; Waters and Moustacchi 1974; Prakash 1975) and can induce both nuclear and cytoplasmic gene mutations (Patrick et al., 1964; Moustacchi 1969). Mutation induction in bacteria is believed to arise from the repair of UV-induced lesions in the DNA by 'error-prone' enzymes (Witkin 1976). Such "mutagenic" repair has been postulated for mutant induction in yeast (Von Borstel and Hastings 1977). The recent work of Lawrence and Christensen (1978) suggests that mutation occurs near, but not necessarily at, the site of a UV-induced dimer, and that factors, other than the number of potential dimer sites, are important in determining UV-induced mutation frequencies.

Using the standard strain 507-4/2b the kinetics of the UV-induced cytoplasmic mutation psi⁺ to psi⁻ were investigated. Experimental procedures used are given in 'Materials and Methods'. A comparison was made of the induction kinetics in log phase cells (defined as a population showing 50-80% budded cells).

507-4/2b showed maximum resistance to the lethal effects of UV-light when irradiated in log phase cells which gave an LD₃₇

of 63 J / m^2 (i.e. the exposure dose of UV required for inactivation down to the 37% survival level and represents one lethal hit per haploid genome; Haynes 1975). The LD_{37} for stationary phase 507-4/2b was calculated at 44 J / m^2 (Figure 3.1). Both log phase and stationary phase cells had a resistant shoulder at low UV doses implying a fully functional DNA repair system in 507-4/2b.

The induction of red ("non-suppressed") colonies was scored over a UV dose range of $0-150 \text{ J / m}^2$ for stationary phase cells (Table 3.11) and over a dose range of $0-200 \text{ J / m}^2$ for log phase cells (Table 3.12). The values given in Tables 3.11 and 3.12 are from 3-6 independent experiments performed. Only those colonies producing the dark red pigment, after storage at 4°C , were scored. Pinkish, presumably leaky, colonies were observed at all UV doses but not scored. All mutant colonies were classified as either pure or sectorial colonies. Those colonies without any white parts were counted as pure colonies, although this would lead to a slight underestimate of the number of pure colonies and an overestimate of the number of sectorial colonies since occasionally very fine 'hairline' sectors of white papillae were found which could have arisen spontaneously during growth of the mutant colonies. This would be particularly true of psi⁻ mutants which show a high reversion frequency to prototrophy (white, ADE⁺) because of nuclear allo-suppressor mutations (Young and Cox 1975).

Log-log plots of frequencies of non-suppressed mutants versus UV dose are shown in Figure 3.2. In both log and stationary phase cells mutation frequency increased linearly with dose

FIGURE 3.1 A comparison of the UV-sensitivities of log
and stationary phase cells of the RAD⁺ psi⁺
strain 507-4/2b .

(○) log phase

(●) stationary phase

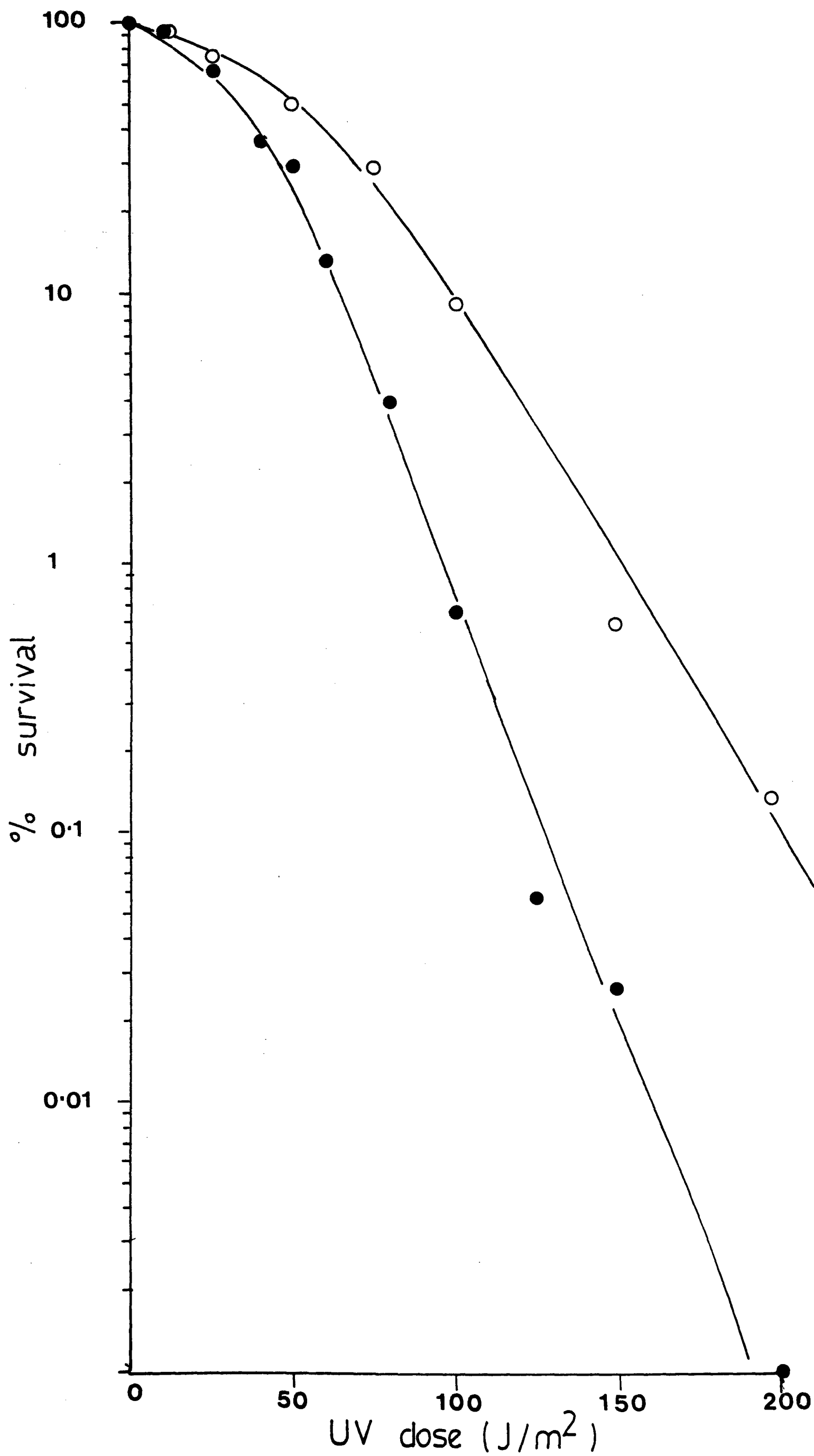


TABLE 3.11 UV-induction of non-suppressed mutants of 507-4/2b; stationary phase cells.

UV DOSE (J/m ²)	% SURVIVAL*1	COLONIES COUNTED	MUTANT COLONIES COUNTED			MUTANTS /10 ³ SURVIVORS
			PURE	SECTORED	TOTAL	
0	100	17,672	2	1	3	0.17
5	98.06	5,434	4	0	4	0.74
10	92.83	8,617	7	6	13	1.51
15	92.75	5,023	9	5	14	2.79
20	74.27	11,739	14	11	25	2.13
25	67.59	4,096	10	3	13	3.17
30	38.54	1,964	6	2	8	4.07
40	36.44	9,214	34	14	48	5.21
50	30.39	8,742	40	15	55	6.29
60	13.22	4,306	24	5	29	6.73
80	3.99	10,385	46	28	74	7.15
100	0.66	6,188	36	22	58	9.37
125	0.06	2,874	19	9	28	9.74
150	0.03	2,964	20	10	30	10.12
200	0.001	111	1	0	1	9.01

*1 The figures are averages from several independant experiments.

TABLE 3.12 UV-induction of non-suppressed mutants of 507-4/2b; log phase cells.

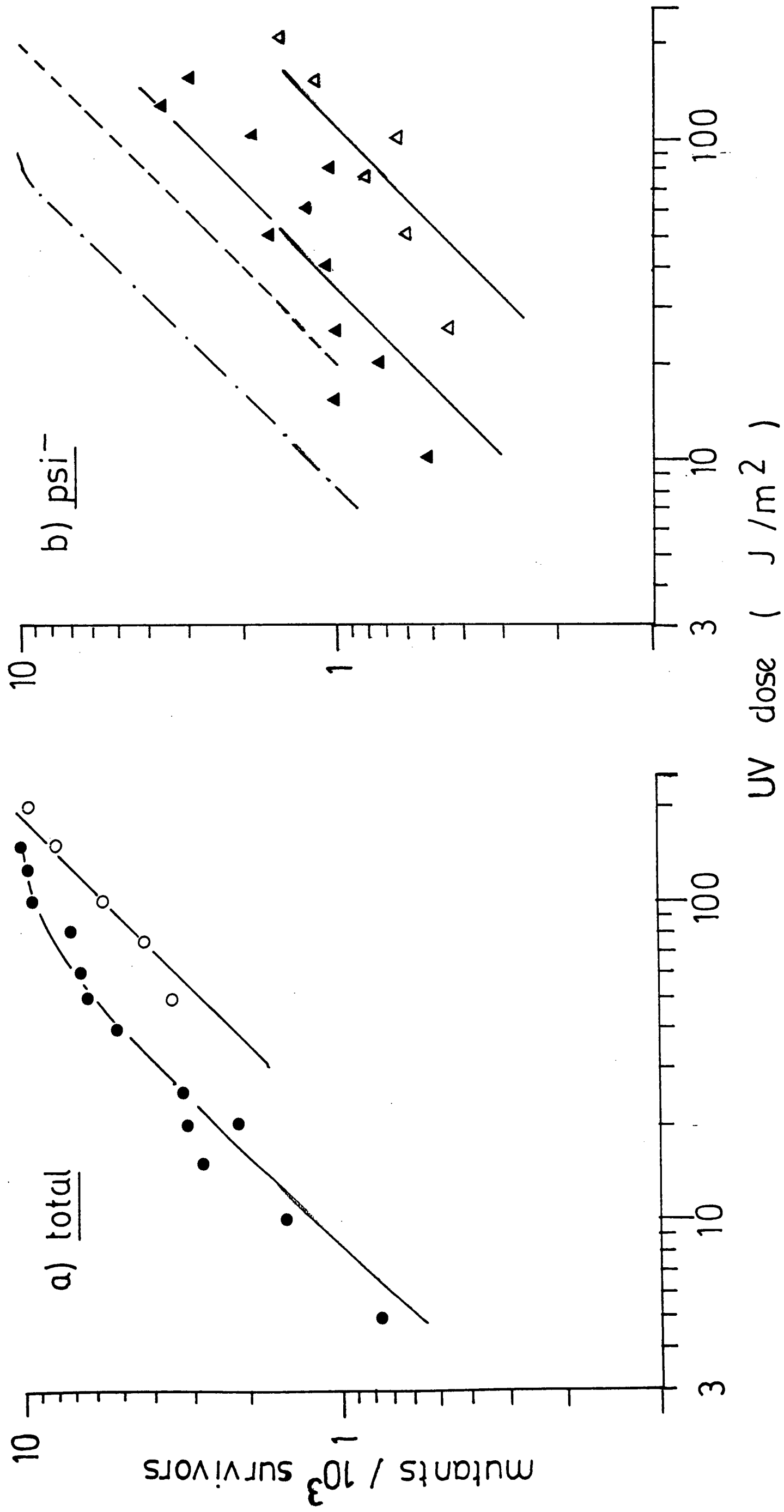
UV DOSE (J/m ²)	% SURVIVAL* 1	COLONIES COUNTED	MUTANT COLONIES COUNTED			MUTANTS/10 ³ SURVIVORS
			PURE	SECTORED	TOTAL	
0	100	11,377	1	2	3	0.27
5	93.34	1,575	0	0	0	-
10	94.81	2,247	0	0	1	0.44
25	73.95	2,285	5	2	7	3.06
50	49.40	7,374	10	16	26	3.53
75	29.43	8,210	14	21	35	4.26
100	9.12	8,282	17	31	48	5.80
150	0.61	3,102	12	13	25	8.06
200	0.14	7,575	49	26	75	9.90
250	0.003	190	1	1	2	10.52

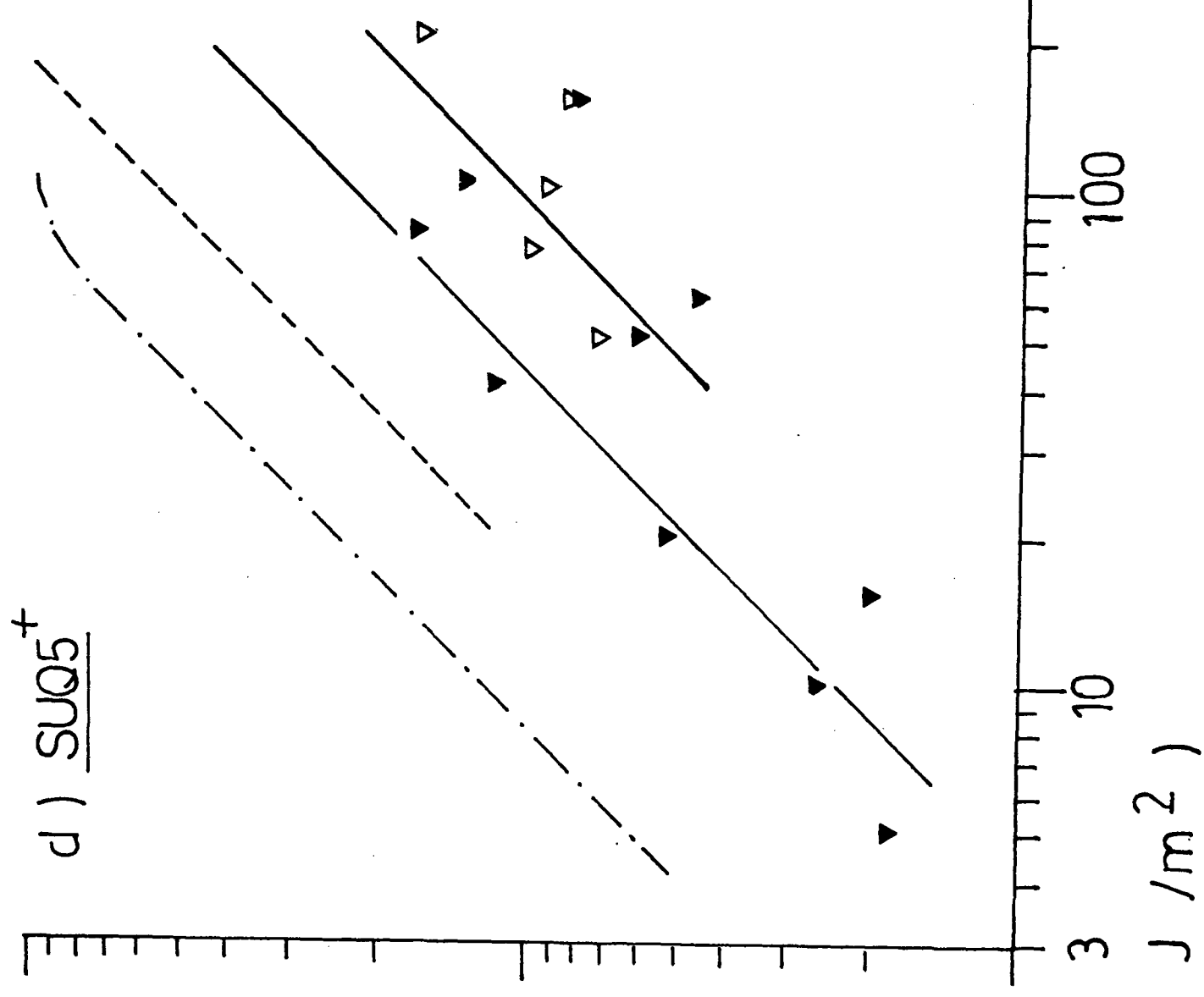
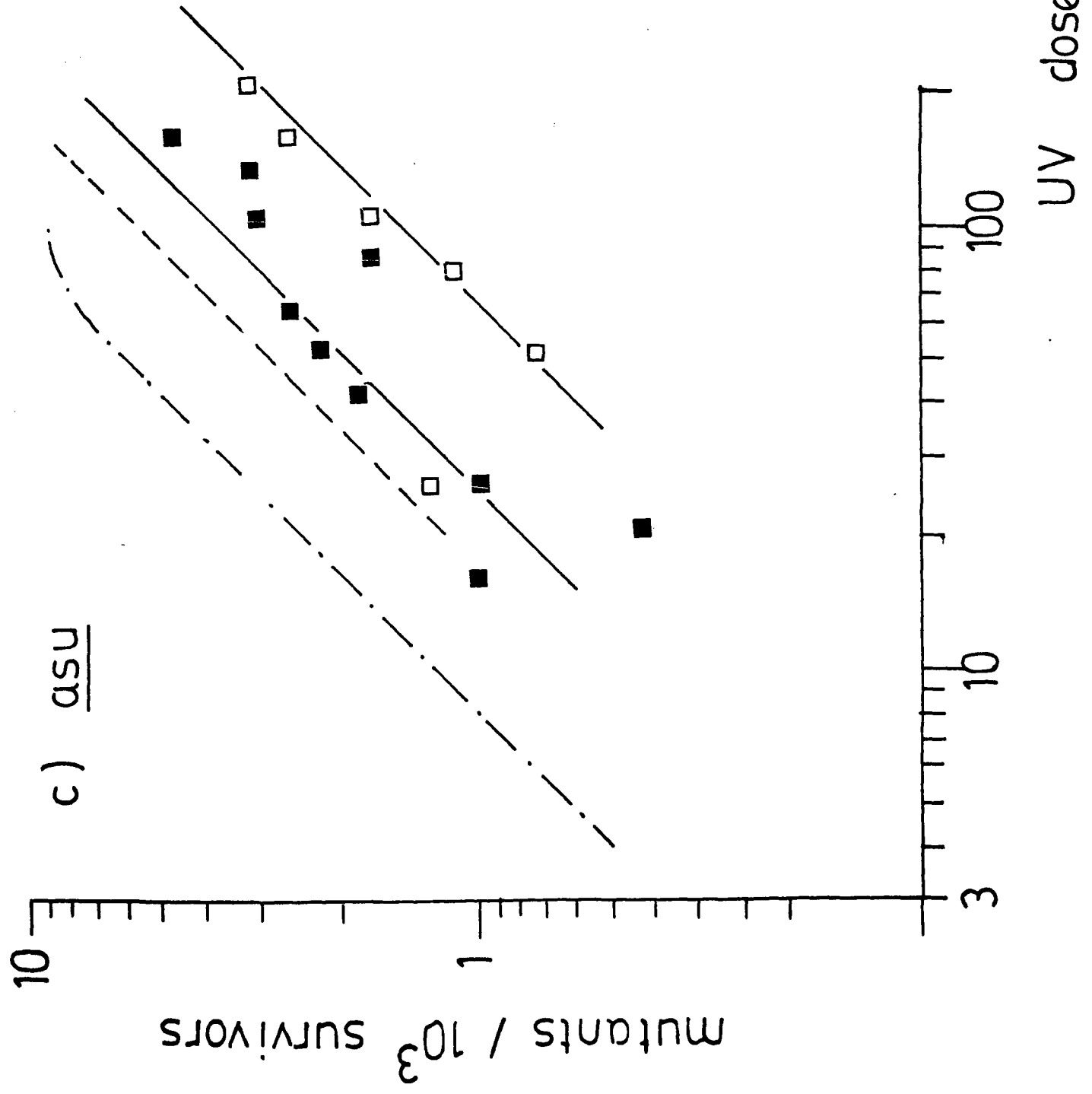
*₁ The figures given are averages from several independant experiments.

FIGURE 3.2 The frequency of non-suppressed mutants
as a function of UV dose for log and
stationary phase cells of 507-4/2b.

- a) total non-suppressed mutants, (●) stationary
(○) log
- b) psi^- mutants, (▲) stationary
(△) log
- c) asu mutants (■) stationary
(□) log
- d) SUQ5^+ mutants (▼) stationary
(▽) log

In figures b) to c) — · — · — · — represents total non-suppressed mutants from stationary phase, — — — — — represents total non-suppressed mutants from log phase cells.





plateauing at high doses (in fact a slight decline was seen in stationary phase cells at very high doses of UV). These 'mutational dose-response' curves can be classified on the basis of the power (n) by which mutation frequency (mutant per survivors) increases with dose (Eckardt and Haynes 1977a). For both log and stationary phase cells $n=1$ i.e. they show linear induction kinetics implying that a 'single-hit' event is responsible for the mutation to non-suppression. Such linear induction kinetics have also been reported for other forward mutation systems in yeast (Eckardt and Haynes 1977b). The overall ratio of whole:sectoried mutant colonies in stationary phase cells was 2:1 whilst in log phase cells an excess of sectoried over whole occurred at doses up to 150 J / m^2 .

To see if all classes of mutation giving rise to the mutant phenotype, in particular the cytoplasmic psi⁻ mutation, showed linear induction kinetics, the non-suppressed mutants were cloned on YC and their genotypes determined followed by complementation analysis. (Table 3.13 and Table 3.14). Both pure and sectoried colonies were analysed. All the predicted classes of mutant were found (Table 3.13 and Table 3.14) and could occur as both pure or sectoried mutant colonies. The mutational-dose response curves were plotted for several classes of mutation (Figure 3.2). All including the psi⁻ mutation showed approximately linear induction kinetics.

The log-log plots demonstrate that the UV-induced cytoplasmic psi⁻ mutation is a 'single-hit' event and that the frequency of psi⁻ mutants per 10^3 survivors per unit dose of UV was lower in irradiated log phase cells, compared to stationary phase cells,

TABLE 3.13 Frequencies of UV-induced mutants in stationary phase 507-4/2b: breakdown into different classes of mutant.

DOSE (J/m ²)	MUTATION FREQUENCY (PER 10 ³ SURVIVORS)*1								
	TOTAL*2	psi ⁻	pnm	PNM/ASU	asu	SQ5 ⁺	ade1	ade2.x	NM
0	0.17(30)	-	0.06(1)	0.06(1)	-	0.06(1)	-	-	-
10	1.51(12)	0.50(4)	0.50(4)	0.13(1)	0.13(1)	0.25(2)	-	-	-
15	2.79(14)	1.00(5)	0.20(1)	0.20(1)	1.00(5)	0.20(1)	-	0.20(1)	-
20	2.13(21)	0.71(7)	0.51(5)	-	0.41(4)	0.51(5)	-	-	-
25	3.17(13)	0.98(4)	0.49(2)	0.24(1)	0.98(4)	-	0.24(1)	0.24(1)	-
40	5.21(40)	1.04(8)	0.39(3)	0.39(3)	1.82(14)	1.17(9)	0.26(2)	0.13(1)	-
50	6.29(42)	1.65(11)	0.75(5)	0.75(5)	2.25(12)	0.60(4)	0.45(3)	0.15(1)	0.15(1)
60	6.73(44)	1.22(8)	0.46(3)	1.07(7)	2.60(17)	0.46(3)	0.46(3)	0.46(3)	-
80	7.13(70)	1.02(10)	1.22(12)	0.41(4)	1.73(17)	1.73(17)	0.51(5)	0.51(5)	-
100	9.37(42)	1.78(8)	0.67(3)	1.34(6)	3.12(14)	1.34(6)	0.22(1)	0.89(4)	-
125	9.74(18)	3.25(6)	1.62(3)	0.54(1)	3.25(6)	-	0.54(1)	0.54(1)	-
150	10.12(25)	2.83(7)	0.40(1)	0.81(2)	4.86(12)	0.80(2)	-	0.40(1)	-
5	0.74(4)	0.56(3)	-	-	-	0.18(1)	-	-	-

TABLE 3.14 Frequencies of UV-induced mutants in log phase 507-4/2b: breakdown into different classes of mutant.

(J/m ²)	TOTAL ^{*2}	MUTATION FREQUENCY (PER 10 ³ SURVIVORS) ^{*1}						NM (Sterile)
		psi ⁻	pnm	PNM/ASU	asu	SQ5 ⁺	ade1	ade2.1
0	0.27(3)	0.18(2)	-	-	0.09(1)	-	-	-
25	3.06(7)	0.44(1)	0.88(2)	-	1.31(3)	-	-	0.44(1)
50	3.53(24)	0.59(4)	0.15(1)	0.88(6)	0.74(5)	0.74(5)	-	0.44(3)
75	4.26(27)	0.79(5)	0.49(3)	0.49(3)	1.15(7)	0.98(6)	0.33(2)	0.16(1)
100	5.80(37)	0.63(4)	0.47(3)	1.10(7)	1.72(11)	0.94(6)	-	0.94(6)
150	8.06(7)	1.15(10)	1.27(11)	1.04(9)	2.76(24)	0.81(7)	0.46(4)	0.58(5)
200	9.90(60)	1.48(9)	1.15(7)	1.15(7)	3.30(20)	1.65(10)	0.33(2)	0.82(5)

*1 The figures in brackets are the number of mutants analysed.

*2 The total frequency is that calculated in TABLE 3.12. The figure in brackets is the number of non-suppressed mutants analysed by complementation.

as was found for all classes of forward nuclear mutation scored. The psi⁻ mutant could arise as either a pure or sectorized mutant clone.

The kinetics of UV-induction of the cytoplasmic "petite" mutation were also determined in both log and stationary phase cells (Table 3.15). When plated on double-logarithmic paper, as mutation frequency versus dose, markedly different kinetics were seen between these and other induction kinetics observed (Figure 3.3). In both types of cell biphasic induction kinetics were obtained, being linear ($n=1$) at doses up to approximately $35 \text{ J} / \text{m}^2$ but shifting to higher order kinetics ($n > 1$) at higher doses. The mutation frequency per unit dose of UV was lower in log phase cells than in stationary phase cells at all doses as observed for the other classes of mutation. It should be noted that only whole petite colonies were scored in these experiments.

3.4 Nitrosoguanidine Mutagenesis

Nitrosoguanidine (N-methyl-N' -nitro-N-nitrosoguanidine, NTG) is an alkylating agent and a potent mutagen. It directly attacks DNA resulting in the alkylation of at least 8 sites in DNA and ultimately leads to depurination and strand breakage (Strauss et al., 1975). The most frequent point of attack is the N-7 of guanine giving rise to ionised guanine which could have a tendency to mispair with thymine instead of cytosine giving a GC $\rightarrow$ AT transition (Auerbach 1976). Selective GC $\rightarrow$ AT transitions have been shown, by reversion analysis of iso-1-cytochrome C mutants, to be the major base substitution induced in yeast (Prakash and Sherman 1973) by this mutagen.

TABLE 3.15 Frequencies of UV-induced petite mutants in log and stationary phase 507-4/2b cells.

DOSE (J/m ²)	STATIONARY		LOG	
	TOTAL COLONIES TESTED	% PETITE	TOTAL COLONIES TESTED	% PETITE
0	5540	0.31	4769	0.82
5	1724	0.41	1178	0.34
10	2516	0.68	1082	0.55
15	1566	1.40	-	-
20	3608	1.47	-	-
25	533	5.25	1137	1.41
30	983	3.97	-	-
40	2458	3.09	-	-
50	1847	6.88	1394	4.45
60	2334	10.84	-	-
75	-	-	1307	6.89
80	2039	17.41	-	-
100	2926	27.55	1129	8.33
125	301	36.21	-	-
150	970	62.58	425	23.29
200	111	65.76	505	41.19
250	-	-	54	53.70

- = not tested.

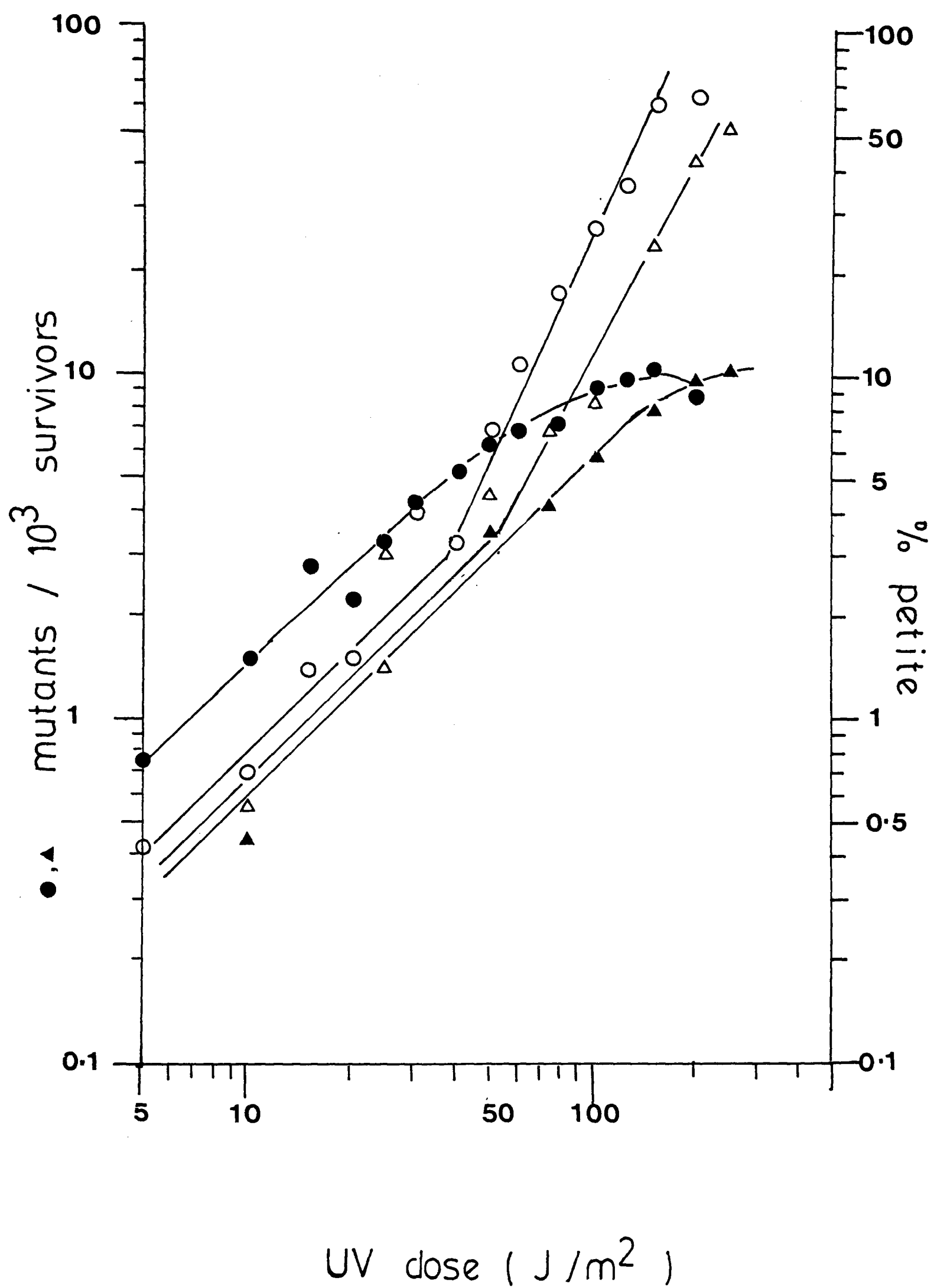
FIGURE 3.3 The frequency of cytoplasmic petite mutants as a function of UV dose for log and stationary phase cells of 507-4/2b compared to total non-suppressed mutation frequency.

(Δ) log phase , petite

($\circ$) stationary phase , petite

($\blacktriangle$) log phase , non-suppressed mutants

($\bullet$) stationary phase , non-suppressed mutants



NTG has been used to induce both nuclear and cytoplasmic mutants in yeast (Megnet 1965; Nordström 1967; Dawes and Carter 1974). Induction of nuclear mutants is maximal during the nuclear S period of the cell cycle in both bacteria (Cerdá-Olmedo et al., 1968) and yeast (Dawes and Carter 1974; Kee and Haber 1975). NTG selectively mutates the replicating region of DNA leading to a high frequency of linked double mutations (Guerola et al., 1971) and this finding has been used to test for temporal order in the replication of the genome in yeast (Burke and Fangman 1975). The molecular basis for the replication dependence of NTG mutagenesis is unknown, but it has been suggested, by in vitro studies with bacteria, that NTG inactivates both the polymerase and exonuclease activity of DNA polymerase III* leading initially to a high frequency of errors at the growing point of DNA (Jiménez-Sánchez 1976) and finally to a complete stopping of DNA growing points (Jiménez-Sánchez and Cerdá-Olmedo 1975).

The following experiments were undertaken to see if NTG could mutate psi⁺ to psi⁻ and whether there was co-mutation of a nuclear or cytoplasmic gene.

1. NTG Mutagenesis in haploid strains

Two haploid psi⁺ strains of different origin were used in these experiments : 507-4/2b and 465/1b. The genotype of 465/1b is SUQ5 ade2.1 his5.2 ura3.1 psi⁺ and was found to be a very weak killer (K^w). The protocol for NTG mutagenesis is given in 'Materials and Methods'.

Both strains were treated with a number of different concentrations of NTG and, for 507-4/2b, an experiment was carried out with 1mg/ml of NTG at 4°C. The results obtained are given

in Table 3.16 and the data plotted in Figure 3.4. In some cases, to obtain large numbers of mutants, mutagenised cells were also plated onto YC to give approximately 1500 colonies / plate e.g. 507-4/2b, 25 μ g/ml at 28°C. As with the UV studies only red mutant colonies were scored, leaky pink colonies being discarded from the calculations.

All conditions of treatment with NTG gave very high mutation frequencies (20-35 non-suppressed mutants per 10³ survivors) compared to UV-irradiation (maximum 10 per 10³ survivors). It was noticeable with both 507-4/2b and 465/1b that the lowest NTG concentration used (12.5 μ g/ml) gave the highest mutation frequency with survival levels being almost 50%. No increase in mutation frequency was observed when higher concentrations of NTG were used.

65 non-suppressed mutants of 465/1b, induced by 25 μ g/ml of NTG, were recloned on YC. Those which were HIS⁺ (a total of 34) were discarded and complementation analysis performed on the remaining 31 mutants (Table 3.17). All classes of mutant, including at least 4 psi⁻ mutants, were found. The psi⁻ mutants were all able to grow at 37°C and on OM2; no co-mutation was observed. The one spontaneous mutant was an SUQ5⁺ mutant.

172 NTG-induced, non-suppressed mutants of 507-4/2b, from the 5 different conditions of treatment used, were classified by complementation analysis (Table 3.17). All the predicted classes of mutant were found and at least 25 were cytoplasmic psi⁻ mutants. Of these 25, 2 were petite and 2 were ts at 37°C. All grew well on OM2. No one set of conditions was specific for induction of any one class of mutation.

TABLE 3.16 Induction of non-suppressed mutants in two unrelated SUQ5 ade2.1 psi⁺ strains by nitrosoguanidine.

NTG MUTAGENESIS		PLATE INCUBATION TEMP. °C	465/1b				507-4/2b			
			% SURVIVAL	COLONIES SCORED	MUTANTS COLONIES WHOLE SECTORED	MUTANTS PER 10 ³ SURV.	% SURVIVAL	COLONIES SCORED	MUTANT COLONIES WHOLE SECTORED	MUTANTS PER10 ³ SURV.
0	28	28	100	1453	1	0	100	945	1	1.1
12.5	28	28	22.9	458	9	5	48.7	545	9	36.7
25	28	28	2.9	3358	36	30	3.8	1495	14	21.4
37.5	28	28	1.4	27	0	0	2.3	439	8	31.9
0	4	28		NT			100	781	0	-
1000	4	28		NT			0.9	466	2	34.3
0	28	23		NT			100	482	0	-
25	28	23		NT			7.0	4292	67	27.0

NT = not tested.

TABLE 3.17 Nature of non-suppressed mutants induced by NTG in 507-4/2b and 465/1b.

psi- STRAIN	TREATMENT			NUMBER OF MUTANTS EXAMINED	NO. OF PETITES	12°C (CS)	37° (ts)	*2 om2	MUTANT CLASSIFICATION							
	NTG CONC ^N (u/gml)	MUTAGENESIS TEMP (°C)	INCUBATION TEMP (°C)						ade1	ade2.x	asu	SQ5 ⁺	PNM/ASU	psi ⁻	pnm	STERILE
0	TOTAL FROM ALL EXPERIMENTS			1	0	0	0	0	1	0	0	0	0	0	0	
12.5	28	28	28	16	3	6	1	1	2	0	0	1	4	(8)*1	1	
25	28	28	28	41	7	NT	8	1	1	4	9	12	11	3	0	
37.5	28	28	28	11	3	5	3	3	0	0	2	1	5	0	1	
1000	4	28	28	14	0	0	0	NT	0	0	5	3	1	3	0	
25	28	28	28	90	22	9	14	4	6	14	20	10	3	17	3	
TOTAL (NOT INCL. 0)					172	35	(20)	26	9	9	18	36	27	24	(23) (23)	5
0	28°	28°	28°	1	0	0	0	0	0	0	0	1	0	0	0	
25	28°	28°	28°	31	15	NT	2	1	0	0	4	9	7	(10)*1	0	

NT = not tested.

*1 'kar complementation test' carried out 2 years after initial isolation of mutants; for 507-4/2b. 3 were dead 2 psi- and 3 pnm; for 507-4/2b, 4 were dead, 4 psi- and 2 pnm.

*2 the number of non-suppressed mutants that failed to grow on OM (containing all 10 amino acid and base supplements; see materials and methods).

NTG was originally shown by Nordström (1967) to be a potent inducer of the cytoplasmic petite mutation with 50% of cells, surviving treatment with 2mg/ml NTG in growth medium for 100 minutes, being petite. Following treatment of 507-4/2b with 25µg/ml NTG for 45 minutes in buffer 22.3% of the survivors (67/301 colonies tested) were petite against 0.5% (2/374 colonies tested) for the untreated control culture. When colonies were plated at 23°C a large number of mutants were found to be ts ; 47/312 (15.1%) tested were unable to grow at 37°C whilst a further 32 (10.3%) showed very poor growth at 37°C. All 482 untreated colonies tested grew well at this temperature.

4 ts, non-suppressed mutants of 507-4/2b were further characterised to see if the mutation giving rise to the non-suppressed phenotype co-segregated in tetrads with the ts phenotype in a cross to a TS⁺ mutant. Each mutant was crossed to the standard SUQ5⁺ ade2.1 psi⁺ tester 465/2a and a number of tetrads dissected from the diploids. (Table 3.18)

W4 ; was a pnm mutant. This was confirmed by crossing to a standard SUQ5 ade2.1 psi⁺ strain (MT193/3b). In the latter cross the ts and the pnm mutations were not linked (PD=0, NPD=1, TT=4).

W13; was a psi⁻ mutant which also carried a single nuclear ts mutation which was unlinked to SUQ5 (PD=3, NPD=0, TT=7).

S7 ; was an SUQ5⁺ mutant with a single nuclear ts mutation. From this analysis it could not be determined whether the two mutations were linked.

TABLE 3.18 Genetic analysis of NTG-induced ts non-suppressed mutants of 507-4/2b.

MUTANT NO.	CROSSED TO*	DIPLOID PHENOTYPE	TETRAD ANALYSIS			SEGREGATION IN COMPLETE TETRADS					NATURE OF MUTANT	
			NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS	SUPPRESSED;NON-SUPP			ts:TS ⁺			
						2:2	1:3	0:4		2:2		1:3
W4	465/2a	WHITE ADE ⁺ HIS ⁺	9	94.4	7	1	3	3	7	0	0	prn
W4	MT193/3b	WHITE ADE ⁺ HIS ⁺	5	100	5	5	0	0	5	0	0	
W13	465/2a	WHITE ADE ⁺ HIS ⁺	12	89.6	10	10	0	0	10	0	0	
S7	465/2a	RED ADE ⁻ HIS ⁻	10	100	10	0	0	10	9	1	0	SUQ5 ⁺
S12	465/2a	WHITE ADE ⁺ HIS ⁺	6	95.8	5	0	5	0	1	3	1	asu

* See text for genotypes

S12 ; was an asu mutant which failed to give a clear 2ts:2TS⁺ segregation. This suggested that two unlinked mutations were present in S12 which together gave the ts phenotype. Of the 5 ts spores obtained one was suppressed.

The ts phenotype of W4, W13 and S12 was therefore not linked to the mutation giving the non-suppressed phenotype. All ts mutations were recessive. 3 further ts mutants, when crossed to 465/2a, gave diploids which failed to sporulate.

2. NTG Mutagenesis in diploid, triploid and tetraploid strains

A preliminary analysis of the ability of NTG to induce psi⁻ mutants in psi⁺ strains with ploidies greater than 1 was undertaken. Non-suppressed mutants in such strains should be the result of either a psi⁺ to psi⁻ mutation or induction of an ASU or PNM nuclear mutation. Recessive mutations giving rise to the non-suppressed phenotype in haploids i.e. pnm, asu, ade1, ade2.x and SUQ5⁺ would not be expressed.

i) Strain Construction

	507-4/2b	<u>α</u>	<u>SUQ5</u>	<u>ade2.1</u>	<u>his5.2</u>	<u>can1.100</u>		<u>ura3.1</u>	<u>psi</u> ⁺
MT	=	x							
35			<u>a</u>	<u>SUQ5</u>	<u>ade2.1</u>	<u>his5.2</u>	<u>can1.100</u>	<u>trp5.48</u>	<u>ura3.1</u> <u>psi</u> ⁺

To obtain mutants homozygous for mating type (either αα or aa) a log phase culture of MT35 was treated with 3% EMS, a mutagen which induces mitotic recombination at a high frequency (Yost et al., 1967). The protocol is given in 'Materials and Methods'. This gave 12.6% survival. 206 mutagenised colonies were picked and streaked across an α and a ura1 haploid testers (α = g218/3b, a = g218/7c) on YC plates. After 24 hours, to allow mating, the cross

streaks were replica plated onto OM - ura. Because MT35 was homozygous for ura3.1 mating was detected by confluent growth on OM - ura on the distal side of the cross streak. 4 αα and 3 aa diploids were detected in this way. One of the αα diploids showing strong mating (HM2) was used further.

A triploid strain (MT54) was constructed by crossing HM2 to 518/1c (a SUQ5 ade2.1 his5.2 can1.100 trp5.48 ura3.1 psi⁺) and a tetraploid strain (MT57) was constructed by crossing HM2 with an aa mutant of MT35 (HM3). The diploid strains were stable for their ADE⁺ HIS⁺ CAN^S ρ⁺ K⁺ phenotypes. The tetraploid strain gave rise spontaneously to colonies with pink, and occasionally red, non-suppressed sectors. Only MT35 and MT57 could be induced to sporulate; 10 tetrads dissected from MT35 gave 95% spore germination with all the spores being suppressed; 14 tetrads dissected from MT57 gave 71.4% spore germination with all the spores being suppressed.

ii) Mutagenesis

MT35, HM2, MT54 and MT57 were treated with 0 - 25 μg/ml NTG at 28°C, plated onto YC and incubated at 28°C. The results obtained are given in Table 3.19 and are plotted on Figure 3.4 with the haploid strains' results. MT35 was more resistant to the lethal effects of NTG than any of the other three strains which, although showing similar sensitivities to each other, were more resistant than the two haploid strains 507-4/2b and 465/1b. Surprisingly the frequencies of non-suppressed mutants was higher in the strains with ploidies greater than one compared to the two haploid strains with HM2 being the least mutable and MT54 giving the highest induced mutation frequencies. For each strain the maximum mutation frequency was reached at the lowest NTG concentration used (12.5 μg/ml).

TABLE 3.19 Induction of non-suppressed mutants in diploid, triploid and tetraploid psi⁺ strains by NTG.

STRAIN	NTG ug/ml	COLONIES COUNTED	% SURVIVAL	NO. OF NON-SUPPRESSED COLONIES		FREQUENCY OF NS COLONIES /10 ³ SURVIVORS
				WHOLE	SECTORED	
MT35 (2N)	0	5851	100	0	0	-
	12.5	1281	86.9	0	72	56.2
	25* ₁	3681	60.3	49	116	44.8
	37.5	1801	39.4	19	85	57.7
HM2 (2N)	0	466	100	0	0	-
	12.5	501	43.0	2	16	35.9
	25	382	32.8	4	12	41.9
	37.5	2125	18.2	21	44	30.6
MTS4 (3N)	0	1043	100	0	0	-
	12.5	655	58.5	11	40	77.9
	25* ₁	3792	26.3	52	201	66.7
	37.5	469	20.9	2	13	32.0
MT57 (4N)	0	1053	100	4	6	9.5
	12.5	629	68.9	12	26	60.4
	25	365	35.0	3	18	57.5
	37.5	221	18.1	2	11	58.8

*₁ a total of 3 independant experiments; all other data represents one experiment only.

FIGURE 3.4 Nitrosoguanidine induced mutation to non-suppression
and lethality in psi⁺ strains of different ploidies.

- (○) 507-4/2b, mutation
- (●) 507-4/2b, cell viability

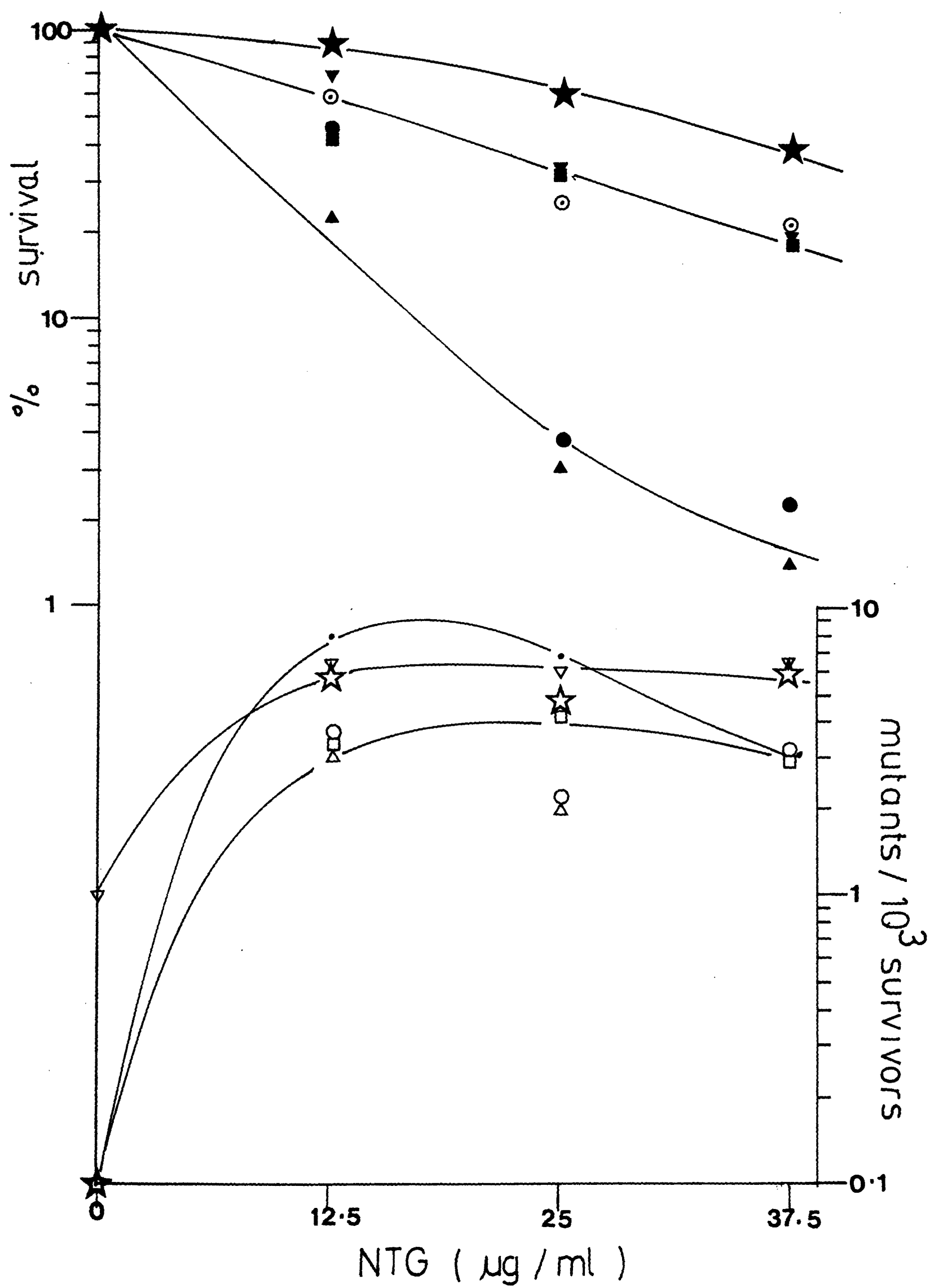
- (△) 465/1b, mutation
- (▲) 465/1b, cell viability

- (□) HM2, mutation
- (■) HM2, cell viability

- (☆) MT35, mutation
- (★) MT35, cell viability

- (⊙) MT54, mutation
- (•) MT54, cell viability

- (▽) MT57, mutation
- (▼) MT57, cell viability



To determine whether the high mutation frequencies observed in these strains was due to a high frequency of induction of psi⁻ mutants, in strains of high ploidy, an attempt was made to classify non-suppressed mutants of MT35, HM2 and MT57.

iii) Mutant Classification

(1) MT35 ; 57 non-suppressed mutants (induced by 25 µg/ml) were recoloned on YC and classified. 50 were ade⁻ his⁻ with 26 being resistant to 30 µg/ml can. 3/57 (5.3%) were petite whilst all 57 grew well on OM2. 40 of the ade⁻ his⁻ mutants were analysed further by allowing them to sporulate on FMA medium for 7 days. Only those which showed > 5% asci were then suspended in 100µl of a 1% helicase solution and left at room temperature for 2-3 hours to dissolve away the ascal wall. This was followed by 30 seconds agitation on a vortex mixer ('Whirlimixer'). This resulted in total disruption of all asci. The helicase suspension was then spread onto YC medium and incubated for 5 days at 28°C. This 'random spore analysis' gave one of three results:

- (a) all red clones: taken to indicate the diploid was psi⁻.
- (b) a great majority of red colonies but with less than 1% white colonies; these mutants were assumed to be heterozygous for PNM (i.e. PNM/+) elimination of psi⁺ by PNM normally never reaching 100% (See Chapter 7).
- (c) large numbers of white colonies together with the red colonies; these mutants were assumed to be heterozygous for a dominant antisuppressor (ASU).

Table 3.20 gives the frequencies with which these different 'classes' were observed. Of the 26 analysed by random spore analysis 13 appeared to be psi⁻, 3 PNM/+ and 10 ASU/+ diploids.

TABLE 3.20 Results of a 'random spore analysis' of 40 NTG - induced non-suppressed mutants of MT35*1.

SPORULATION	NUMBER	COLOUR OF COLONIES ON YC			
		RED	RED+ < 1%W	RED+WHITE	RED+PINK*3
GOOD (> 20%)	20	8	3	9	0
FAIR (5-20%)	6	5	0	0	1
POOR (< 5%)	5	-	-	-	-
NONE	9*2				

*1 MT35 showed good sporulation and exclusively white colonies on random spore analysis.

*2 3 of these were petite.

*3 presumed to be heterozygous for a weak ASU.

- not tested .

In an attempt to confirm the predictions above tetrads were dissected from diploids falling into each of the 3 classes (Table 3.21). It was apparent from these results that NTG treated cells, if they managed to sporulate, produced almost exclusively inviable spores. Each of the single viable spores from N33 and N34 complemented as psi⁻ mutants whilst the single spore from N38 was suppressed.

This analysis demonstrated the psi⁻ mutants probably represented approximately 50% of the non-suppressed mutants induced by NTG but that analysis of such non-suppressed mutants is severely hampered by poor spore germination induced by NTG mutagenesis.

(2) HM2 ; 59 mutant colonies were recloned on YC of which 35 were ade⁻ his⁻. None were petite and one showed very poor growth at 37°C. All grew well on OM2. All 35 ade⁻ his⁻ mutants showed no complementation with an a ade1 tester strain but 5 showed strong complementation with the a ura1 tester, 20 showing very weak complementation and 10 failing to complement. 16 of the ura1-complementing mutants were also crossed to an a SUQ5 ade2.1 psi⁻ (468/1a) tester. All showed weak complementation with 468/1a but no complementation with 465/2a. This unusual complementation pattern suggests that gene dosage may result in false positives and/or false negatives, e.g. the ade2.1/ade2.1/+, +/+ade1, triploid was ade⁻. No firm conclusions could be drawn from these results about the nature of the mutants although the fact that complementation was seen with 468/1a suggests they were psi⁺.

(3) MT57 ; The phenotypes of 70 non-suppressed mutants of MT57 were determined ; 45 were ade⁻ his⁻ and 25 ade⁻ HIS⁺,

TABLE 3.21 Tetrad analysis of non-suppressed mutants of MT35 induced by 25µg/ml NTG.

STRAIN	DIPLOID GENOTYPE	SPORULATION	RANDOM SPORE ANALYSIS	TETRAD DISSECTED	NUMBER OF						% VIABILITY
					VIABLE SPORES						
					4	3	2	1	0		
MT35	ade ⁺ his ⁺ can ^s	GOOD	WHITE	10	8	2	0	0	0	0	95.0
N-33	ade ⁻ his ⁻ can ^s	GOOD	RED	10	0	0	0	1	9	9	2.5
N-34	ade ⁻ his ⁻ can ^s	GOOD	RED	10	0	0	0	1	9	9	2.5
N-35	ade ⁻ his ⁻ can ^s	GOOD	RED	10	0	0	0	0	10	10	0
N-31	ade ⁻ his ⁻ can ^r	GOOD	RED+WHITE	10	0	0	0	0	10	10	0
N-36	ade ⁻ his ⁻ can ^r	GOOD	RED+WHITE	10	0	0	0	0	10	10	0
N-38	ade ⁻ his ⁻ can ^r	FAIR	RED+WHITE	10	0	0	0	1	9	9	2.5
N-32	ade ⁻ his ⁻ can ^s	FAIR	RED+<1%WHITE	10	0	0	0	0	10	10	0

all being CAN^s. None were ts or petite. Further analysis of 18 of the ade⁻ his⁻ tetraploid mutants was undertaken by allowing them to sporulate on FMA and dissecting tetrads from those which showed good sporulation (> 20% asci) of which there were 10. 4 of the remaining 8 failed to sporulate, the other 4 showing very poor sporulation (< 1% asci). The results of the tetrad analyses are given in Table 3.22. Again spore germination was drastically reduced compared with the non-mutant parent.

Only two mutants, N7 and N8, failed to give any suppressed spores. Of the 5 non-suppressed spores from these two mutants, one was an aa diploid, and the remaining 4 (3 aa, 1 aa) failed to complement any of the standard tester strains.

These results suggest that the majority of the non-suppressed tetraploid mutants were psi⁺, but as observed with MT35, spore germination was drastically reduced by treatment with NTG.

3. Conclusions

NTG can induce the psi⁺ to psi⁻ mutation at a high frequency. It is also a potent inducer of both nuclear and cytoplasmic mutation. No identifiable co-mutation occurred with the psi⁻ mutation, i.e. temperature sensitivity, cold sensitivity, petite or auxotrophic requirement. NTG also induced very high frequencies of non-suppressed mutants in diploid, triploid and tetraploid psi⁺ strains which were significantly more resistant to the lethal effects of NTG than the two haploid strains used. Similar results were obtained in a preliminary study with MT35 and HM2 using another alkylating agent EMS (data not shown). This suggests that diploid yeast cells are able to repair damage by alkylating agents more efficiently than haploid cells, but that most of the repair is error-prone, resulting in a high frequency

TABLE 3.22 Tetrad analysis of non-suppressed tetraploid mutants induced by NTG.

MUTANT NO.	TOTAL TETRAIDS	NUMBER OF SPORES VIABLE					SPORE VIABILITY (%)	SPORE PHENOTYPES*2		
		4	3	2	1	0		WHITE	PINK	RED
MT57	14	4	5	4	1	0	71.4	40	0	0
N3	6	0	0	1	3	2	20.8	4	0	1
N5	6	0	0	0	2	4	8.3	4	0	1
N7	10	0	0	0	3	7	7.5	0	0	3
N8	10	0	0	0	2	8	5.0	0	0	2
N10	6	0	1	0	0	5	12.5	3	0	0
N12	6	0	0	1	2	3	16.6	3	0	1
N14	6	0	0	0	3	3	12.5	3	0	0
N16	6	0	1	2	2	1	37.5	7	0	2*1
N17	6	1	3	1	1	0	66.6	5	5	6
N20	6	0	0	5	0	1	33.3	8	0	2

*₁ Included one red spore with a $\frac{1}{4}$ white sector.

*₂ white spores were fully suppressed for ade2.1 and his5.2;
pink spores showed poor suppression of ade2.1 but strong
suppression of his5.2; red spores were ade⁻ his⁻.

of induced mutation. Although NTG-induced non-suppressed mutants of MT35 gave very poor spore germination, NTG was apparently able to induce the psi⁺ to psi⁻ mutation in a diploid psi⁺ strain. Experiments with a diploid homozygous for mating type (HM2) demonstrated that such a strain cannot be used to study psi mutagenesis because gene dosage effects hamper the interpretation of complementation analysis.

3.5 Preliminary analysis of other nuclear mutagens

(i) Formaldehyde (FA)

Formaldehyde (FA) is mutagenic in both prokaryotes and eukaryotes (for review see Auerbach et al., 1977). It has recently been shown that FA can induce both nuclear and cytoplasmic mutation in yeast (R. Chanet personal communication) and, by analysis of sedimentation profiles in alkaline sucrose gradients, FA has been shown to produce single strand breaks in nuclear DNA of yeast via an, as yet undetermined, metabolic pathway (Magana-Schwenke et al., 1978).

Some preliminary experiments were conducted with the strain 507-4/2b to see if FA could induce psi⁻ mutants. Log phase cells were washed once in phosphate buffer (pH 7.0) and resuspended in an equal volume of buffer containing either 50mM or 100mM FA. The viable cell titre at time 0 was 10⁶ cells/ml. Incubation was carried out at room temperature (18°C) in a 20ml McCartney bottle sealed tightly to prevent evaporation during FA treatment. 1ml samples were taken after several different periods of incubation, diluted appropriately, and plated onto YC. The YC plates were incubated at 28°C.

At the two concentrations of FA used a significant decrease in cell viability with increasing incubation time was observed for 507-4/2b (Figure 3.5). Cells held in buffer for 90 mins. at room temperature with FA showed a slight loss of viability (100% to 89.9% survival).

After an initial decline in the frequency of petites among survivors following incubation, in either 50mM or 100mM FA for up to 30 min., a slight increase in the petite frequency was observed with further incubation (Figure 3.5, Table 3.23). For 50mM FA, even after 90 mins. incubation, the frequency of petites never reached that for the control value. These 'induction curves' can be explained by postulating that petite strains are more sensitive to the lethal effects of FA. This has recently been confirmed (Chanet et al., 1976).

Only 3 non-suppressed mutants were obtained following incubation with FA (Table 3.23); for 50mM FA one was psi⁻, the other an asu mutant; for the 100mM the mutant was SUQ5⁺.

Under the conditions used, which resulted in a significant decline in cell viability, FA appeared to be a very weak nuclear and cytoplasmic mutagen. Only one psi⁻ mutant was obtained out of 11,848 colonies screened following treatment with FA at two different concentrations.

(ii) Benomyl

Benomyl (methyl-1- (butylcarbamoyl)-2-benzimidazole carbamate) is a benzimidazole derivative widely used as a systematic fungicide. It is mutagenic in Salmonella typhimurium (Seiler 1972) and in excision-defective (uvr⁻), but not excision-proficient (uvr⁺), strains of Escherichia coli (Kappas et al., 1976) although no evidence for induction of point mutations was detected in the

FIGURE 3.5 The effect of formaldehyde on cell viability
and the frequency of petite mutants in the
strain 507-4/2b.

a) cell viability

(□) control
(○) 50mM FA
(△) 100mM FA

b) petite induction

(■) control
(●) 50mM FA
(▲) 100mM FA

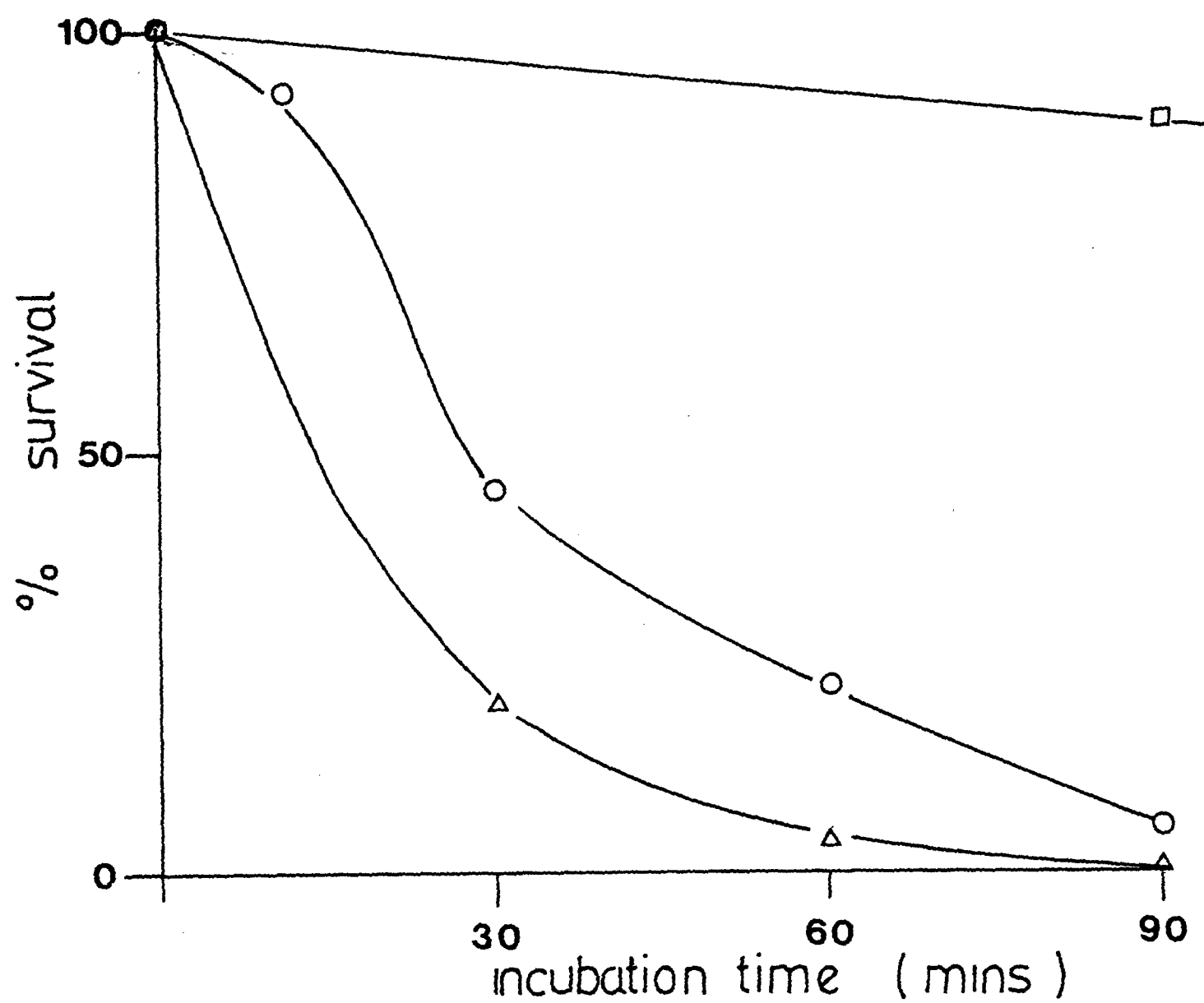
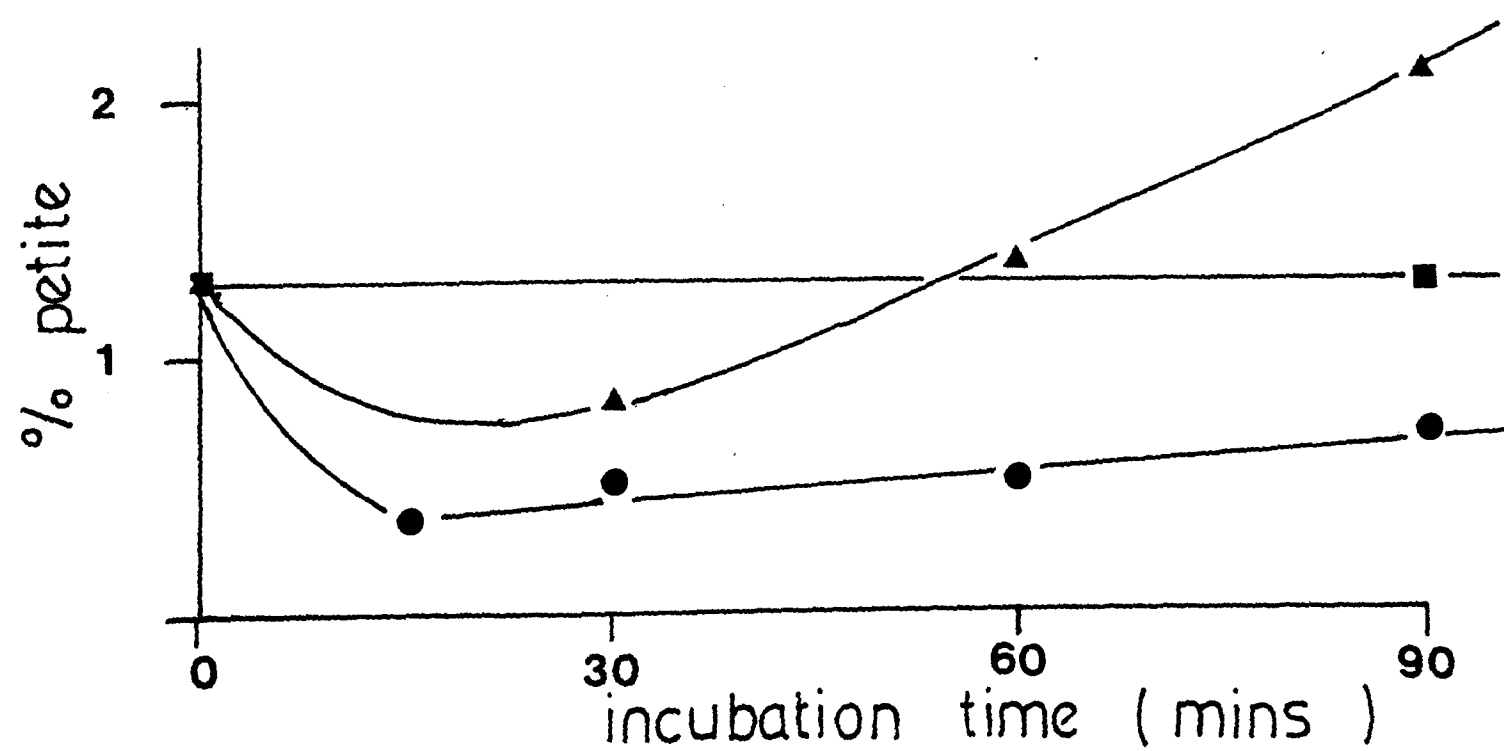
a) cell viabilityb) petite induction

TABLE 3.23 The effect of formaldehyde on the frequency of non-suppressed and petite mutants of 507-4/2b.

FA CONCENTRATION									
50mM					100mM				
INCUBATION TIME (min)	PETITE				PETITE				
	COLONIES	RED	TOTAL	% rho ⁻	COLONIES	RED	TOTAL	% rho ⁻	
	COLONIES	COLONIES	COLONIES	SCORED	COLONIES	COLONIES	COLONIES	SCORED	
0	3743	0	1603	1.3	as for 50mM				
15	779	0	282	0.35	NT				
30	1758	1	574	0.52	1497	1	368	0.82	
60	1709	0	196	0.51	1412	0	948	1.37	
90	3740	1	742	0.67	953	0	717	2.09	

NT = not tested.

eukaryote Aspergillus nidulans (Hastie 1970). Kappas et al., (1976) have suggested that benomyl may be a new type of base analogue that, when incorporated into DNA, is detected by UV-endonuclease and provokes mutations, not by misincorporation during normal DNA replication, but by misrepair of gaps in newly synthesised DNA.

As part of a study on the genetic effects of benomyl on Saccharomyces cerevisiae (see Appendix 2) its ability to induce mutation of psi⁺ to psi⁻ was looked at in a RAD⁺ and a rad1 (excision-defective) strain i.e. 507-4/2b (α SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺) and MT141-4/6d (α SUQ5 ade2.1 his5.2 ura3.1 rad1.1 psi⁺). Benomyl (kindly supplied by Du Pont (UK) Ltd.) and its commercial form Benlate, which is 50% benomyl and 50% unspecified "inert" ingredients, were dissolved in DMSO to a final stock concentration of 10mg/ml prior to each experiment. The final concentration of DMSO in the liquid YC medium was kept at < 0.5%, which has no effect on psi⁺ phenotype (see Chapter 6). The YC + benomyl or Benlate cultures were inoculated with stationary phase cultures of the two strains to a final cell titre of c. 4×10^5 /ml. These cultures were then grown for up to 6 days at 28°C, with constant shaking, prior to diluting and plating onto YC plates, to check for mutation.

Only benomyl (25µg/ml) and Benlate (25 and 50µg/ml) had any significant effect on growth of 507-4/2b (Figure 3.6, Table 3.24) in the YC culture. 2 non-suppressed colonies (1 asu and 1 psi⁻) were found after growth in the presence of 25µg/ml and 5 (3asu, 1 psi⁻, 1 SUQ5⁺) after growth in the presence of 50µg/ml benlate (Table 3.24) suggesting a weak mutagenic effect of benomyl in

FIGURE 3.6 The effect of benomyl (25 $\mu\text{g}/\text{ml}$) and Benlate
(50 $\mu\text{g}/\text{ml}$) on the growth of the strain 507-4/2b.

- (●) YC control
- (▲) YC + 25 $\mu\text{g}/\text{ml}$ benomyl
- (■) YC + 50 $\mu\text{g}/\text{ml}$ Benlate

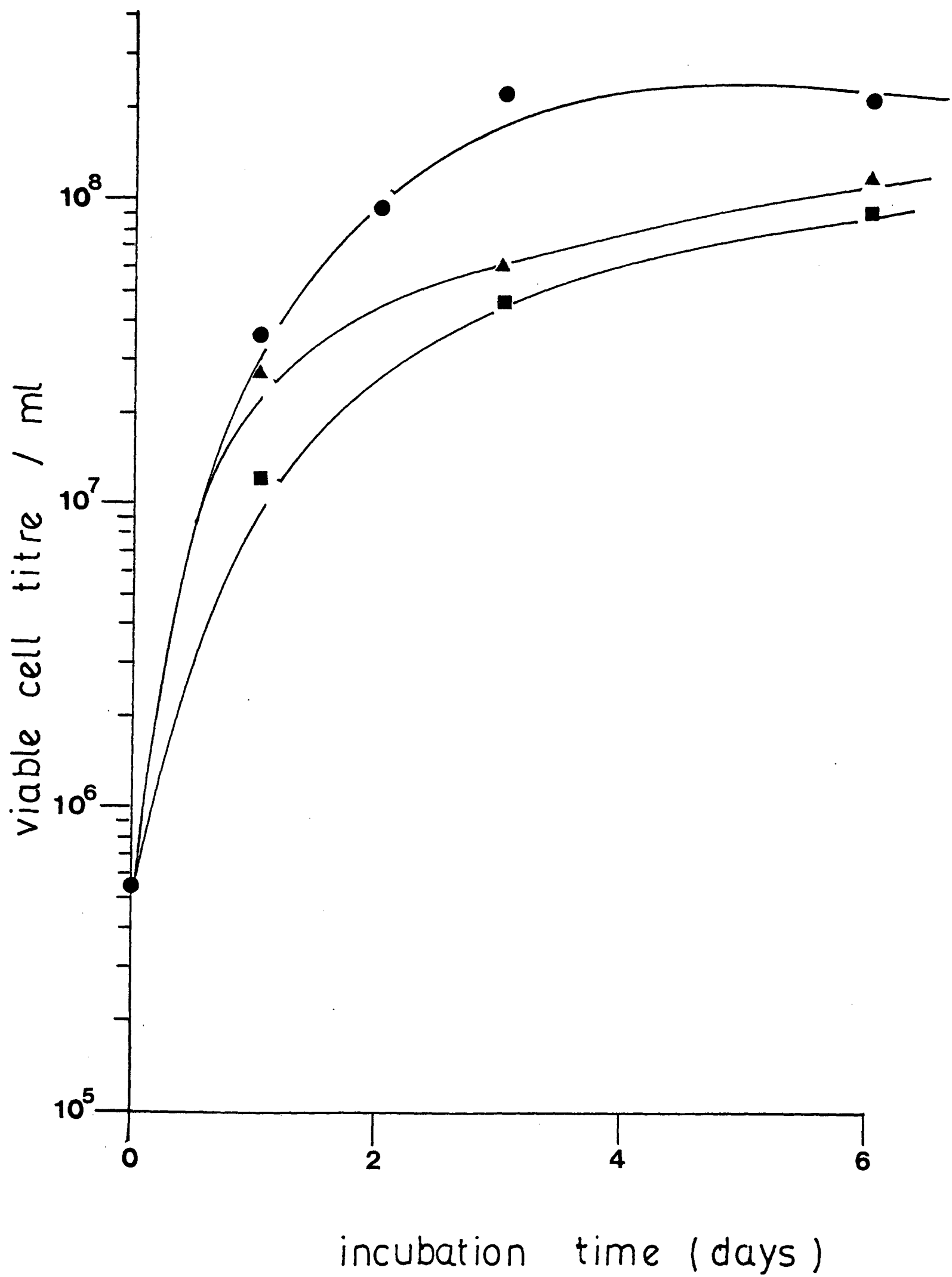


TABLE 3.24 Effect of benomyl and BENLATE on 507-4/2b during growth in liquid YC medium.

INCUBATION TIME (days)	NUMBER OF COLONIES SCORED AND VIABLE CELL TITRE*					
	CONTROL (YC ALONE)	BENOMYL		BENLATE		
		5µg/ml	10µg/ml	25µg/ml	50µg/ml	
0	2743	-	-	-	-	
1	(5.3x10 ⁵)					
1	2114	1197	818	667	1093	1544(3)
	(3.5x10 ⁷)	(9.6x10 ⁷)	(6.1x10 ⁷)	(1.11x10 ⁷)	(3.6x10 ⁷)	(2.9x10 ⁷)
2	1134	NT	NT	NT	NT	NT
	(9.4x10 ⁷)					
3	3702	591	515	1195(2)	322	1315(2)
	(2.3x10 ⁸)	(2.0x10 ⁸)	(1.7x10 ⁸)	(4.8x10 ⁷)	(6.4x10 ⁷)	(6.2x10 ⁷)
6	1843	NT	NT	1921	NT	2435
	(2.2x10 ⁸)			(9.6x10 ⁷)		(1.3x10 ⁸)

NT = not tested.

* the number of non-suppressed mutant colonies is given in square brackets.

507-4/2b. A significant increase in the fraction of petite mutants was observed after 6 days growth in 25µg/ml benomyl (16.3% ;803 colonies scored) and 50µg/ml benlate (6.8%; 717 colonies scored) compared to 0.2% (1117 colonies scored) in a control culture incubated for 6 days. This increase was not due to DMSO; a control culture of YC + 1% DMSO had 0.3% petites (1295 colonies scored) after 6 days growth.

Benomyl (5 µg/ml or 25µg/ml) had an inhibitory effect on growth of the rad1 mutant MT141-4/6d but no evidence of benomyl inducing mutation in this strain was obtained (Table 3.25). 25µg/ml benomyl increased the frequency of petites by a factor of x10, 5µg/ml having no significant effect (Table 3.25). MT141-4/6d was K⁺ but no K⁻ mutants were found in the control (722 colonies scored) or in the benomyl cultures (1969 colonies scored).

To summarise, benomyl and benlate may be slightly mutagenic at the concentrations used, but tests with higher concentrations of the fungicide in a more sensitive mutation assay system are required before a definite statement of the mutagenicity of benomyl can be made. 2 of the 7 non-suppressed mutants obtained were psi⁻, but because of the low numbers of colonies scored, one is unable to state that these were induced by the fungicide. No significant enhancement of mutation by benomyl was seen in a rad1 strain defective in excision-repair.

3.6 Discussion

Analysis of the response to mutagens has been one of the most powerful methods of studying the nature of genetic material at the cellular level. Such an analysis of the cytoplasmic psi⁺ to psi⁻ mutation was undertaken to gain an insight into the

TABLE 3.25 Effect of benomyl on an excision-defective psi⁺ strain MT141-4/6d during growth in liquid YC medium.

BENOMYL μg/ml		INCUBATION TIME	
		3 DAYS	6 DAYS
0	COLONIES SCORED	1452	1475
	REDS	0	0
	VIABLE CELL TITRE	8.7×10^7 /ml	7.4×10^7 /ml
	% PETITES* ₁	0.2(664)	0.2(566)
5	COLONIES SCORED	1477	2761
	REDS	1	0
	VIABLE CELL TITRE	4.9×10^7 /ml	6.9×10^7 /ml
	% PETITES	0.8(256)	0.3(628)
25	COLONIES SCORED	1322	1327
	REDS	0	0
	VIABLE CELL TITRE	8.8×10^6 /ml	5.7×10^7 /ml
	% PETITES	3.0(466)	3.0(623)

*₁ figure in parentheses is the total no. of colonies scored.

molecular nature of the $\bar{\psi}$ factor using two well known mutagenic agents, UV-irradiation and NTG, both of which are potent mutagens for the induction of both cytoplasmic and nuclear mutations. In particular an extensive analysis of the kinetics of induction of the ψ^- mutation was undertaken using UV as the mutagenic agent.

In terms of kinetic of induction, induction frequency per survivor, effect of the position in the cell cycle of the cells at a time of irradiation and the occurrence of whole and sectored mutant clones, the $\psi^+ \rightarrow \psi^-$ mutation showed almost identical characteristics to the UV-induction of forward nuclear gene mutation in the same cells. It differed considerably from UV-induction of the cytoplasmic petite mutation e.g. the kinetics of induction for the petite mutation were biphasic rather than linear as observed for the other mutations, including ψ^- . The frequency of induced petite mutants was at least 10^2 higher than forward nuclear gene mutation or the ψ^- mutation.

These results are consistent with UV-inducing pre-mutational lesions in DNA leading to the ψ^- mutation. Further evidence for this will be presented in the next chapter (Chapter 4).

The linear UV-induction kinetics for the ψ^- mutation over the dose range tested demonstrate that this mutation is a single-hit event. This suggests that there is either only one copy/cell of the $\bar{\psi}$ genome or there is one "master" $\bar{\psi}$ genome/cell against which all other copies ("slaves") are repaired. This will be discussed further in Chapter 9.

ψ^- mutants were picked up both as whole and sectored mutant colonies. For G1 (stationary phase) cells, of 81 UV-induced ψ^- mutants, 57 (70.4%) formed whole mutant colonies and

24 (29.6%) sectoried mutant colonies. This compares favourably to the nuclear mutations scored in G1 cells; 215/322 (66.8%) were whole mutant colonies and 107/322 (33.2%) were sectoried mutant colonies. In G1 cells the pre-mutational lesion, leading to the psi⁻ mutation, is therefore more likely to lead to a pure mutant colony. Very little is known about pure mutant colony formation in yeast although recent studies have suggested that conversion of non-mutant-mutant heteroduplex base pairs to mutant-mutant homoduplexes by some mode of DNA repair is an essential feature of the process (Hannan, Duck and Nasim 1976; Eckardt and Haynes 1977b). The repair of UV-induced damage in the psi⁻ genome may therefore be under similar control to that induced in nuclear DNA. The experiments reported in Chapter 4 were an attempt to confirm this hypothesis directly.

CHAPTER 4

/P̄SI/ MUTAGENESIS : REPAIR OF UV-INDUCED /P̄SI/ DAMAGE

- 4.1 Introduction
- 4.2 Photoreactivation
- 4.3 /P̄Si/ mutagenesis in repair-deficient mutants
- 4.4 Discussion

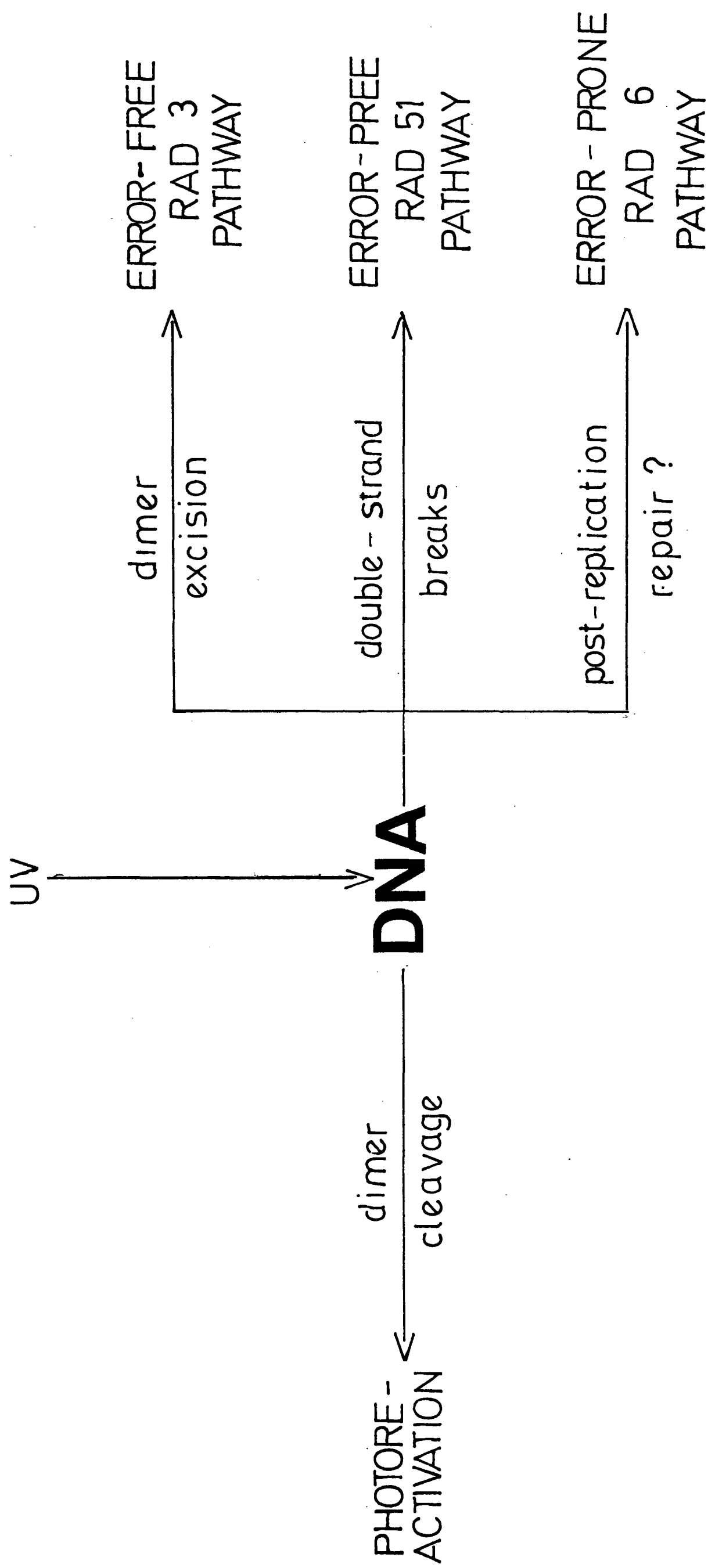
4.1 Introduction

UV-irradiation damages DNA by dimerising adjacent pyrimidines on the same strand; the principal products of the reaction are thymine-thymine dimers ($\hat{T}T$) but some cytosine-cytosine ($\hat{C}C$) and cytosine-thymine ($\hat{C}T$) dimers are also produced. If left in the DNA these lesions are lethal inhibiting both replication and transcription. Normally, however, a large fraction of these dimers are removed soon after irradiation by some mode of "error-free" DNA repair. Those which escape this "error-free" repair may undergo a process of fixation or conversion to informationally altered DNA base sequences through some mode of "error-prone" repair and/or recombination repair giving rise to mutation, recombination or a lethal lesion.

0.1 J/m² of UV-light applied to yeast will generate 24 pyrimidine dimers ($\hat{P}yPy$) per haploid genome (Unrau et al., 1973) and haploid wild type yeast cells can tolerate up to 30 J/m² (approximately 6000 dimers per genome) without showing any cell death. Because any unrepaired dimer in yeast is lethal (Wheatcroft et al., 1975) efficient repair systems must operate in yeast to excise the dimers. At doses higher than 30 J/m² these repair systems become saturated with dimers, which are formed in DNA in proportion to dose (Unrau et al., 1973) resulting in cell death and mutation because of inactivation of the repair systems.

Mainly genetic evidence has shown that yeast has both "error-free" and "error-prone" modes of repair of DNA damage (for review see Cox 1977a). Figure 4.1 outlines the major pathways found in

FIGURE 4.1 Pathways for the repair of UV-induced nuclear
DNA damage in yeast.



yeast for the repair of UV-induced nuclear DNA damage.

Mutants blocked in one or other of these pathways have been found (for review see Cox 1977a). Many such mutants are very sensitive to the lethal effects of UV.

UV-irradiation also leads to the appearance of $\hat{\text{PyPy}}$ in yeast mitochondrial DNA which are partially responsible for petite induction by UV light (Waters and Moustacchi 1974). These dimers are not normally excised from mitochondrial DNA (Waters and Moustacchi 1974; Prakash 1975). Genetic evidence (Moustacchi 1969, 1972, 1973) indicates that the repair of UV-induced damage in mitochondrial DNA is controlled by some, but not all, of the nuclear DNA repair pathways and that mitochondrial DNA-specific repair systems also exist.

UV-irradiation can mutate psi^+ to psi^- (see Chapter 3). Assuming this is a consequence of UV-induced damage to the [psi] genome, is this damage repairable by nuclear DNA repair systems, or do other repair systems exist in yeast for the repair of such damage? The experiments to be described in this Chapter were designed to answer these questions and to confirm that the [psi] genome is DNA.

4.2 Photoreactivation

Both prokaryote and eukaryote cells possess a highly specific enzyme capable of binding to UV-induced pyrimidine dimers ($\hat{\text{PyPy}}$) in DNA and splitting them in situ. The enzyme has an apparently strict requirement for activation by near UV or white light, the maximum activation for the yeast enzyme in vivo being obtained with light of wavelength 370-410nm (Anders et al., 1976). This phenomenon is termed photoreactivation (PHR) and a stationary phase haploid yeast cell contains approximately 270 molecules of the photoreactivating

enzyme (DNA photolyase, deoxyribodipyrimidine photolyase, PRE) (Yasui and Laskowski 1975). Exponentially growing cells contain much less PRE (Boling and Setlow 1967).

PHR, first demonstrated in yeast by Warshaw (1952) leads to an increase in survival and loss of both nuclear and cytoplasmic mutation (Patrick et al., 1964). This photorepair is never complete with the photoreactivable sector values, for different strains of yeast, ranging between 40 and 66% (Moustacchi et al., 1977) suggesting that other lesions apart from the $\hat{\text{PyPy}}$ may be involved in UV-induced cell death and mutation. $\hat{\text{PyPy}}$ formed in both nuclear and mitochondrial DNA are photoreactivable in yeast (Unrau et al., 1971; Waters and Moustacchi 1974) to the same extent.

The only substrate for the photoreactivating enzyme is the $\hat{\text{PyPy}}$ and the removal of some biological effect by PHR means that $\hat{\text{PyPy}}$ are involved (Setlow and Carrier 1964). Therefore PHR can be used as a direct probe into the involvement of $\hat{\text{PyPy}}$ in a mutational event. A reduction in the frequency of psi⁻ mutants induced by UV-light, following PHR, would be confirmation of the at least partial involvement of $\hat{\text{PyPy}}$ in the psi⁺ to psi⁻ mutational event and add weight to the hypothesis that /psi has a DNA genome.

Results

Using the haploid strain 507-4/2b the effect of PHR on UV-induced mutations giving the non-suppressed phenotype, and in particular the psi⁺ to psi⁻ mutation, was looked at in both log phase and stationary phase cells. In both cases an irradiated cell suspension (c. 5×10^6 cells/ml in saline), in a closed plastic petri dish, were exposed for 1 hour at room temperature

to a 500w Phillips 'Photoflood' bulb at a distance of 60cm. A second petri dish, containing 25ml of a 5% (W/V) copper sulphate solution, was placed on top of the cell suspension to prevent overheating. Stationary phase cells were UV-irradiated with 125 J/m^2 whilst log phase cells were given 200 J/m^2 giving approximately the same levels of survival (0.06% and 0.11% respectively).

In both types of cell a substantial increase in survival was seen after PHR together with a decrease in the frequency of non-suppressed mutants (per 10^3 survivors) (Table 4.1). In stationary phase cells premutational lesions leading to whole mutant colony formation appeared to be more photoreactivable than those leading to sectorial mutant colonies i.e. there was a 64% reduction in the frequency of whole mutant colonies after PHR compared to only 20.8% decrease in the frequency of sectorial mutant colonies. In log phase cells the difference was much less (76.9% to 71.1%) although the reduction in mutant frequency (whole + sectorial), at 74.9%, was much higher than that observed for stationary phase cells (49.6%). This implies that PHR of premutational lesions was more efficient in log phase cells. However the increase in survival levels after PHR was less in log phase cells; a 60-fold increase to a 75-fold increase for stationary phase cells.

The effect of PHR on UV-induction of the cytoplasmic petite mutation was also looked at in 507-4/2b (Table 4.2). From these results it appears that the premutational lesion is non-photoreactivable in log phase cells but is photoreactivable in stationary phase cells where a 23.3% reduction in the frequency of the petite mutation was seen after PHR.

TABLE 4.1 The effect of photoreactivation on mutation frequency and survival in log₀ and stationary phase cells of 507-4/2b.

TREATMENT*1	LOG PHASE				STATIONARY PHASE			
	NUMBER COLONIES	SURVIVAL (%)	NON-SUPPRESSED COLONIES		NUMBER COLONIES	SURVIVAL (%)	NON-SUPPRESSED COLONIES	
			NUMBER WHOLE	SECTORED			NUMBER WHOLE	SECTORED
				FREQUENCY/10 ³ SURVIVORS				FREQUENCY/10 ³ SURVIVORS
UNIRRADIATED	1363	100	0	0	1877	100	1	0
UV-IRRADIATED	4237	0.11	34	18	2305	0.06	16	8
UV-IRRADIATED +PHR	4871	6.62	9	6	7999	4.60	20	22
				3.08				5.25

*1 for details see text.

*2 the figures given represent a total of 3 experiments: % survival is the average.

TABLE 4.2

The effect of photoreactivation on the frequency of the UV-induced petite mutation in log and stationary phase cells of 507-4/2b.

TREATMENT*1	LOG			LOG		
	COLONIES TESTED	PETITE	% PETITE	COLONIES TESTED	PETITE	% PETITE
UNIRRADIATED	706	4	0.57	1110	1	0.09
UV-IRRADIATED	459	195	42.48	894	306	34.23
UV-IRRADIATED +PHR	325	172	52.92	834	219	26.26

*1 See text for details.

This is in contrast to the recent observation by Chanet and Heude (1974) who found that, using synchronous culture studies, at all stages of the cell cycle white light decreased the frequency of UV-induced petite mutants.

Complementation analysis was performed on UV-induced non-suppressed mutants obtained both before and after PHR. To obtain large numbers of mutants for this analysis irradiated cells, both before and after PHR, were plated onto YC to give approximately 2000 colonies per plate. The frequencies for each class of mutation are given in Table 4.3 and plotted as a histogram in Figure 4.2. In both log and stationary phase cells a decrease in the frequency of psi⁻ mutants was observed after PHR; a 92% decrease in log phase cells and a 50.1% decrease in stationary phase cells. Combining the data from both types of cells, and expressing it in terms of colony numbers, ¹³/5099 colonies were psi⁻ before PHR, ¹⁷/27995 colonies were psi⁻ after PHR. The psi⁻ mutants were found as both whole and mutant sector colonies before and after PHR. For all classes of nuclear mutation the frequencies were reduced by PHR in both log and stationary phase cells.

4.2.2 Conclusion

The psi⁺ to psi⁻ mutational event is photoreactivable as demonstrated for several classes of forward nuclear mutation in the same strain. This demonstrates that UV-induced $\hat{P}yPy$ are involved in the mutational event giving rise to the psi⁻ mutation, thus showing that the /psi/ genome is a nucleic acid, presumably DNA (but see Discussion). PHR of the psi⁻ premutational lesion is more efficient in log phase cells as is PHR of the nuclear premutational lesions.

TABLE 4.3 The effect of photoreactivation on UV-induced nuclear and cytoplasmic mutation frequency 507-4/2 b.

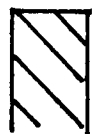
MUTATION	MUTANT FREQUENCY /10 ³ SURVIVORS*1			
	LOG		STATIONARY	
	-PHR	+PHR	-PHR	+PHR
WHITE → RED	12.27(46)	3.08(61)	10.41(18)	5.25(43)
PSI ⁺ → PSI ⁻	1.87(7)	0.15(3)	3.43(6)	1.71(14)
PNM ⁺ → pnm	1.33(5)	0.35(7)	1.73(3)	0.37(3)
ASU ⁺ → ASU	4.27(16)	0.81(16)	3.43(6)	1.71(14)
ASU ⁺ /PNM ⁺ → PNM/ASU	1.60(6)	0.56(11)	-	-
SUQ5 → SUQ5 ⁺	2.13(8)	0.71(14)	-	-
rho ⁺ → rho ⁻	424.8(195)	529.2(172)	342.3(894)	262.6(834)

*1 Only those mutation classes for which at least 3 mutants were scored are given. The figure in brackets is the number of mutants scored.

FIGURE 4.2 The effect of photoreactivation on UV-induced mutation in log and stationary phase cells of 507-4/2b ; a histogram representation of total, psi⁻ and asu mutations.

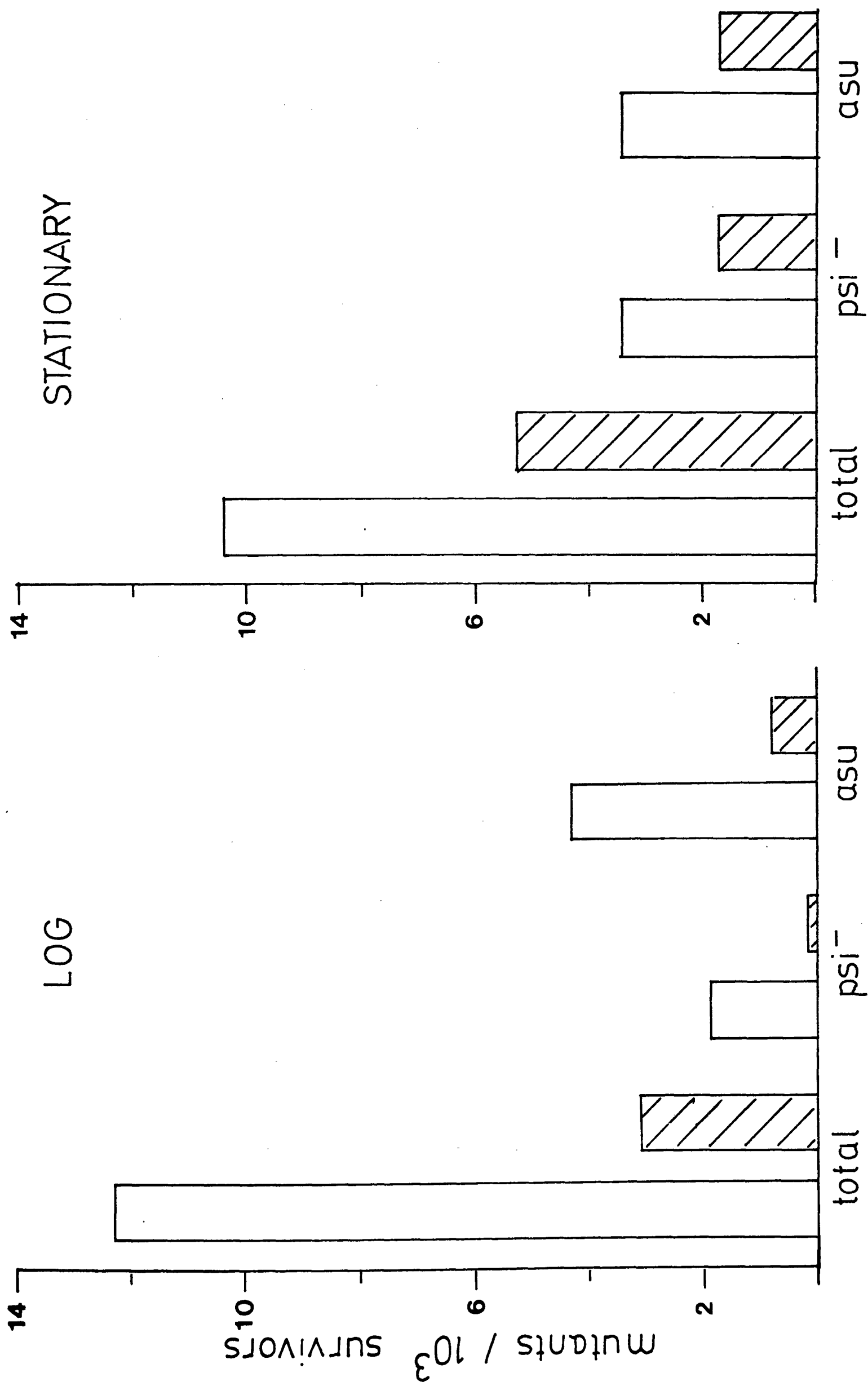


no photoreactivation



following photoreactivation

- see text for details.



4.3 /Psi/ Mutagenesis in repair-deficient mutants

4.3.1 Introduction

Excluding PHR there exist three competitive pathways for the repair of UV-induced nuclear DNA damage in yeast (Cox and Game 1974, see Figure 4.1), involving at least 20 genetic loci. Mutants defective at one or other of these loci have been isolated which result in one or other of the repair pathways being blocked; rad1, rad2, rad3 and rad4 in the RAD3 excision repair pathway; rad6, rad9, rad18, rev1, rev2, rev3 and possibly umr1 - umr7 in the putative error-prone, possible recombinational RAD6 repair pathway and rad50 and rad51 in the minor, presumably error-free RAD50 pathway (for review see Cox 1977a).

Pyrimidine dimers induced by UV-light in the DNA of mutants blocked in the RAD3 pathway are repaired by the RAD6 error-prone pathway and consequently are very sensitive to the lethal effects of UV-light and show enhanced UV-light mutagenesis (Hunnable 1972; Lawrence and Christensen 1976). In mutants blocked in the RAD6 pathway all genetic damage is repaired by the error-free RAD3 pathway and they show a marked reduction in UV-induced mutability (Lawrence and Christensen 1976) although not all of these mutants are UV-sensitive (Lemontt 1977).

UV-induced /psi/ mutation was looked at in strains blocked in either the RAD3 or RAD6 pathway to see if psi mutation frequency is enhanced and depressed respectively. Similar studies with UV-induced petite mutagenesis have indicated that UV-induced petite mutation frequencies do not always follow the pattern predicted in such mutants suggesting that UV-induced damage to mitochondrial DNA is also repaired by

mitochondrial DNA-specific repair pathways (Moustacchi 1969, 1972). If $\bar{\psi}$ mutagenesis were enhanced in mutants blocked in the RAD3 pathway, and depressed in mutants blocked in the RAD6 pathway, this would indicate that UV-induced damage to the $\bar{\psi}$ genome was repaired by the pathways responsible for the correction of nuclear DNA damage and would be circumstantial evidence for the $\bar{\psi}$ genome being DNA.

4.3.2 $\bar{\psi}$ mutation in mutants blocked in RAD3 pathway

UV-induced mutation was studied in strains carrying either the rad1 or rad3 mutation both of which are defective in excision-repair.

(a) rad1

Originally isolated by Cox and Parry (Cox and Parry 1968), strains carrying this mutation have been shown to be defective in the ability to excise UV-induced $\hat{P}yPy$ (Unrau et al., 1971). The UV-induced reversion of nuclear alleles, as a function of UV dose, is higher in rad1 strains than in strains wild type for this locus (RAD⁺) (Moustacchi 1969; Lawrence and Christensen 1976). The rad1 mutation has no significant effect on spontaneous mutability (Brychcy and Von Borstel 1977).

The original strain carrying the rad1 mutation (i.e. 197/2d) was obtained from Dr B. Cox and derivation of strains used in this study are given in Figure 4.3 together with their full genotypes.

The RAD⁺ control for these experiments was 507-4/2b. Log phase cells of both RAD⁺ and rad1 strains were irradiated. Figure 4.4 gives the UV survival curves for all 3 strains used. Each point is an average of at least 2 experiments except for MT141-2/4b (SUQ5 ade2.1 rad1.1 psi⁺) which is just one experiment.

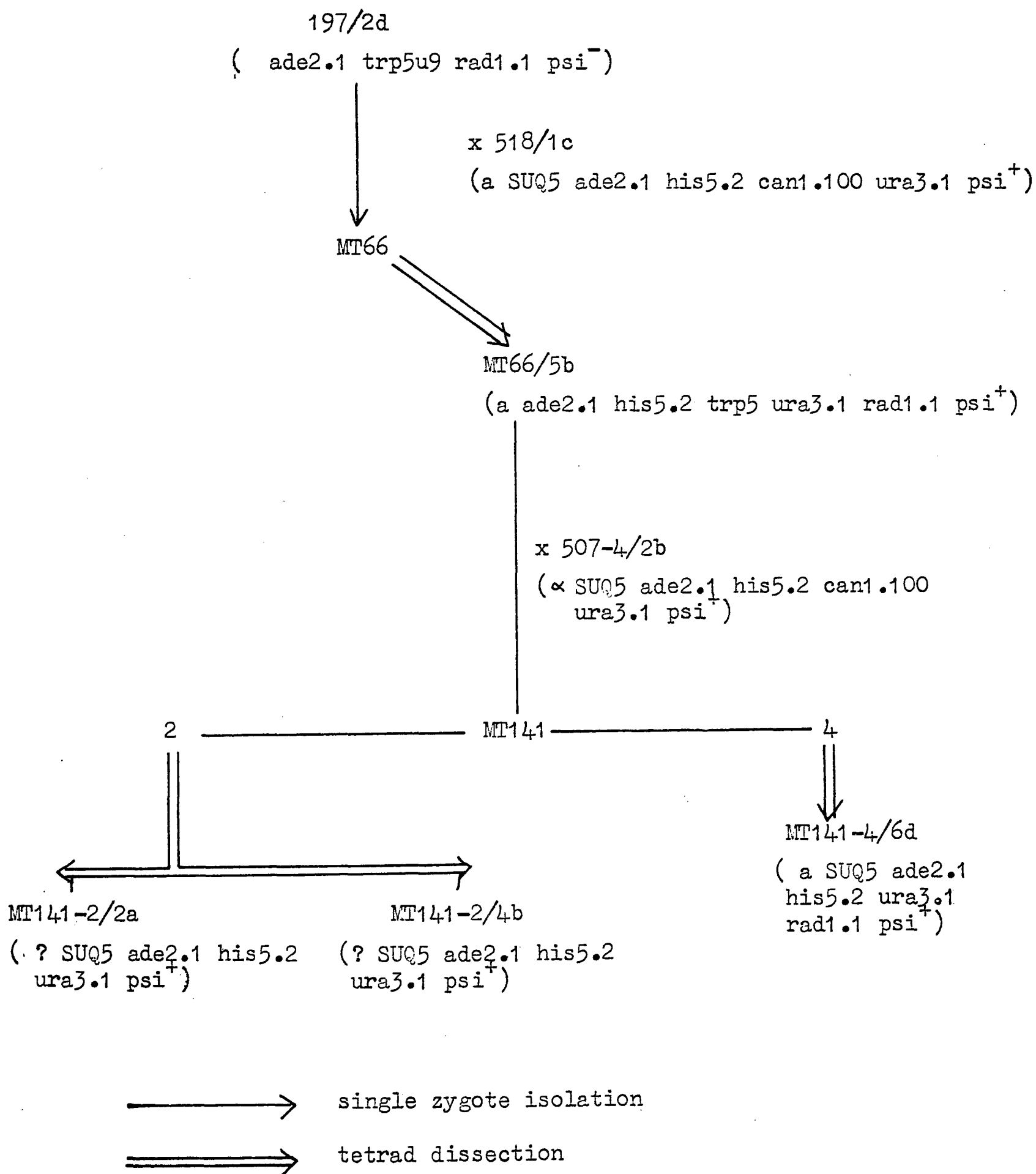
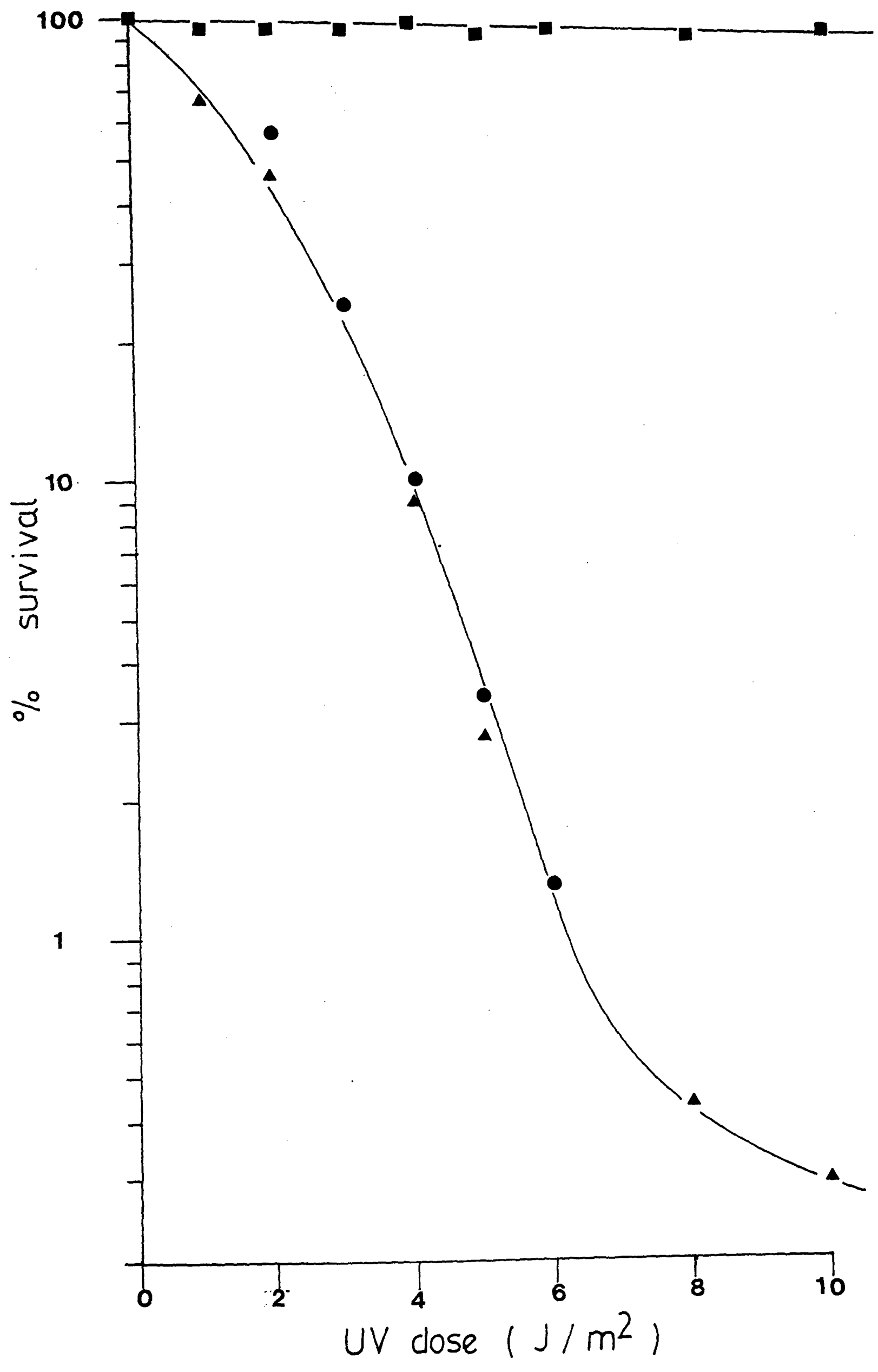
FIGURE 4.3 Derivation of rad1.1 carrying strains used in this study.

FIGURE 4.4 The UV-sensitivity of two rad1.1 psi⁺ strains
(MT141-2/4b , MT141-4/6d) compared to the RAD⁺
psi⁺ strain 507-4/2b.

- (■) 507-4/2b
- (●) MT141-2/4b
- (▲) MT141-4/6d



The average LD_{37} for the rad1 strains was 2.3 J / m^2 . Both rad1 strains showed slight 'shoulders' to their survival curves at very low UV doses. The induction of non-suppressed mutants in these 3 strains was studied over a dose range of $0-10 \text{ J / m}^2$ and the results are given in Table 4.4.

Both rad1 strains showed a high frequency of induced mutation at a given UV dose compared to the RAD⁺ control strain and, plotted as log mutation frequency versus log dose (Figure 4.5), the induction kinetics were linear for the rad1 and RAD⁺ strains.

Attempts to classify the mutations giving rise to the non-suppressed phenotype in strain MT141-2/4b were hampered by the discovery that, of 128 non-suppressed mutants screened by crossing with an α ade1 and an a ade1 tester strain, 25 (19.5%) failed to complement either, 34 (26.6%) complemented α ade1, 44 (34.4%) a ade1 and 26 (20.3%) complemented both α and a ade1. MT141-2/4b therefore appeared to carry a mutation affecting mating type inter-conversion, a normally rare event in these heterothallic strains (Hawthorne 1963; Herskowitz et al., 1977). 6 tetrads from the diploid MT141-2 and 4 from MT141-4 (a second diploid clone from the same cross) all showed 2 spore which mated with both α and a strains and 2 which only mated with a strains. This mutation therefore seemed to lead to a high frequency of conversion of a mating type to α mating type as is observed with certain homothallic strains of yeast (for a review see Herskowitz et al., 1977). MT141-4/6d (SUQ5 ade2.1 rad1.1 psi⁺) showed normal α mating type response and non-suppressed mutants were analysed in this strain (Table 4.5).

TABLE 4.4 Frequencies of UV-induced mutants in a RAD^+ and 2 different rad 1 mutant strains.

UV DOSE (J/m ²)	RAD ⁺ : 507-4/2b (SUQ5 ade 2.1 psi ⁺)				rad 1 MT141-2/4b (SUQ5 ade 2.1 psi ⁺)				rad 1 MT141/4/6d (SUQ5 ade 2.1 psi ⁺)			
	COLONIES	NO. MUTANTS*		MUTANTS /10 ³ SURVIVORS	COLONIES	NO. MUTANTS		MUTANTS /10 ³ SURVIVORS	COLONIES	NO. MUTANTS		MUTANTS /10 ³ SURVIVORS
		W	S			W	S			W	S	
0	11,377	1	2	0.26	1,008	1	3	3.97	3,838	3	0	0.78
1	1,610	0	0	0	832	0	3	3.61	2,293	3	7	4.36
2	4,078	0	2	0.49	1,525	0	13	8.52	9,220	5	37	4.56
3	1,639	0	0	0	785	0	5	6.37			-	
4	3,810	0	2	0.52	5,082	13	38	10.04	6,526	14	30	6.74
5	1,575	0	0	0	2,169	1	22	10.60			-	
6	3,796	1	0	0.26	10,340	26	84	10.64	8,284	37	53	10.86
8	2,170	0	3	1.38		-			3,201	21	24	14.06
10	2,247	0	1	0.44		-			4,642	37	29	14.22

* W = whole ,

S = sectored mutant colonies.

FIGURE 4.5 The frequency of non-suppressed mutants as function of UV dose for a RAD^+ psi^+ strain (507-4/2b) and rad1.1 psi^+ strains (MT 141-4/6d and MT141-2/4b).

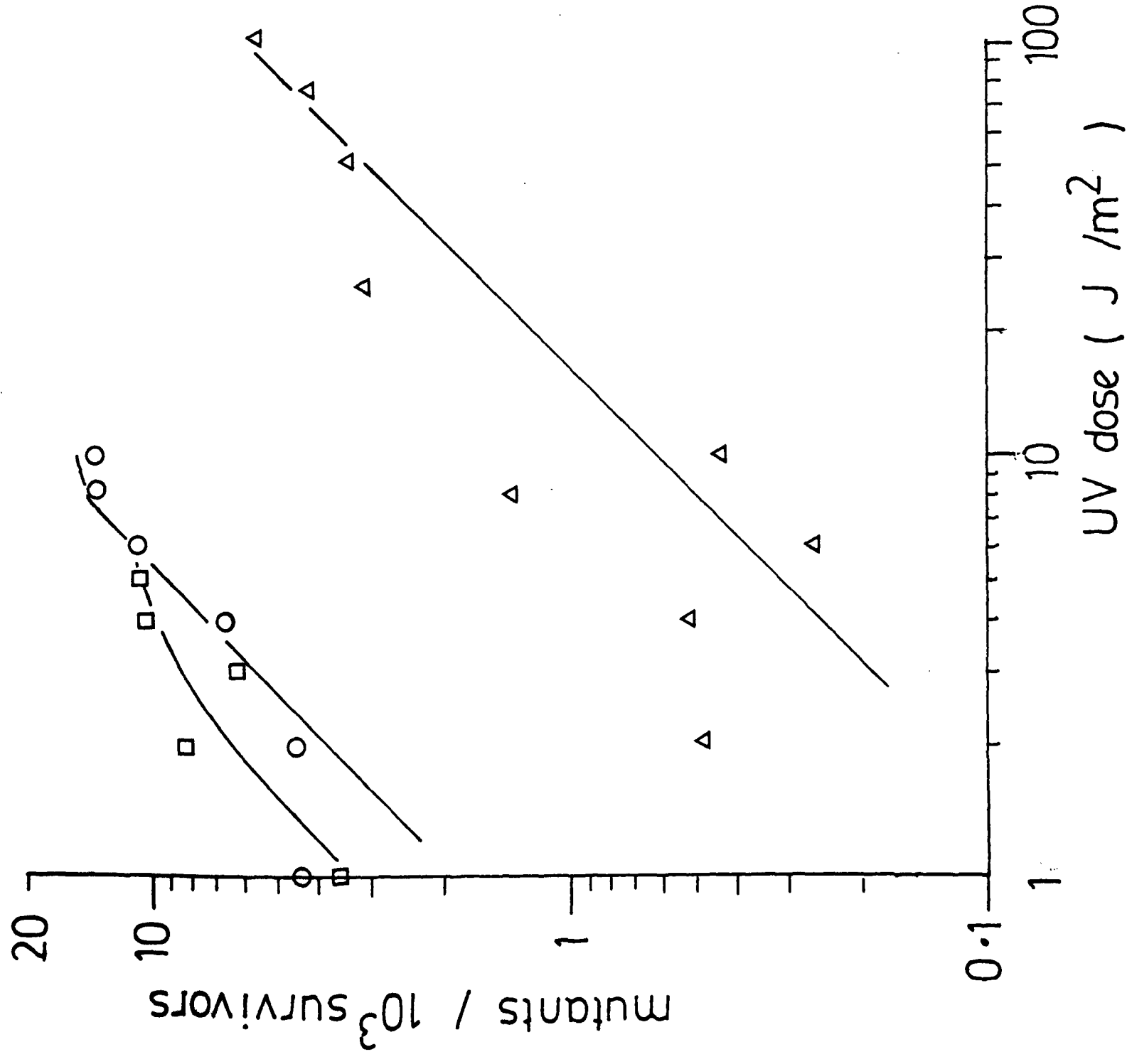
- (Δ) 507-4/2b RAD^+ psi^+
- ($\square$) MT141-2/4b rad1.1 psi^+
- ($\circ$) MT141-4/6d rad1.1 psi^+

FIGURE 4.6 The frequency of different classes of mutant giving the non-suppressed phenotype as a function of UV dose for the rad1.1 mutant MT141-4/6d.

- ($\bullet$) psi^-
- (Δ) pnm
- ($\blacksquare$) asu
- ($\circ$) PNM/ASU

--- represents data for the psi^- mutation in the RAD^+ strain 507-4/2b.

4.5



4.6

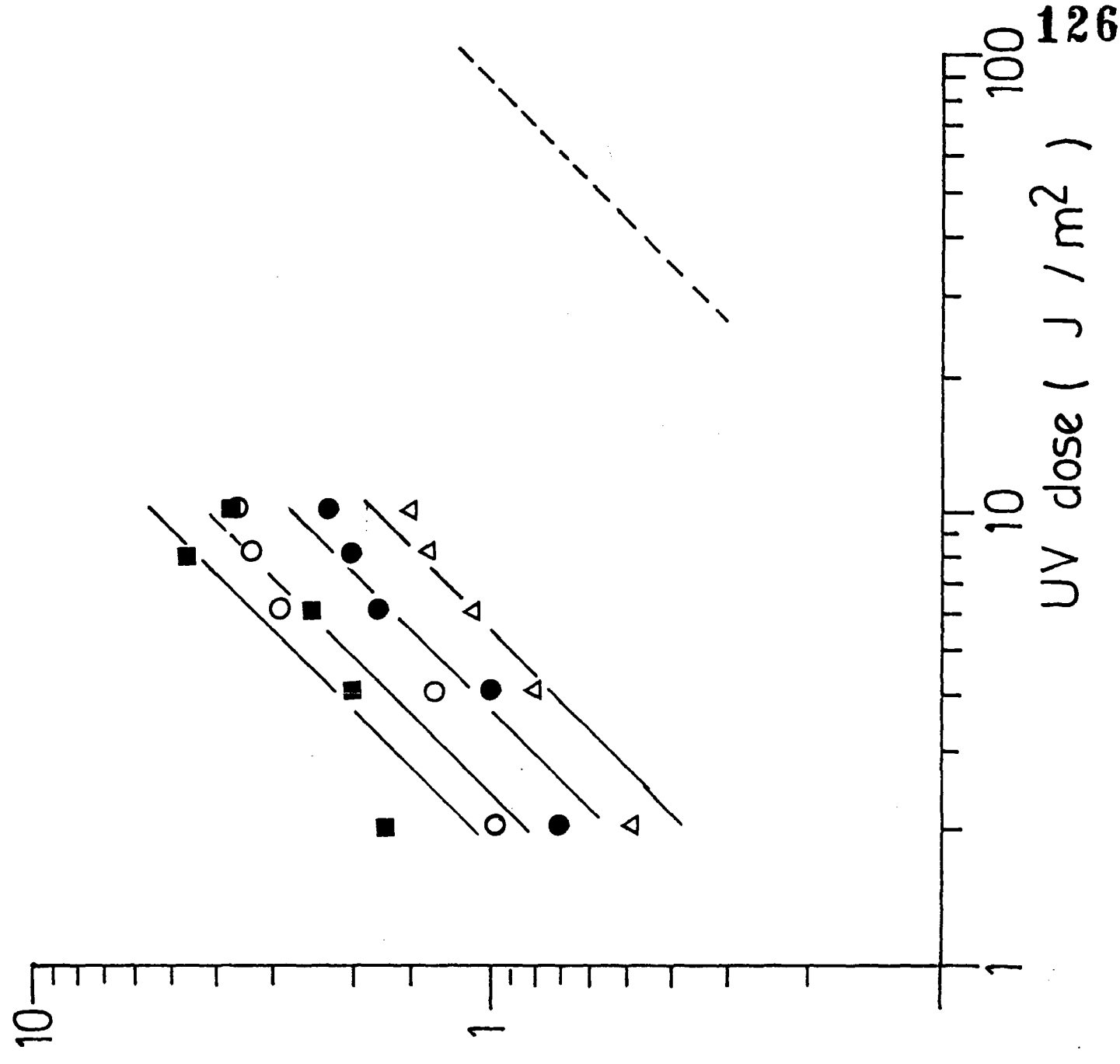


TABLE 4.5 UV-induced mutation frequencies of the different classes of mutation giving the non-suppressed phenotype in MT141-4/6d (SUQ5 ade 2.1 rad 1.1 psi⁺).

DOSE (J/m ²)	TOTAL*2 MUTATION FREQUENCY /10 ³ SURVIVORS	NUMBER*1 MUTANTS ANALYSED	p ⁻	ts	MUTATION FREQUENCY /10 ³ SURVIVORS*2							
					ade1	ade2.x	psi ⁻	pnm	asr	PNM/ASU	SUQ5 ⁺	(NON-MATTER)
0	0.78(3)	3	0	0	0	0	0	0	0.52(2)	0.26(1)	0	
2	4.56(42)	19	0	0	0	0.48(2)	0.72(3)	0.48(2)	1.68(7)	0.96(4)	0.24(1)	0
4	6.74(44)	41	1	2	0.49(3)	0.82(5)	0.99(6)	0.82(5)	1.97(12)	1.32(8)	0.33(2)	0
6	10.86(90)	49	1	2	0.22(1)	0.66(3)	1.77(8)	1.11(5)	2.44(11)	2.88(13)	1.33(6)	0.44(2)
8	14.06(45)	21	1	0	0	2.00(3)	2.00(3)	1.34(2)	4.68(7)	3.35(5)	0	0.67(1)
10	14.22(66)	19	1	0	0	0.75(1)	2.24(3)	1.50(2)	3.74(5)	3.74(5)	1.50(2)	0.75(1)

*1 includes both whole and sectorred mutant classes.

*2 number of mutants in brackets.

The log-log plot (Figure 4.6) showed that induction kinetics for the psi⁻ mutation were linear ($n = 1$), as found in the RAD⁺ strain (see Figure 3.2) and that, per unit dose of UV-light, the frequency of psi⁻ mutants was substantially higher than in the RAD⁺ control strain. This was also true for other classes of mutation tested (Figure 4.6). Psi⁻ mutants were recovered as both whole and sectorized mutant clones in the rad1 strain at all doses of UV.

Of the 7 non-suppressed mutants isolated from the log phase RAD⁺ strain 507-4/2b (SUQ5 ade2.1 psi⁺), after UV doses between 2 and 10 J / m², 4 were asu, 1 was SUQ5⁺, 1 pnm and 1 PNM/ASU; all 7 were ts⁺ and grande.

The ratio of whole sectorized mutant colonies in rad1 (i.e. MT141-4/6d) ranged from 1:7 at 1 J / m² to 6:4 at 10 J / m² a pattern approximately observed in the RAD⁺ strain, not at equivalent UV doses, but at the same levels of survival. Total mutation frequency was slightly higher in the rad1 strain MT141-4/6d than the RAD⁺ strain at equivalent levels of survival.

b) rad3

Originally isolated by Cox and Parry among a series of UV-sensitive mutants (Cox and Parry 1968), the RAD3⁺ locus has been postulated as the first step in the excision-repair pathway (Cox and Game 1974), and recent biochemical evidence has shown that rad3 mutants are defective in dimer excision (Prakash 1977). Rad3 mutants show an enhanced UV-induced mutation rate per unit dose of UV-light compared to a RAD3⁺ strain (Hunnable 1972; ^wLarence and Christensen 1976) as found in rad1 mutants. Rad3 mutants also show an enhanced spontaneous mutation rate i.e. are "mutators" (Brychcy and Von Borstel 1977).

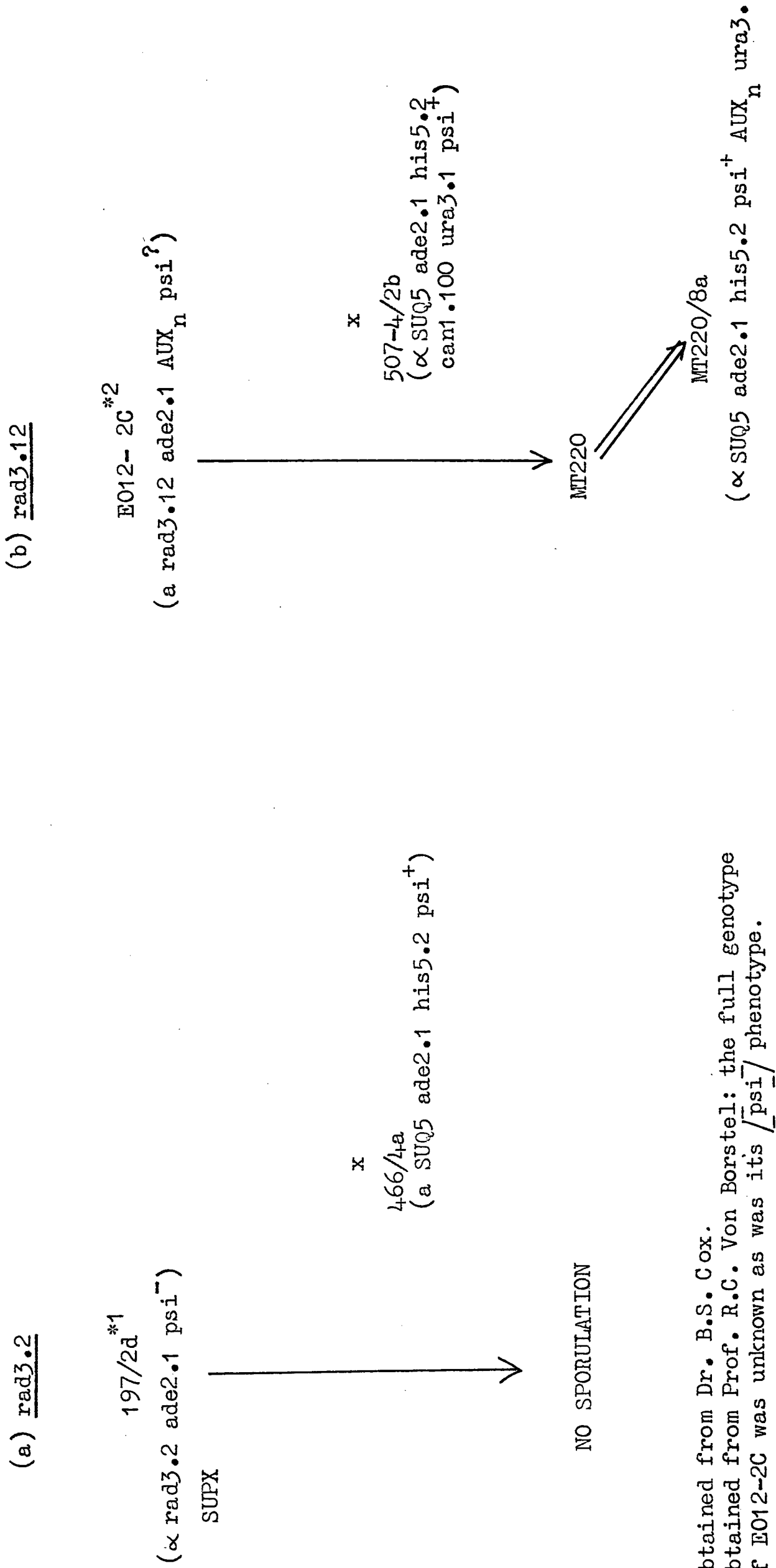
Figure 4.7 outlines the derivation of the rad3 strain used in this study. A UV-fluence of $50 \text{ J} / \text{m}^2$ had to be used to distinguish rad3.12 spores from RAD3⁺ spores in tetrads from MT220. The rad3.12 is a leaky allele of rad3 with respect to UV-sensitivity (J.C. Game personal communication). An attempt to isolate a strain carrying a non-leaky rad3 allele (i.e. rad3.2) was unsuccessful because all 6 diploids, isolated from the cross between 197/2d (rad3.2) and 466/4a, failed to sporulate (see Figure 4.7).

The SUQ5 ade2.1 rad3.12 psi⁺ strain constructed, MT220/8c, had an LD₃₇ of $6.2 \text{ J} / \text{m}^2$ and was significantly more sensitive to UV than the RAD3⁺ control (507-4/2b). In a preliminary experiment on the survival of MT220/8c, irradiating cells on YC plates directly rather than in saline, an LD₃₇ of $2.6 \text{ J} / \text{m}^2$ was obtained (Figure 4.8). Similarly the RAD⁺ strain 507-4/2b was more sensitive to the lethal effects of UV when irradiated directly on YC plates (Figure 4.8) with an LD₃₇ of $30 \text{ J} / \text{m}^2$ against an LD₃₇ of $42 \text{ J} / \text{m}^2$ when irradiated in saline.

It should be noted that in all experiments rad3.12 and control RAD3⁺ strains were irradiated as stationary phase cells, whilst rad1 strains were irradiated as log-phase. No difference in UV-sensitivity is seen when comparing log and stationary phase cultures of excision-defective strains (Ferguson 1975).

UV-mutagenesis was measured over a dose range of $0-60 \text{ J} / \text{m}^2$ in MT220/8c (SUQ5 ade2.1 rad3.12 psi⁺) and the results are given in Table 4.6. An enhanced mutation frequency per unit dose of UV was seen compared to the RAD⁺ control and the kinetics of induction (Figure 4.9) were linear up to a dose of $15 \text{ J} / \text{m}^2$.

FIGURE 4.7 Derivation of rad3 strains used in this study.



*1 obtained from Dr. B.S. Cox.

*2 obtained from Prof. R.C. Von Borstel: the full genotype of E012-2C was unknown as was its psi phenotype.

FIGURE 4.8 The UV-sensitivity of the rad3.12 psi⁺ strain MT220/8c following irradiation in saline or directly on YC plates ; compared to the RAD⁺ strain 507-4/2b.

- (○) 507-4/2b RAD⁺ , irradiated in saline
- (●) 507-4/2b RAD⁺ , irradiated on YC plates
- (□) MT220/8c rad3.12 , irradiated in saline
- (■) MT220/8c rad3.12 , irradiated on YC plates

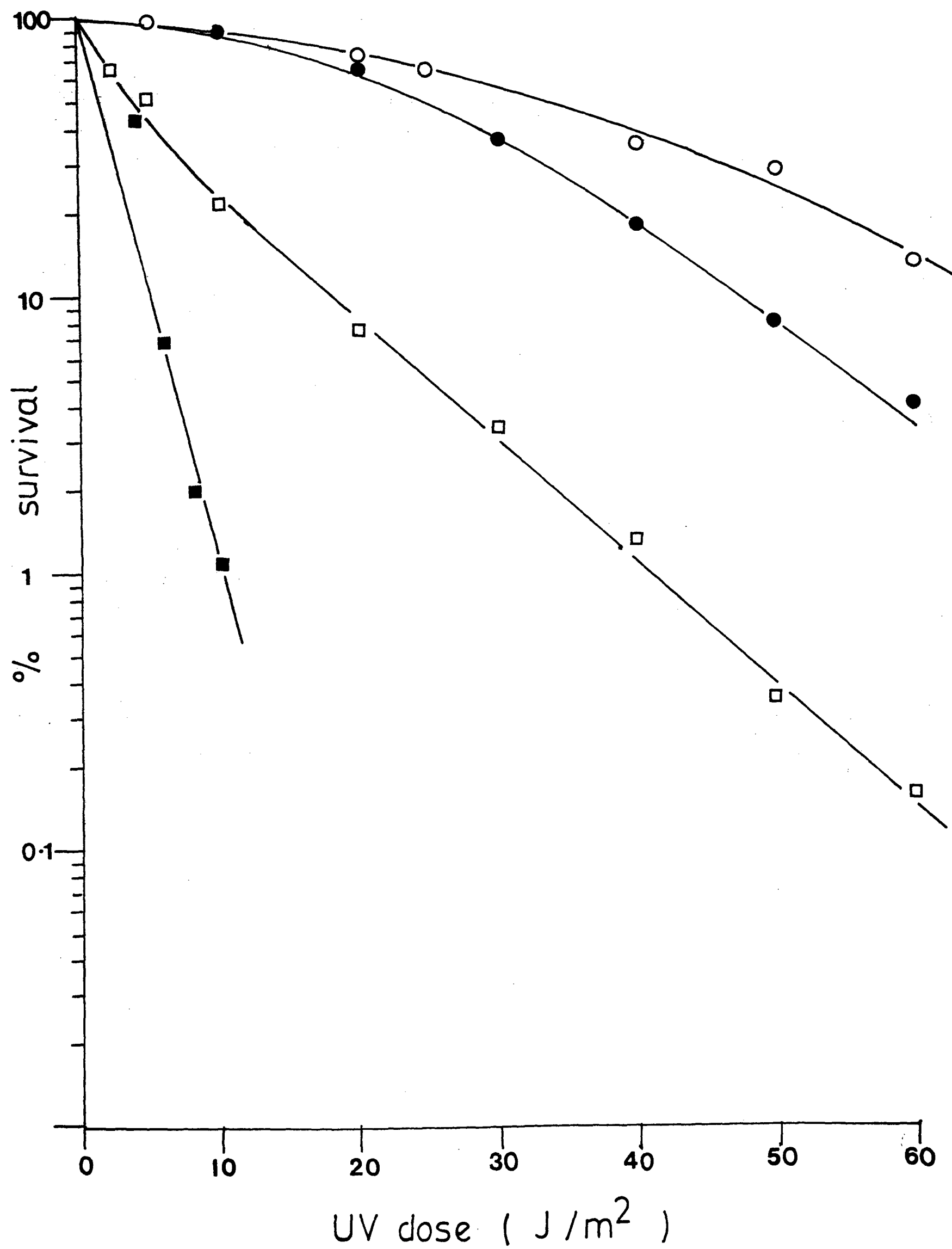


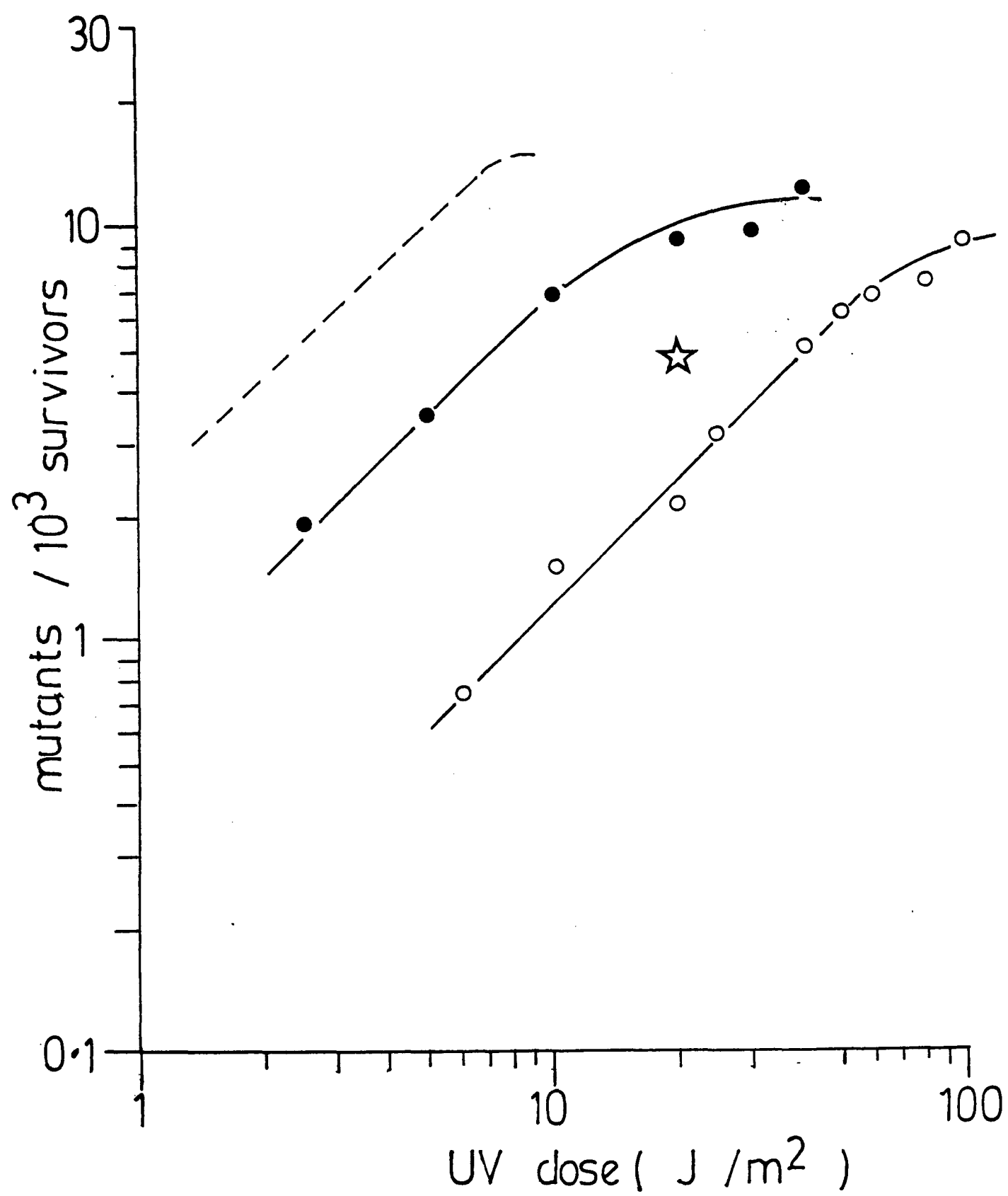
TABLE 4.6 Induction of non-suppressed mutants of
MT220/8c (SUQ5 ade 2.1 rad 3.12 psi⁺) by UV.

UV DOSE (J/m ²)	NUMBER OF EXPERIMENTS	% SURVIVAL	COLONIES	MUTANTS		TOTAL MUTANTS. FREQUENCY/10 ³ SURVIVORS
				WHOLE	SECTORED	
0	5	100	11 244	3	2	0.44
2.5	1	65.5	1 030	1	1	1.94
5	2	53.0	3 970	6	8	3.53
10	2	21.9	5 037	18	16	6.75
20	2	7.7	9 493	46	42	9.27
30	2	3.4	4 645	27	18	9.69
40	2	1.4	5 976	31	42	12.22
50	1	0.4	203	1	1	9.85
60	1	0.2	522	3	3	11.49

FIGURE 4.9 The frequency of non-suppressed mutants as a function of UV dose for a rad3.12 psi⁺ strain (MT220/8c) and a RAD⁺ psi⁺ strain (507-4/2b).

- (○) 507-4/2b RAD⁺ psi⁺ total
- (●) MT220/8c rad3.12 psi⁺ total
- (☆) psi⁻ mutation for MT220/8c

----- represents the data for total non-suppressed mutants of the rad1.1 strain MT141-4/6d.



At doses higher than $15 \text{ J} / \text{m}^2$ a levelling off was observed. A total of 58 non-suppressed mutants isolated, after $20 \text{ J} / \text{m}^2$, from MT220/8c were analysed by complementation (Table 4.7). Both whole (30 mutants) and sectorised (28 mutants) mutant colonies were analysed. The psi⁻ mutation frequency was approximately $\times 5$ greater in the rad3.12 strain and the total mutation frequency was $\times 4$ greater than the RAD⁺ control. All other classes occurred with a higher frequency in the rad3.12 strain.

5 spontaneous non-suppressed mutants of MT220/8c (SUQ5 ade2.1 rad3.12 psi⁺) were obtained (Table 4.6); 3 were psi⁻ and two were asu mutants.

Among the 58 non-suppressed mutants analysed (Table 4.7) 46 showed very high frequencies of reversion to ADE⁺ presumably because rad3.12 is a mutator. 12 however were stable for the ade⁻ phenotype. 7 of these were the ade2.x and ade1 mutants whilst the remaining 5 were SUQ5⁺ mutants. The 7 other SUQ5⁺ mutants isolated gave high frequencies of reversion to ADE⁺. It is not clear why some presumptive second-site mutants in the SUQ5 gene should fail to revert to ADE⁺.

At all doses tested the ratio of whole sectorised mutant colonies was approximately 1:1 which is in contrast to that observed in the rad1 and RAD⁺ strains (see Table 4.4).

c) Petite induction in excision-defective rad mutants

The induction of the cytoplasmic petite mutation by UV-light, in rad mutants defective in excision-repair, was originally looked at by Moustacchi who found that the RAD1⁺ and RAD4⁺ gene products were not necessary for repair of mitochondrial DNA damage whilst the RAD2⁺ gene product was (Moustacchi 1972).

TABLE 4.7 UV-induced mutation frequency in rad3.12 for different classes of mutation giving rise to the non-suppressed phenotype.

MUTATION	MUTANTS /10 ³ SURVIVORS *1	
	rad3.12	RAD3 ⁺
TOTAL	9.27(88)	2.13(41)
psi ⁻	3.84(24)	0.71(7)
pnm	0.96(6)	0.51(5)
asu	1.12(7)	0.41(4)
PNM/ASU	0.32(2)	-
SUQ5 ⁺	1.92(12)	0.51(5)
ade1	0.16(1)	-
ade2x	0.96(6)	-

*1 figure in brackets is the number of mutants classified. The data for RAD3⁺ was taken from TABLE 3.13. All mutants were isolated after a UV dose of 20J/m².

She was however unable to determine the role of the RAD3⁺ and RAD22⁺ gene products because of the high spontaneous petite frequencies of the rad3 and rad22 strains used (Moustacchi 1972).

The UV-induction of petite mutants was looked at in MT141-4/6d (rad1) and MT220/8c (rad3.12) (Table 4.8). The frequency of petite mutants among survivors was higher in both the rad1 and rad3.12 strain compared to the RAD⁺ control over the dose ranges measured (Figure 4.10). However a large number of nuclear genes are required to maintain growth on glycerol and other non-fermentable carbon sources, the so-called 'segregational petites', e.g. pet1 - pet46. The observed increase in frequencies could therefore be due to nuclear rather than cytoplasmic mutations, a point not considered by Moustacchi in her original analysis of rad1 (Moustacchi 1972).

4.3.3 /P̄si7 mutation in mutants blocked in the RAD6 pathway

UV-induced /p̄si7 mutagenesis was measured in psi⁺ strains carrying either the rev3.1, umr1 or rad6 mutations, all of which block the RAD6 error-prone pathway.

(a) rev3

Originally isolated by Lemontt among a series of mutants showing a reduction of UV-induced reversion of the highly UV-revertable ochre allele arg4.17 (Lemontt 1971). The rev3 gene, which lies in the RAD6 pathway, confers a UV-sensitive phenotype and the REV3⁺ gene product appears to be necessary for all kinds of mutagenic event at all sites within the nuclear genome (Lawrence and Christensen 1976).

Two rev3 mutants were used in this study. One, 494/5a, was obtained from Dr B. Cox. Figure 4.11 shows the derivation of the second strain, MT196/3a. The former proved to be quite

TABLE 4.8 Induction by UV of petite mutants in excision-defective strains.

UV DOSE J/m ²	RAD ⁺ *1			rad 3.12		
	COLONIES SCREENED	% ϕ^-	COLONIES SCREENED	% ϕ^-	COLONIES SCREENED	% ϕ^-
0	5540	0.31	3405	0.41		
2.5	-		765	1.04		
5	1724	0.41	1244	1.37		
10	2516	0.68	2002	2.80		
20	3608	1.47	1166	4.72		
30	983	3.97	258	4.26		
40	2458	3.09	175	6.28		
50	1847	6.88	197	13.70		
60	2334	10.84	412	14.56		

UV DOSE J/m ²	RAD ⁺ *1			rad 1		
	COLONIES SCREENED	% ϕ^-	COLONIES SCREENED	% ϕ^-	COLONIES SCREENED	% ϕ^-
0	1189	0.25	850	0.47		
2	1147	0.26	1329	1.65		
4	1178	0.34	901	2.66		
6	1087	0.28	825	2.55		
8	1074	0.37	878	2.39		
10	1082	0.55	77	6.49		

*1 RAD⁺ data from TABLE 3.15

FIGURE 4.10 The induction by UV light of the petite mutation in rad1.1 (MT141-4/6d) and a rad3.12 (MT220/8c) strain compared to RAD⁺ (507-4/2b).

a) (■) 507-4/2b
(▲) MT141-4/6d

b) (■) 507-4/2b
(●) MT220/8c

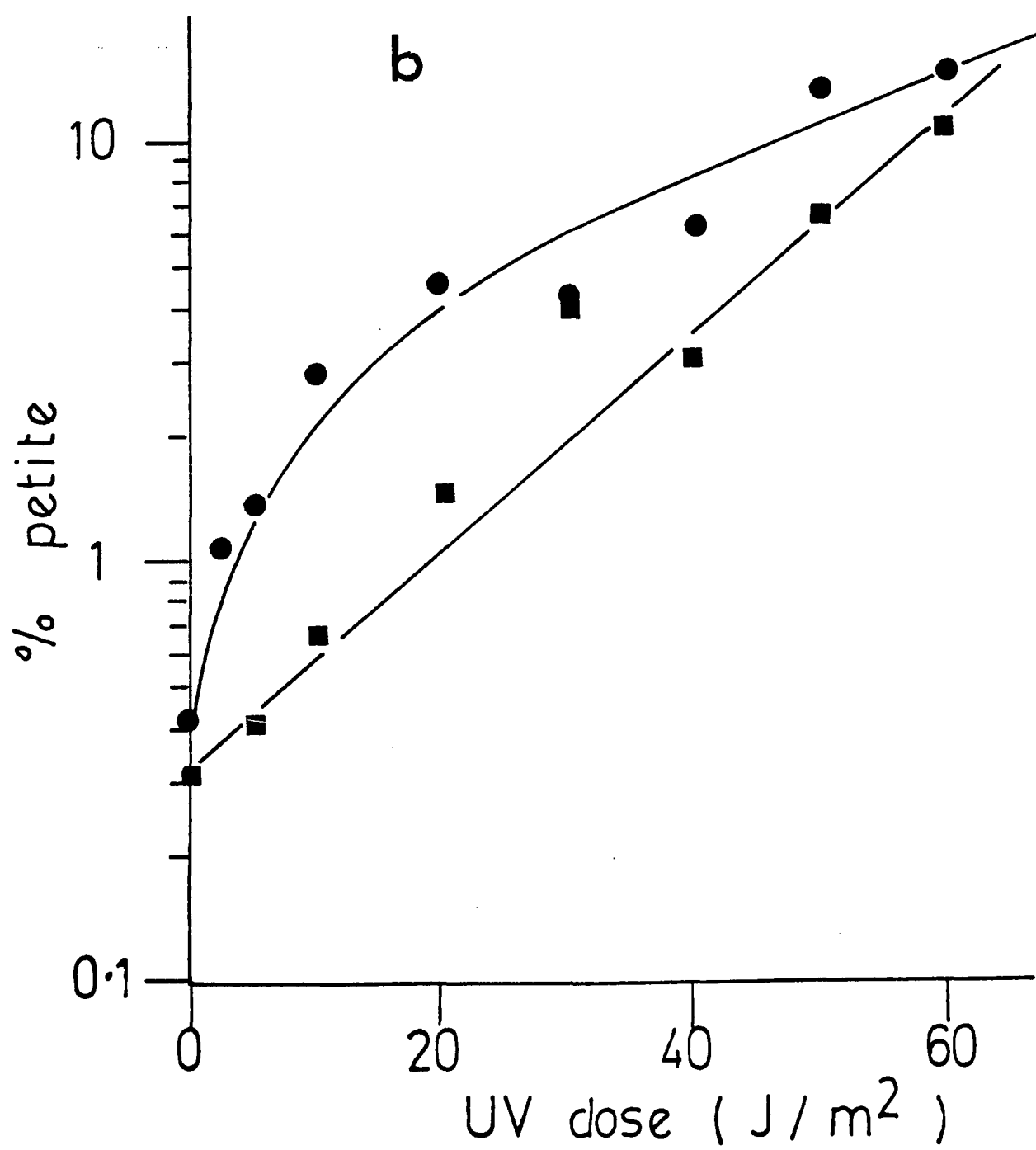
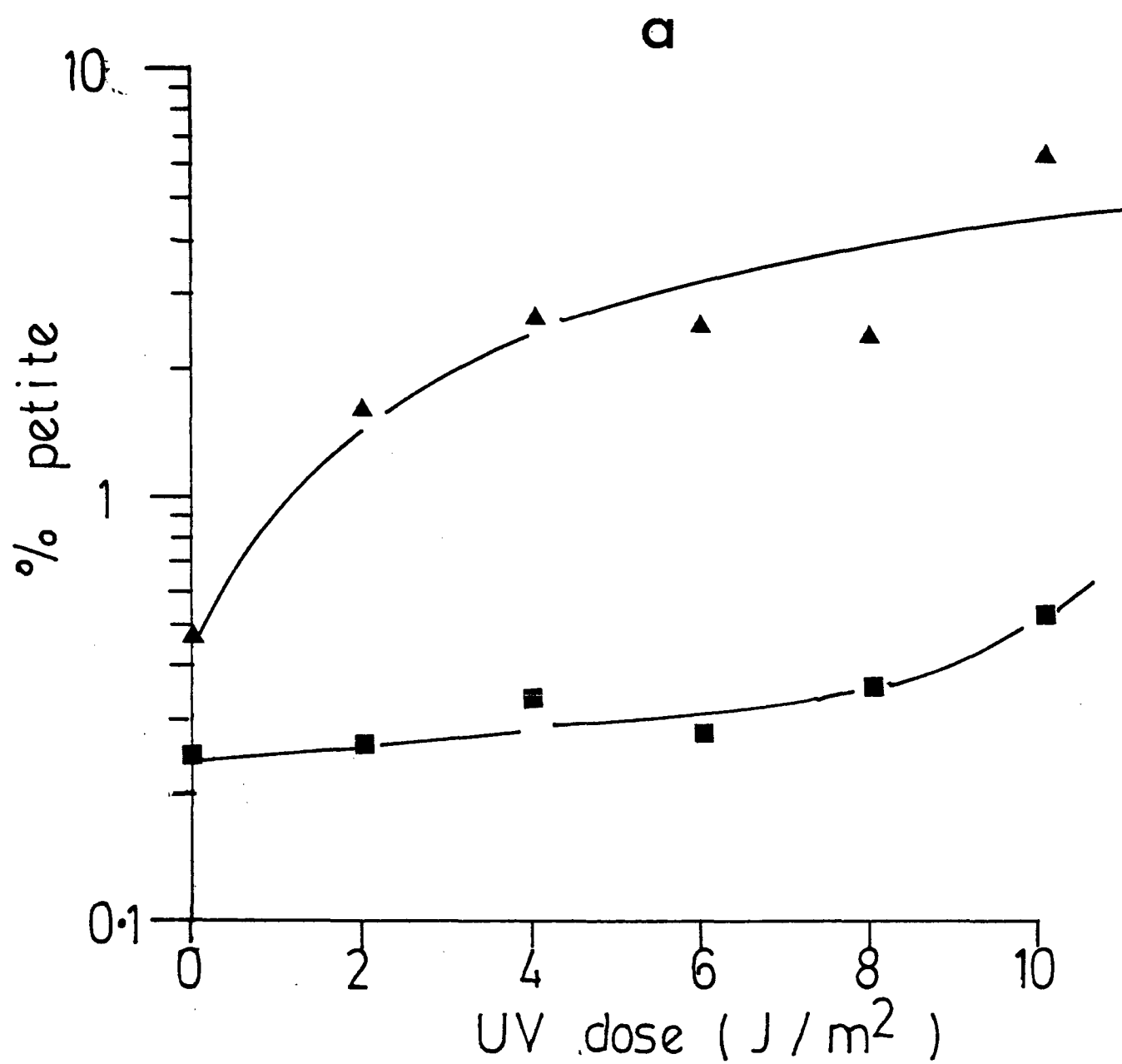
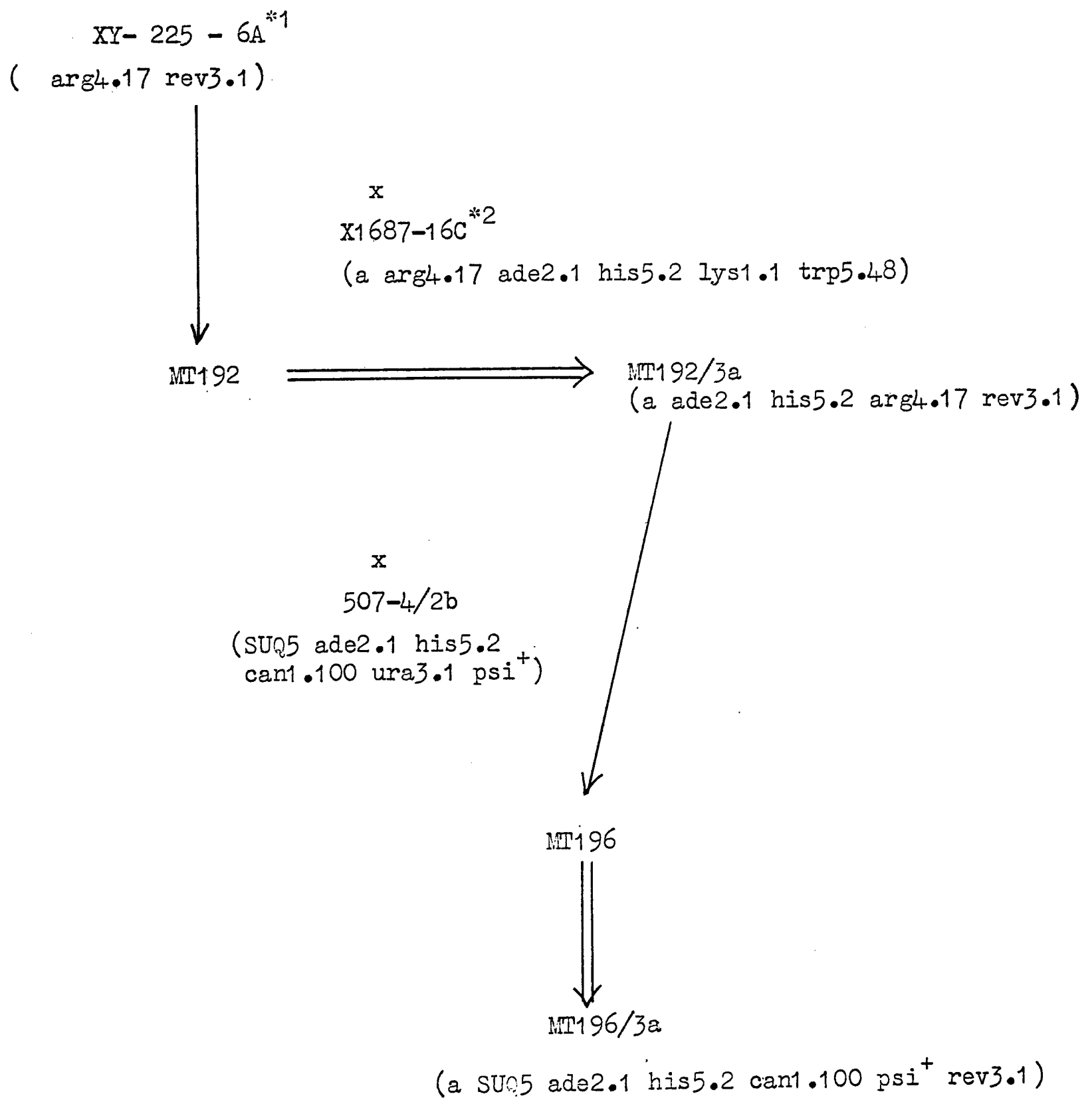


FIGURE 4.11 Construction of strain of genotype SUQ5 ade2.1 his5.2can1.100 psi⁺ rev3.1.

*1 obtained from Dr. J.F. Lemontt.

*2 obtained from Dr. F. Eckardt.

→ single zygote isolation.

⇒ tetrad dissection.

resistant to the lethal effects of UV and consequently the latter strain was constructed and shows the expected UV-sensitive phenotype: 494/5a had an LD_{37} of 29 J / m^2 whilst MT196/3a had an LD_{37} of 3.2 J / m^2 (Figure 4.12). Both strains were in stationary phase when UV-irradiated. UV-induction of non-suppressed mutants was measured in both strains and compared to the results obtained with 507-4/2b (REV3⁺).

UV-induction of non-suppressed mutants in both rev3 strains was drastically reduced over the whole UV dose range used (Table 4.9). At lower doses of UV ($< 25 \text{ J / m}^2$) the mutation rate appeared to be slightly higher than at doses greater than 25 J / m^2 for MT196/3a; the data is summarised in Table 4.10.

A total of 24 non-suppressed mutants of MT196/3a were re-cloned and analysed by complementation;

<u>TOTAL</u>	<u>psi⁻</u>	<u>pnm</u>	<u>asu</u>	<u>PNM/ASU</u>	<u>SUQ5⁺</u>	<u>ade1</u>	<u>ade2.x</u>
24	1	3	7	5	2	0	6

- all were IS⁺ and 10 were petite. Therefore the induction of psi⁻ mutants in rev3 strains is drastically reduced compared to the REV3⁺ strain.

b) umr1

This is one of the 7 non-allelic mutants which show a reduced capacity for UV-induced forward mutation from CAN1⁺ can1 (Lemontt 1973). The umr1 mutant, unlike the rad or rev mutants, is not very UV-sensitive with a dose reduction factor of approximately 1.2 at 10% survival (Lemontt 1977). It shows reduced UV-revertibility of some, but not all, of the ochre

FIGURE 4.12 The UV-sensitivity of rev3.1 strains.

- (▲) 507-4/2b RAD⁺ psi⁺
- (●) BSC494/5a rev3.1 psi⁺
- (■) MT196/3a rev3.1 psi⁺

Each point is the average of two to six independant experiments.

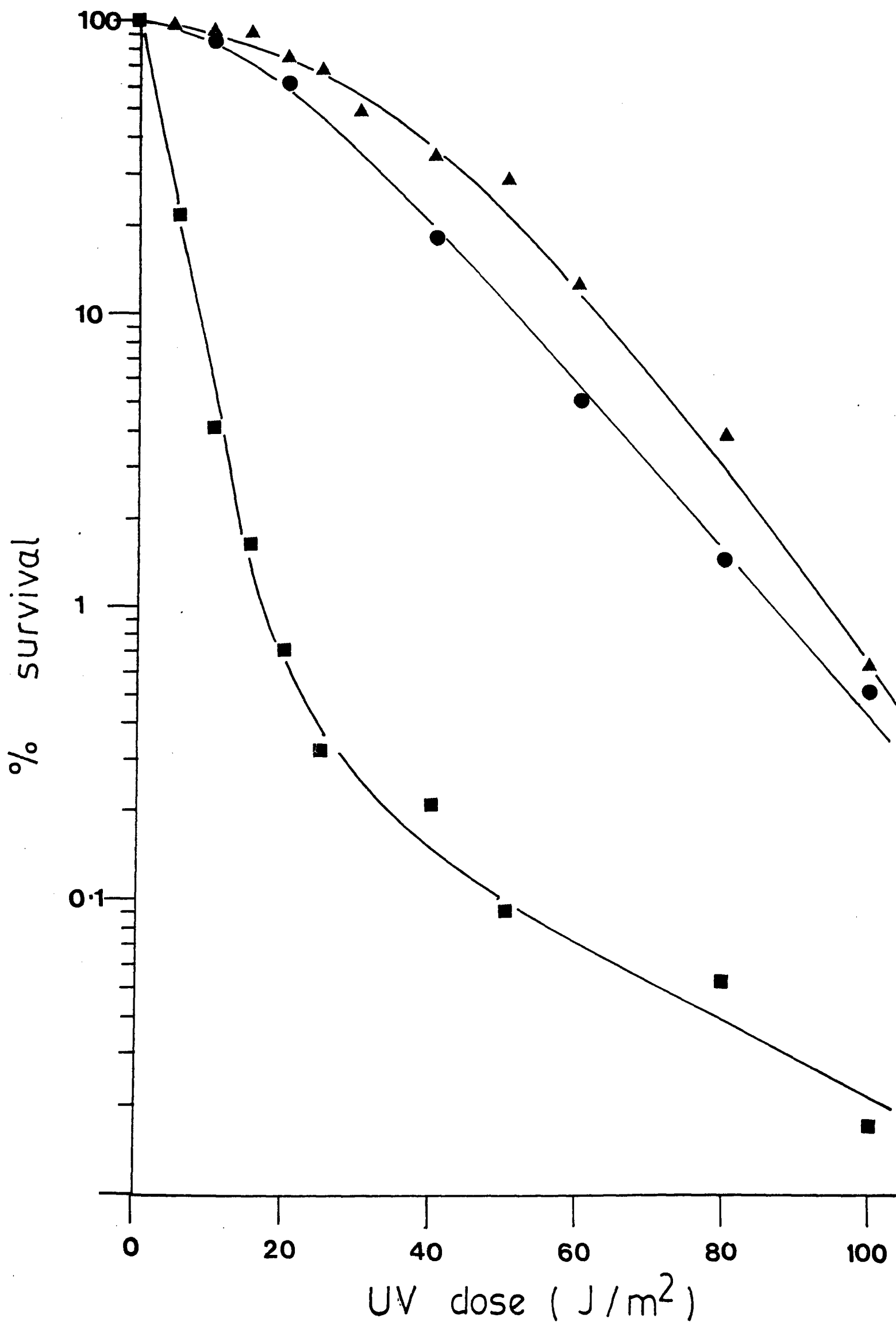


TABLE 4.9 UV-induced mutation in strains carrying the rev3 mutation.

RAD ⁺ *1				BSC494/5a				MT196/3a			
DOSE J/m ²	COLONIES	NO.		TOTAL MUTANTS /10 ³ SURV.	COLONIES	NO.		TOTAL MUTANTS /10 ³ SURV.	COLONIES	NO.	
		NON-SUPPRESSED MUTANTS	WHOLE:SECTORED			NON-SUPPRESSED MUTANTS	WHOLE:SECTORED			NON-SUPPRESSED MUTANTS	WHOLE:SECTORED
0	17 672	2	1	0.17	2 337	0	0	-	12 188	0	1
5	5 434	4	0	0.74			NT		4 006	1	0
10	8 617	7	6	1.51	2 295	0	0	-	3 988	1	0
15	5 023	9	5	2.79			NT		5 086	3	2
20	11 739	14	11	2.13			NT		9 640	7	2
25	4 096	10	3	3.17			NT		3 245	1	0
40	9 214	34	14	5.21	3 258	1	0	0.31	854	2	0
50	8 742	40	15	6.29			NT		5 978	1	2
60	4 306	24	5	6.73	4 507	1	1	0.44			NT
80	10 385	46	28	7.13	3 728	0	2	0.54	8 099	0	0
100	6 188	36	22	9.37	2 961	0	3	1.01	5 510	0	1

NT = not tested.

*1 these are the results given in TABLE 3.13.

TABLE 4.10 Summary of data from TABLE 4.9 for RAD⁺
and MT196/3a.

UV DOSE RANGE (J/m ²)	MUTATION	TOTAL COLONIES	MUTANTS WHOLE:SECTORED		TOTAL MUTANTS /10 ³ SURV.
5 - 25	RAD ⁺	34,909	44	25	1.98
	rev3	25,965	13	4	0.65
40 - 100	RAD ⁺	38,835	180	84	6.80
	rev3	20,441	3	3	0.29

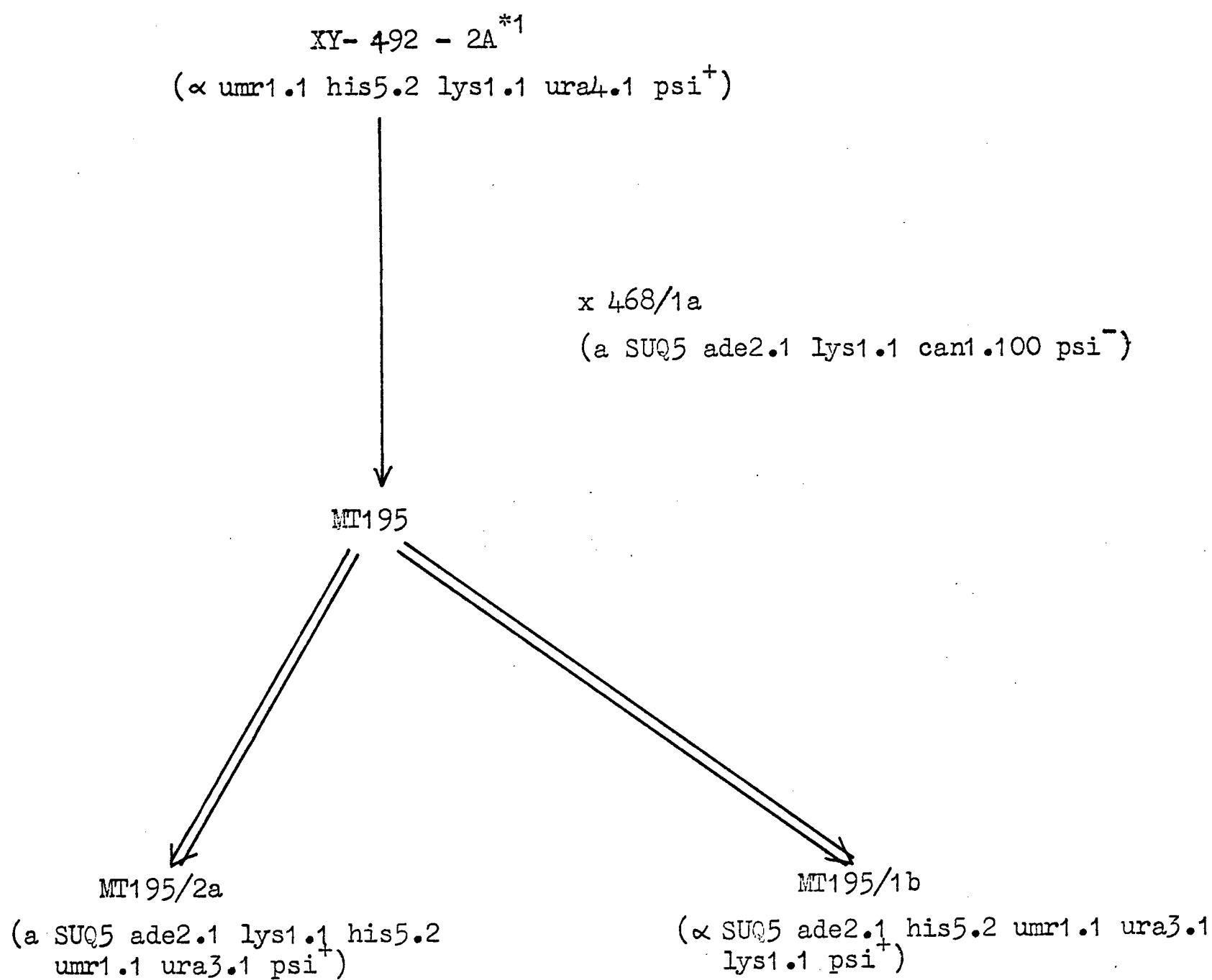
alleles tested (Lemontt 1977). The phenotype of umr1 is consistent with it being in the RAD6 error-prone pathway, or at least a branch of that pathway required for specific mutational events.

/Psi mutagenesis was measured in two psi⁺ strains carrying umr1.1 (MT195/1b and MT195/2a). Figure 4.13 shows how the strains were driven. All strains were in stationary phase when irradiated. MT195/1b had an LD₃₇ of 11 J / m² whilst MT195/2a had an LD₃₇ of 30 J / m² compared to 42 J / m² for the UMR⁺ control (507-4/2b). MT195/2a was more sensitive to UV-light, if irradiated directly on YC plates rather than in saline, at doses above 20 J / m² (Figure 4.14).

The induction by UV of non-suppressed mutants in the two umr1 strains was measured over the dose range 20-100 J / m² and compared with the data previously obtained for stationary phase 507-4/2b (UMR⁺). At each of the doses tested the frequency of non-suppressed mutants was significantly less than the UMR⁺ strain (Table 4.11). A total of 44,706 UMR⁺ colonies were screened after UV-treatment at those 6 doses giving 262 mutants (5.86/10³ survivors) compared to 49 mutants (0.99/10³ survivors) from 49,638 umr1 colonies screened after UV.

A total of 83 non-suppressed umr1 mutants, including 34 isolated by plating irradiated cells to give approximately 2000 colonies per plate, were cloned and classified by complementation analysis (Table 4.12). 21 mutants from MT195/1b, isolated in the first experiment conducted, all failed to mate with any of the tester strains (both a and α). The initial spore clone of MT195/1b was recloned and 4 independent colonies checked microscopically for mating ability; 2 mated with an a ade1

FIGURE 4.13 Derivation of psi⁺ umr1.1 strains used in this study.



*1 obtained from Dr. J.F. Lemontt.

FIGURE 4.14 The UV-sensitivity of umr1.1 strains .

- (Δ) 507-4/2b UMR⁺
- ($\bullet$) MT195/1b umr1.1
- ($\circ$) MT195/2a umr1.1 - irradiated in saline
- ($\blacksquare$) MT195/2a umr1.1 - irradiated on YC plates

The curve for MT195/1b is the average from five independent experiments.

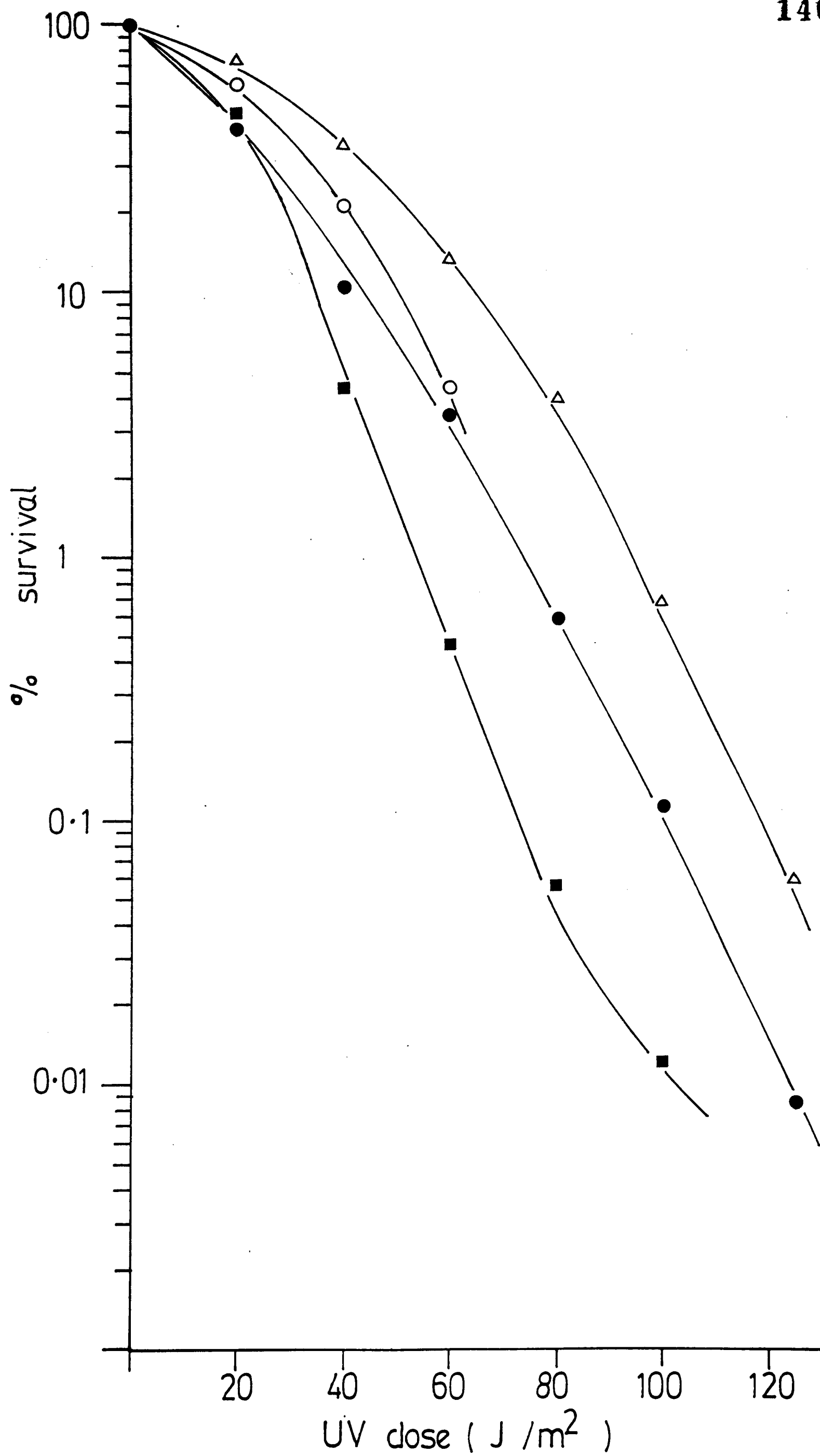


FIGURE 4.11 UV-induction at non-suppressed mutants in strains carrying umr1.

DOSE (J/m ²)	RAD ⁺ (507-4/2b) *1				umr1:1(MT195/1b) *2				umr1.1 (MT195/2a)		
	COLONIES	NO. MUTANTS WHOLE:SECTORED	MUTANTS /10 ³ SURV.		COLONIES	NO. MUTANTS WHOLE:SECTORED	MUTANTS /10 ³ SURV.		COLONIES	NO. MUTANTS WHOLE:SECTORED	MUTANTS /10 ³ SURV.
0	17 672	2	1	0.17	4 171	0	0	-	2 374	0	0
20	11 739	14	11	2.13	6 845	1	5	0.88	1 069	0	0
40	9 214	34	14	5.21	4 206	2	2	0.93	2 610	1	0
60	4 306	24	5	6.73	4 063	4	0	0.98	4 463	2	1
80	10 385	46	28	7.13	7 736	4	2	0.78			NT
100	6 188	36	22	9.37	18 021	13	12	1.39			NT
125	2 874	19	9	9.74	535	0	0	-			NT

NT = not tested .

*1 data from TABLE 3.13.

*2 results given are the sums from 5 independant experiments.

TABLE 4.12 Classes of mutation giving rise to the non-suppressed phenotypes in umr 1 strains .

STRAIN	EXPERIMENT AND UV DOSE(J/m ²)	NUMBER OF MUTANTS	rho ⁻	ts	CLASSES OF MUTATION							SUQ5 ⁺	PNM/ASU	(NON-MATTER)
					ade1	ade2x	psi ⁻	pnm	asu					
MT195/1b (umr1)	EXPT 1 20-100	21	18	0	0	0	0	0	0	0	0	0	0	21
	40	3	3	0	0	0	2	0	0	0	1	1	0	0
	EXPTS 80	23	23	0	0	0	3	4	3	10	3	3	0	0
	2-5													
	100	21	21	1	0	0	3	4	2	9	3	3	0	0
MT195/2a (umr1.a)	40	5	1	0	3	0	0	0	0	1	1	1	0	0
	EXPT 1 80	6	1	0	3	0	0	0	0	2	1	1	0	0
	100	4	0	0	1	0	0	0	0	3	0	0	0	0
	TOTAL umr 1.	83	67	1	7	0	8	8	5	25	9	21	21	
507-4/2b (UMR ⁺)														
	TOTAL UMR ⁺⁺ 1	345	NT	NT	16	18	81	42	106	31	50	1	1	

NT = not tested.

tester strain whilst the other two failed to mate with either an α or an a ade1 tester strain. In subsequent experiments only the α MT195/1b strain was used. It is interesting to note that α umr7 haploids fail to produce α hormone and are unable to mate with a haploids whilst a umr7 haploids show normal mating (Lemontt 1977). Both non-suppressed mutants and independent clones of MT195/2a (a umr1) showed normal mating with α strains. It is therefore possible that umr1 strains have a somewhat 'leaky' umr7 phenotype with respect to mating although Lemontt made no such observation (Lemontt 1977).

Only 8 of the 62 classifiable non-suppressed umr1 mutants were psi⁻. Therefore UV-induced [psi] mutation was reduced in umr1 strains. At 80 J / m² the frequency of psi⁻ mutants / 10³ survivors was 0.10 (1.02 in UMR⁺) and at 100 J / m² the frequency was 0.20 (1.78 in UMR⁺).

(c) rad6

The rad6 mutant (rad6.1) originally isolated by Cox and Parry (1968) is both highly UV- and X-ray-sensitive and blocked in mutation induced by both chemical and physical agents (Prakash 1974; Lawrence and Christensen 1976). The RAD6⁺ gene appears to control an early step of an error-prone pathway and its gene product appears to be necessary for all kinds of mutagenic events at all sites within the nuclear genome so far tested (Lawrence and Christensen 1976). However rad6.1 mutants do show an enhanced level of spontaneous mutation (Hastings et al., 1976). It has recently been shown that rad6.1 mutants are proficient in dimer excision (Ferguson 1975; Prakash 1977).

(i) rad6.1

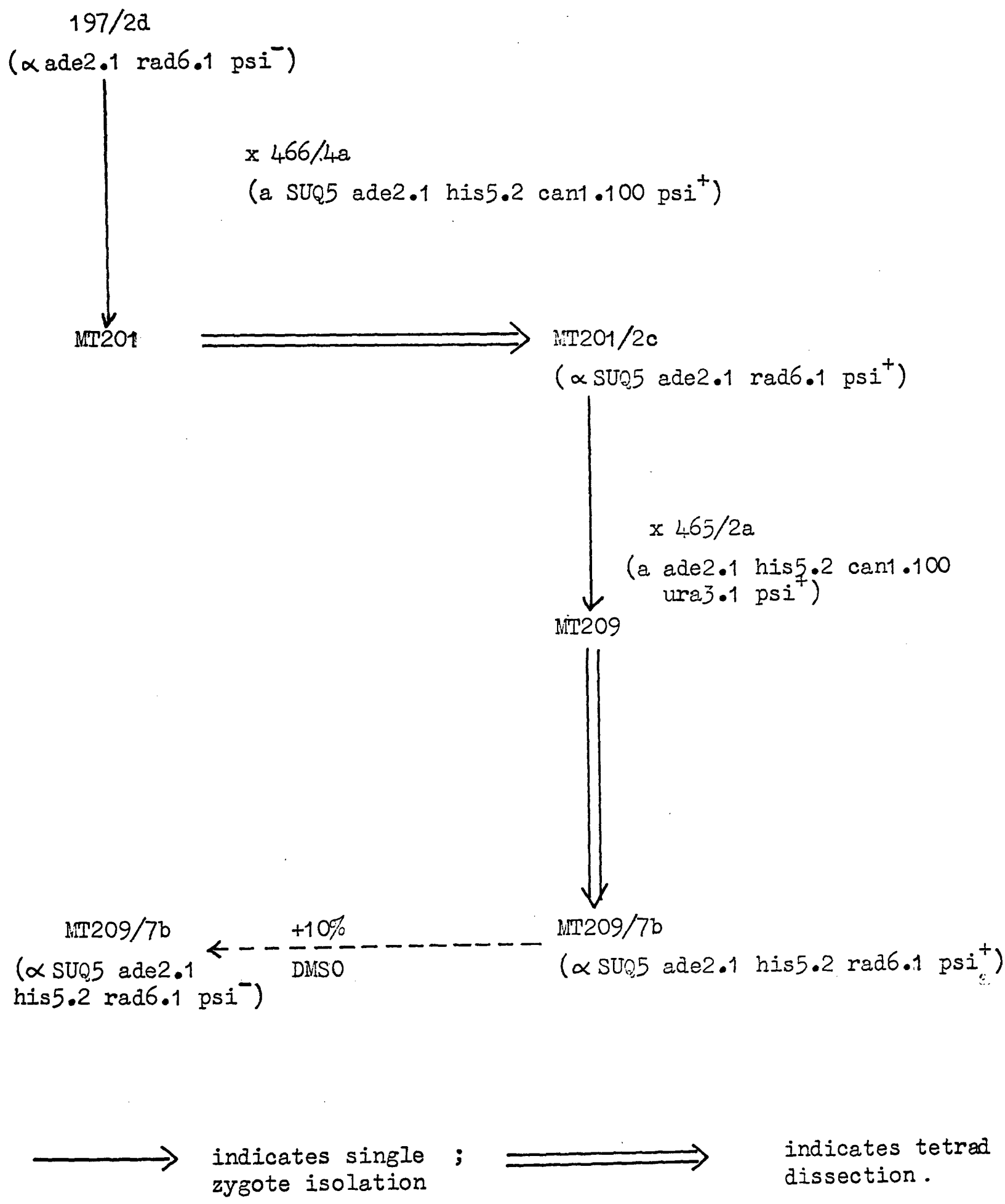
A strain with the required SUQ5 ade2.1 his5.2 rad6.1 psi⁺ genotype was constructed directly from the rad6.1 mutant (197/2d) isolated by Cox and Parry (1968) (Figure 4.15). 12 tetrads dissected from the first diploid isolated (MT201) showed regular segregation patterns, all being 2:2 for ade⁻:ADE⁺. One of the ADE⁺ rad6.1 MT201 segregants (MT201/2c) was crossed with 465/2a to give the diploid MT209 (Figure 4.15). 10 tetrads dissected from MT209 all showed 2ade⁻:2ADE⁺ spores and one of the suppressed rad6.1 segregants, MT209/7b (SUQ5 ade2.1 his5.2 psi⁺) was used to study /psi mutagenesis.

In looking at the segregation of rad6.1, in crosses heterozygous for SUQ5, it was noted that non-suppressed rad6.1 spores were much more UV-sensitive than suppressed rad6.1 spores and doses up to 75 J / m² had to be used to clearly identify rad6.1 segregation in such crosses. This indicated that rad6.1 may be a suppressible ochre mutation, although rad6.1 has previously been reported as an amber mutation (Lawrence et al., 1974).

Consequently survival curves were obtained for various suppressed and non-suppressed rad6.1 haploid strains. Both survival, after irradiating cells directly on plates (Figure 4.16a), or irradiating in saline and then plating (Figure 4.16b), was determined. All cells were in stationary phase at the time of UV-irradiation. The psi⁻ derivative of MT209/7b was obtained by treating MT209/7b psi⁺ with 10% Dimethyl sulphoxide (see Chapter 6) and was confirmed as being psi⁻ by complementation analysis.

MT209/7b psi⁺ was found to be far more resistant to the lethal effects of UV than the parent rad6.1 psi⁻ strain 197/2d but still significantly more sensitive than a RAD⁺ psi⁺ strain (e.g. 466/4a, 507-4/2b). A second strain, with the genotype

FIGURE 4.15 Derivation of rad6.1 mutants used in this study.

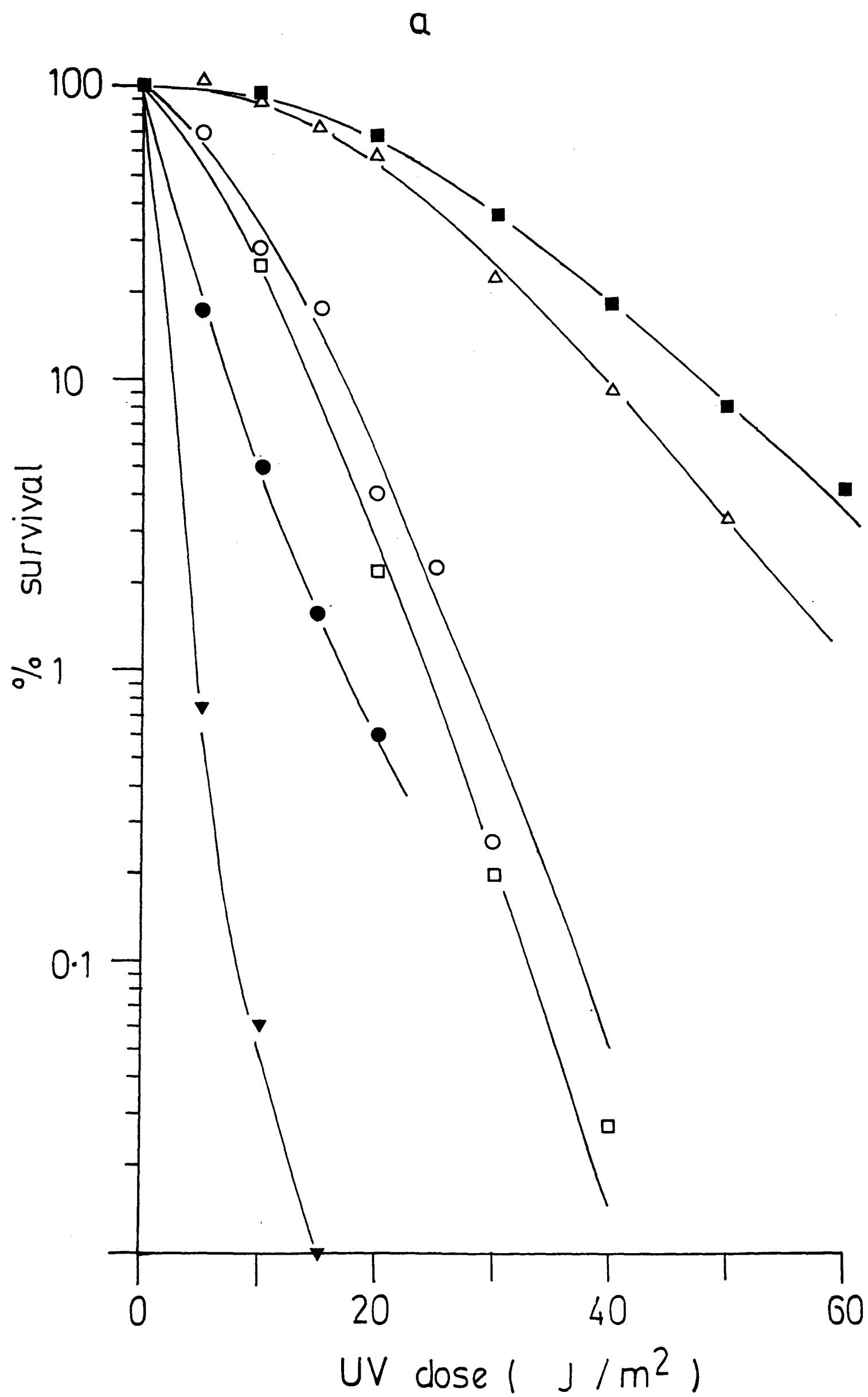


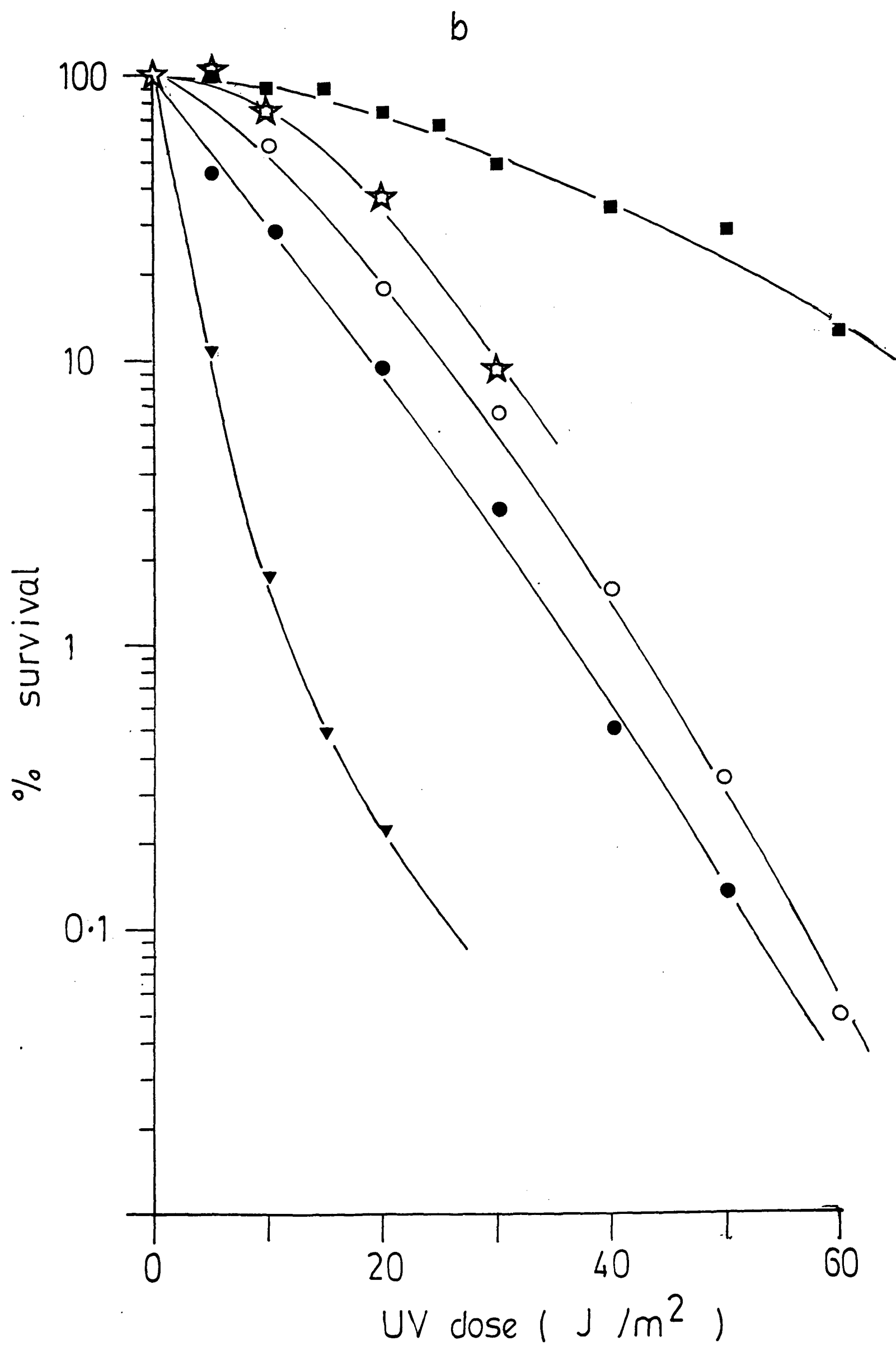
NOTE: Only the genotypes determined are given and is not necessarily the full genotype.

FIGURE 4.16 The UV-sensitivity of suppressed and non-suppressed rad6.1 mutants.

- a) cells irradiated on YC plates.
- b) cells irradiated in saline prior to plating onto YC plates.

(■)	507-4/2b	SUQ5 ade2.1 RAD6 ⁺ psi ⁺
(△)	466/4a	SUQ5 ade2.1 RAD6 ⁺ psi ⁺
(○)	MT209/7b	SUQ5 ade2.1 rad6.1 psi ⁺
(●)	MT209/7b	SUQ5 ade2.1 rad6.1 psi ⁻
(□)	MT201/2c	SUQ5 ade2.1 rad6.1 psi ⁺
(▼)	197/2d	SUQ5 ⁺ ade2.1 rad6.1 psi ⁻
(☆)	MT209/7b	SUQ5 ade2.1 rad6.1 psi ⁻ salx





SUQ5 ade2.1 rad6.1 psi⁺ (MT201/2c) showed similar UV survival kinetics to MT209/7b psi⁺. MT209/7b psi⁻ was more UV-sensitive than MT209/7b psi⁺ and appeared to have no resistant shoulder to its survival curve as was also found for 197/2d. All other strains tested had a resistant shoulder at low UV doses. A strain of MT209/7b psi⁻ carrying an undefined recessive allosuppressor (sal x) was more resistant to UV than MT209/7b psi⁻ SAL⁺ or MT209/7b psi⁺ (Figure 4.16b).

It is apparent from these results that the rad6.1 UV-sensitive phenotype is partly suppressed by SUQ5, this suppression being more efficient in a psi⁺ background. UV-induced mutation in MT209/7b psi⁺ was measured to see if the second major rad6.1 phenotype (i.e. lack of UV-induced mutation) was also suppressed and, if not, whether the UV-induced frequency of psi⁻ mutants was reduced compared to the RAD⁺ (507-4/2b) control.

Table 4.13 gives the data obtained with MT209/7b psi⁺ after irradiating stationary phase cells directly on the YC plates or after irradiating in saline prior to plating onto YC. Mutant frequency increased with dose and, in both cases, the induction kinetics were linear ($n = 1$) (Figure 4.17). At comparative UV doses the frequency of non-suppressed mutants was slightly higher in the rad6.1 strain compared to the RAD⁺ control. The frequency of mutants per unit UV dose was higher in rad6.1 cells irradiated directly on plates. This treatment however resulted in lower survival values compared to irradiation in saline and the frequency of mutants plotted as a function of % survival (not shown) was approximately the same

TABLE 4.13 UV-induction of non-suppressed mutants in MT209/7b psi⁺ after irradiation on YC plates or in saline prior to plating on YC plates.

UV DOSE J/m ²	IRRADIATED ON PLATES			IRRADIATED IN SALINE		
	COLONIES ^{*1}	NO. MUTANTS (WHOLE:SECTORED)	MUTANT FREQUENCY /10 ³ SURVIVOR	COLONIES ^{*1}	NO. MUTANTS (WHOLE:SECTORED)	MUTANT FREQUENCY /10 ³ SURVIVORS
0	12,488(100)	1:0	0.08	3,906(100)	1:0	0.26
5	2,395(71.8)	4:1	2.09		NT	
10	2,397(28.1)	12:2	5.88	6,520(57.5)	9:1	1.53
20	5,004(4.1)	27:4	6.20	7,532(17.8)	15:4	2.52
30	4,179(0.25)	42:9	12.20	5,573(6.6)	31:4	6.28
40	2,125(0.03)	31:8	18.35	6,041(1.6)	38:5	7.11
50		NT		6,221(0.3)	55:10	10.45
60		NT		7,625(0.05)	71:11	10.74

^{*1} % survival in brackets.

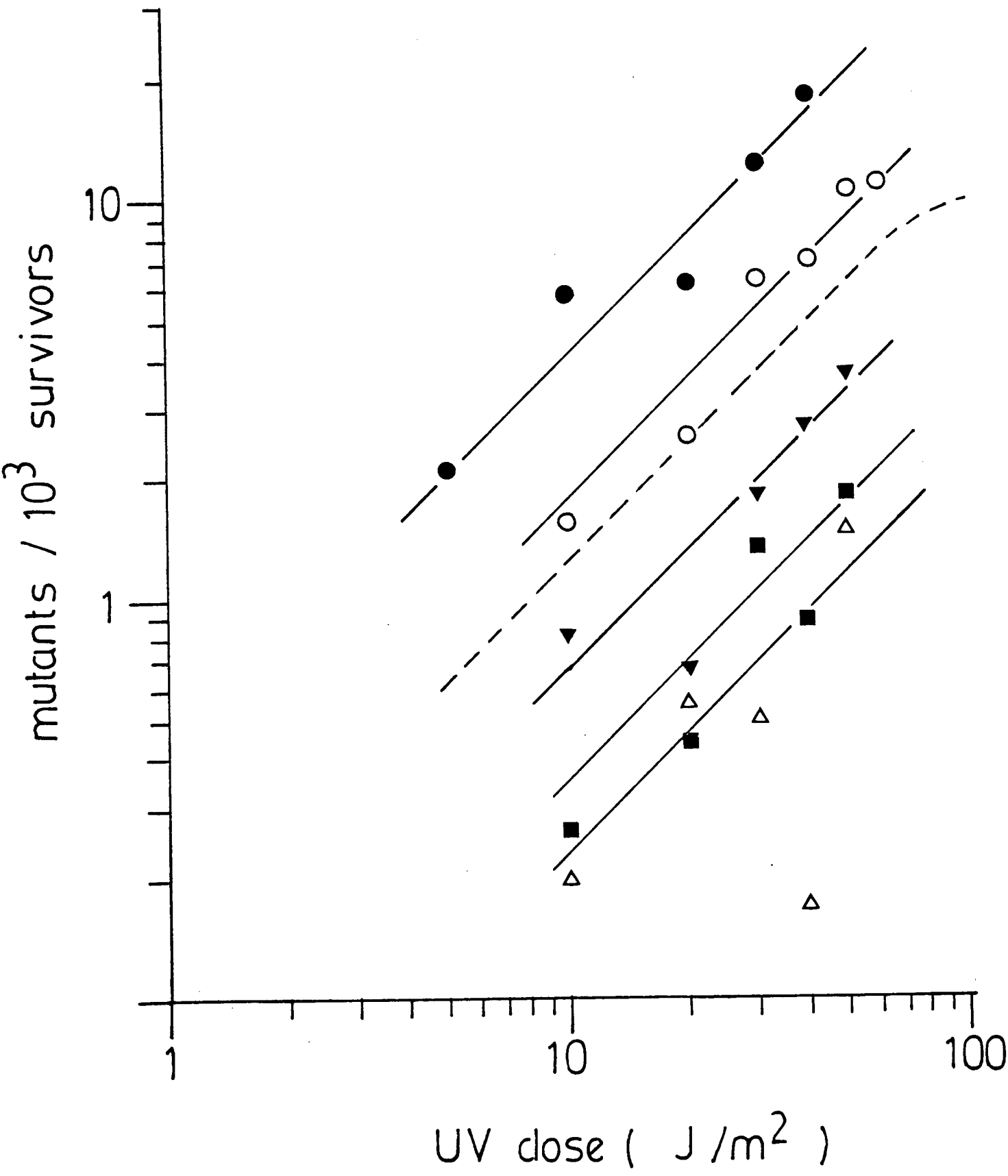
NT - not tested.

NOTE Above figures are the totals from at least 2 independant experiments. % survival is the average of at least 2 independant experiments.

FIGURE 4.17 The frequency of non-suppressed mutants as a function of UV dose of MT209/7b.

- (●) total non-suppressed mutants ; irradiated directly on YC plates
- (○) total non-suppressed mutants ; irradiated in saline prior to plating on YC plates
- (Δ) psi^-
- (▼) SUQ5^+
- (■) PNM/ASU

----- represents data for the RAD^+ strain
507-4/2b ; total non-suppressed , stationary phase cells.



for both treatments at survival levels $> 10\%$. At survival levels $< 10\%$ cells irradiated in saline gave a much lower mutation frequency.

Both whole and sectored mutant colonies were found with the ratio of whole:sector being approximately 5-6:1 in both conditions at all doses used.

Thus the blocked UV-induced mutation phenotype of rad6.1 was also suppressed by SUQ5 and in fact showed a slight enhancement of UV-mutagenesis compared to the RAD⁺ strain. A total of 166 non-suppressed mutants of MT209/7b psi⁺ (134 whole and 34 sectored), following 5 different UV doses, were cloned and classified by complementation analysis (Table 4.14). All classes of mutant were found although, unlike the RAD⁺ control, second-site mutations in the SUQ5 gene (SUQ5⁺) were the major class of mutant present. The induction kinetics for the psi⁻ mutation were linear (Figure 4.17). All mutant classes, including psi⁻, were found as both whole and sectored mutant colonies.

(ii) rad6.3

To determine whether the effect of ochre suppression on the rad6 phenotypes was allele specific or locus specific, a mutant strain carrying a different allele of rad6 (rad6.3) was constructed by crossing JF9-4D (a rad6.3 lys1.1 and/or lys2.1 his1.1 arg4.17 trp2 - obtained from Dr C. Lawrence, University of Rochester, New York) with 507-4/2b (α SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺). 4 complete tetrads, from the resulting diploid (MT221), were isolated and rad6.3 was found to segregate 2:2 in all four. Because the cross was heterozygous for ade2.1 the ADE⁺ haploid segregants with the required genotype were determined by complementation analysis with an SUQ5 ade2.1 sal3.5 PNM1 psi⁻ strain (Materials and Methods). MT221/1b (SUQ5 ade2.1 his1.1

TABLE 4.14 UV-induced frequencies of mutations giving rise to the non-suppressed phenotype in MT209/7b irradiated in saline.

UV DOSE (J/m ²)	NUMBER ANALYSED	NUMBER $\overline{p}$ ts	TOTAL MUTANTS FREQUENCY /10 ³ SURVIVORS	FREQUENCIES OF MUTANT CLASSES /10 ³ SURVIVORS*1							
				ade1	ade2.x	psi ⁻	pnm	asu	PNM/ASU	SUQ5	NM
10	23	0 0	1.53	0	0.07(1)	0.20(3)	0.07(1)	0.07(1)	0.26(4)	0.80(12)	0.07(1)
20	23	0 0	2.52	0	0.22(2)	0.55(5)	0.11(1)	0.55(5)	0.44(4)	0.66(6)	0
30	51	5 0	6.28	0.37(3)	0.25(2)	0.49(4)	0	1.97(16)	1.35(11)	1.85(15)	0
40	41	8 0	7.11	0.35(2)	0.52(3)	0.17(1)	0	2.43(14)	0.86(5)	2.77(16)	0
50	28	5 1	10.45	0.37(1)	0.37(1)	1.49(4)	0.37(1)	2.24(6)	1.87(5)	3.73(10)	0
TOTAL NUMBERS	166	18 1	-	6	9	17	3	42	29	59	1

*1 number of mutants obtained in brackets.

ura3.1 rad6.3 /his5.2 can1.100 arg4.17?7 psi⁺) complemented this strain to give red diploids and was used in the following preliminary experiments on UV-sensitivity and UV-induced mutation.

i. UV-sensitivity

Figure 4.18 gives the survival curves of strains used in this study. All were irradiated in stationary phase. Again the rad6.3 UV-sensitive phenotype appeared to be suppressed, MT221/1b being significantly more resistant to UV than the parent JF9-4D (LD_{37} of 28.5 and 2 J / m² respectively).

ii. UV-induced mutation

Three independent experiments were conducted on stationary phase cells of MT221/1b. The results are summarised in Table 4.15. They clearly demonstrated that the blockage of UV-induced mutation remained in the suppressed rad6.3 strain. Only 18 non-suppressed mutants were found among 27,775 treated cells screened ($0.65 / 10^3$ screened), a figure slightly lower than obtained for the umr1 strains ($0.99 / 10^3$), but slightly higher than measured for rev3 ($0.49 / 10^3$).

6 of the non-suppressed mutants were picked, recloned on YC and analysed. 2 were asu, 2 were ade1, one was an SUQ5⁺ mutant and the last one a PNM/ASU mutant.

Therefore UV-induction of the psi⁺ to psi⁻ mutation is blocked in strains carrying the rad6.3 mutation when compared to the RAD⁺ control (Table 4.15) or a suppressed rad6.1 strain (Table 4.13). The UV-sensitivity and the UV-immutability phenotypes of the rad6 gene appear to be separable functions on the basis of this data.

FIGURE 4.18 The UV-sensitivity of suppressed and non-suppressed rad6.3 mutants.

- (Δ) 507-4/2b SUQ5 ade2.1 RAD6⁺ psi⁺, irradiated in saline
- ($\bullet$) JF9-4D SUQ5⁺ ADE⁺ rad6.3 psi[?], irradiated in saline
- ($\square$) JF9-4D irradiated on YC plates
- ($\blacksquare$) MT221/1b SUQ5 ade2.1 rad6.3 psi⁺, irradiated in saline
- ($\circ$) MT221/1b irradiated on YC plates

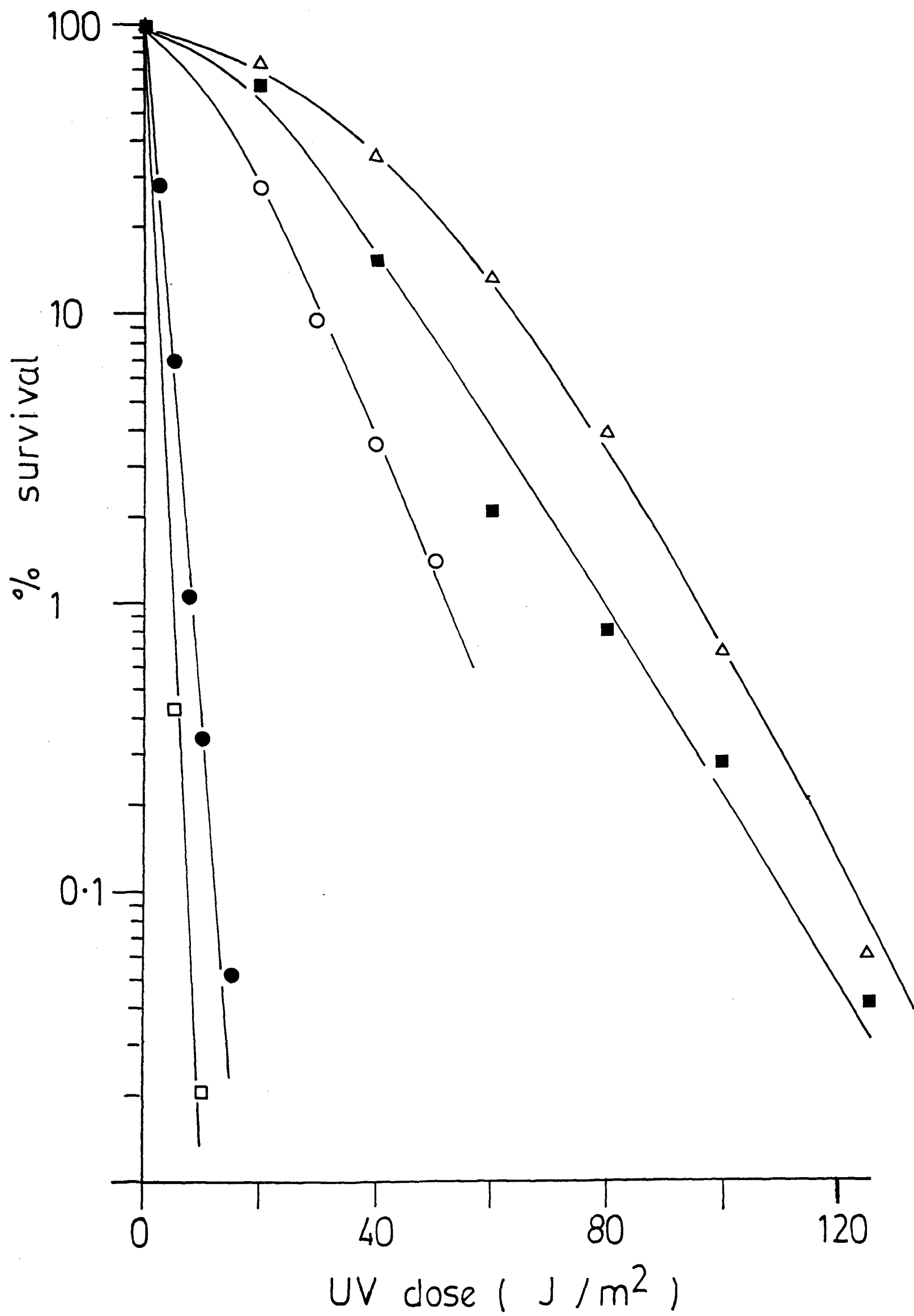


TABLE 4.15 Induction by UV-light of non-suppressed mutants in RAD^+ (507-4/2b) and a $\text{rad } 6.3$ (MT221/1b) mutant.

UV DOSE J/m^2	RAD^+ (507-4/2b) *2			$\text{rad } 6.3$ (MT221/1b)		
	COLONIES*1 SCORED	NO. MUTANTS WHOLE:SECTORED	MUTANT COLONIES FREQUENCY/ 10^3 SURV.	COLONIES*1 SCORED	NO. MUTANTS WHOLE:SECTORED	MUTANT COLONIES FREQUENCY/ 10^3 SURV.
0	17,642(100)	2 1	0.17	3,544(100)	0 0	-
10	8,617(92.8)	7 6	1.51	631(95.4)	0 0	-
20	11,739(74.5)	14 11	2.13	5,922(63.5)	0 0	-
40	9,214(36.4)	34 14	5.21	1,612(16.2)	0 0	-
60	4,306(13.2)	24 5	6.73	1,131(2.0)	1 0	0.88
80	10,385(4.0)	46 28	7.13	7,484(0.8)	3 4	0.94
100	6,188(0.7)	36 22	9.37	10.683(0.3)	4 6	0.94
125	2,874(0.06)	19 9	9.74	312(0.04)	0 0	-

*1 % survival in brackets.

*2 data from TABLE 3.11.

(d) UV-induction of the cytoplasmic petite mutation in strains defective in error-prone repair

In Moustacchi's original study (Moustacchi 1972) the UV-induction of petites was measured in 4 rad mutants now known to be defective in error-prone repair. She concluded that the RAD8⁺ and RAD9⁺ gene products were not involved in the repair of UV-induced mitochondrial genetic damage whilst the gene products of the RAD6⁺ and RAD18⁺ genes were involved in at least the immediate expression of such damage.

In this study UV-induction of petite mutants was measured in strains carrying either the umr1.1, rev3.1, rad6.1 or rad6.3 mutation (Table 4.16).

- i. rev3; although blocked in UV-induced nuclear and /psi⁻7 mutation the strain studied, MT196/3a, was found to be hypersensitive to petite induction by UV-light (Figure 4.19). The kinetics of induction were linear on a log-log plot (Figure 4.20) with $n = 2$ compared to the biphasic induction kinetics seen for RAD⁺ strain where $n \div 1$ up to $30 \text{ J} / \text{m}^2$ and $n = 2$ doses greater than $30 \text{ J} / \text{m}^2$. The possibility that MT196/3a carried a nuclear mutation, unlinked to rev3, which conferred this phenotype cannot be ruled out. Such a nuclear mutation, designated uvs rho5, has recently been reported (Moustacchi et al., 1976).
- ii. umr1; MT195/1b, which was shown to be blocked in UV-induced nuclear and /psi⁻7 mutation, also appeared to be specifically UV-sensitive in petite induction (Figure 4.19). However it had a very high spontaneous petite mutation frequency (25.5%) unlike the rev3 mutant. Preliminary studies with a

TABLE 4.16 UV-induction of the cytoplasmic petite mutation in strains defective in error prone repair.

RAD ⁺ (507-4/2b)*		umr 1 195/1b			umr 1 195/2a			rev3 196/3a			rad 6.1 209/7b psi ⁺			rad6.1 209/7b psi ⁻			rad6.3 JF9-4D		
(J/m ²)	COLONIES %ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻	COLONIES	%ϕ ⁻	%ϕ ⁻
0	5540	0.3	1336	25.5	1315	3.0	3177	2.1	1519	4.1	534	8.0	397	29.7					
5	1724	0.4	-	-	-	-	1266	3.8	-	-	-	-	113	23.9					
10	2516	0.7	-	-	-	-	642	12.8	930	4.2	181	9.9	246	35.3					
15	1566	1.4	-	-	-	-	823	32.0	-	-	-	-	344	44.5					
20	3608	1.5	540	70.6	427	4.2	1577	46.7	1135	5.0	263	8.7	-						
25	533	5.2	-	-	-	-	711	75.5	-	-	-	-	-						
30	983	4.0	-	-	-	-	-	-	284	7.0	240	10.4	-						
40	2458	3.1	1030	88.6	1000	7.4	-	-	987	10.0	201	19.9	-						
50	1847	6.9	-	-	-	-	571	92.8	749	15.8	113	24.8	-						
60	2334	10.8	858	93.1	-	-	-	-	792	19.7	-	-	-						
80	2039	17.4	823	92.0	-	-	-	-	27	33.3	-	-	-						
100	2926	27.6	2181	97.5	-	-	-	-	-	-	-	-	-						
125	301	36.2	208	99.5	-	-	-	-	-	-	-	-	-						

* data from TABLE 3.15.

FIGURE 4.19 The induction of the petite mutation by UV in strains defective in error-prone repair.

(□)	507-4/2b	RAD ⁺	psi ⁺
(○)	MT196/3a	rev3.1	psi ⁺
(△)	MT195/1b	umr1.1	psi ⁺
(●)	MT209/7b	rad6.1	psi ⁺
(▼)	MT209/7b	rad6.1	psi ⁻

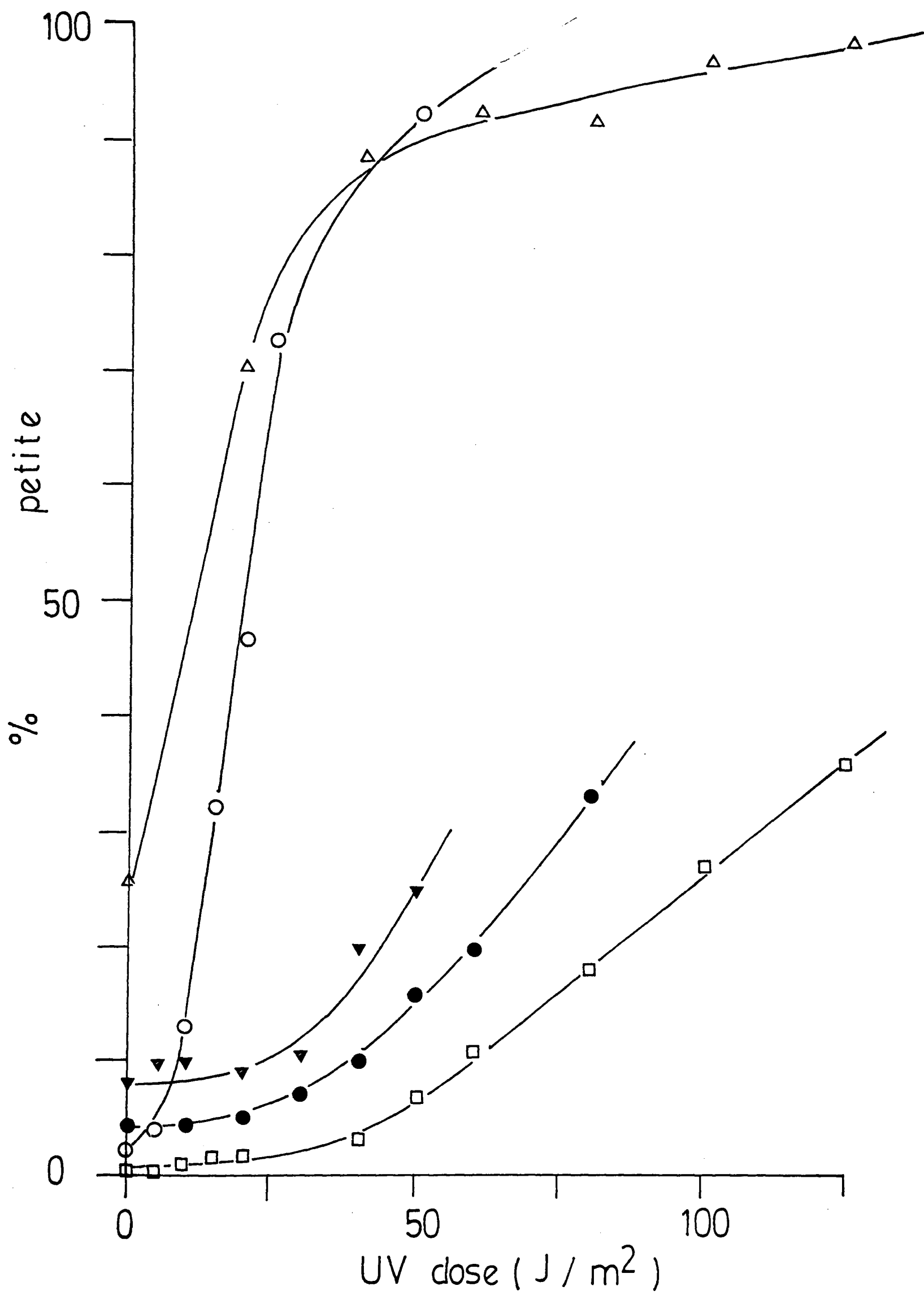
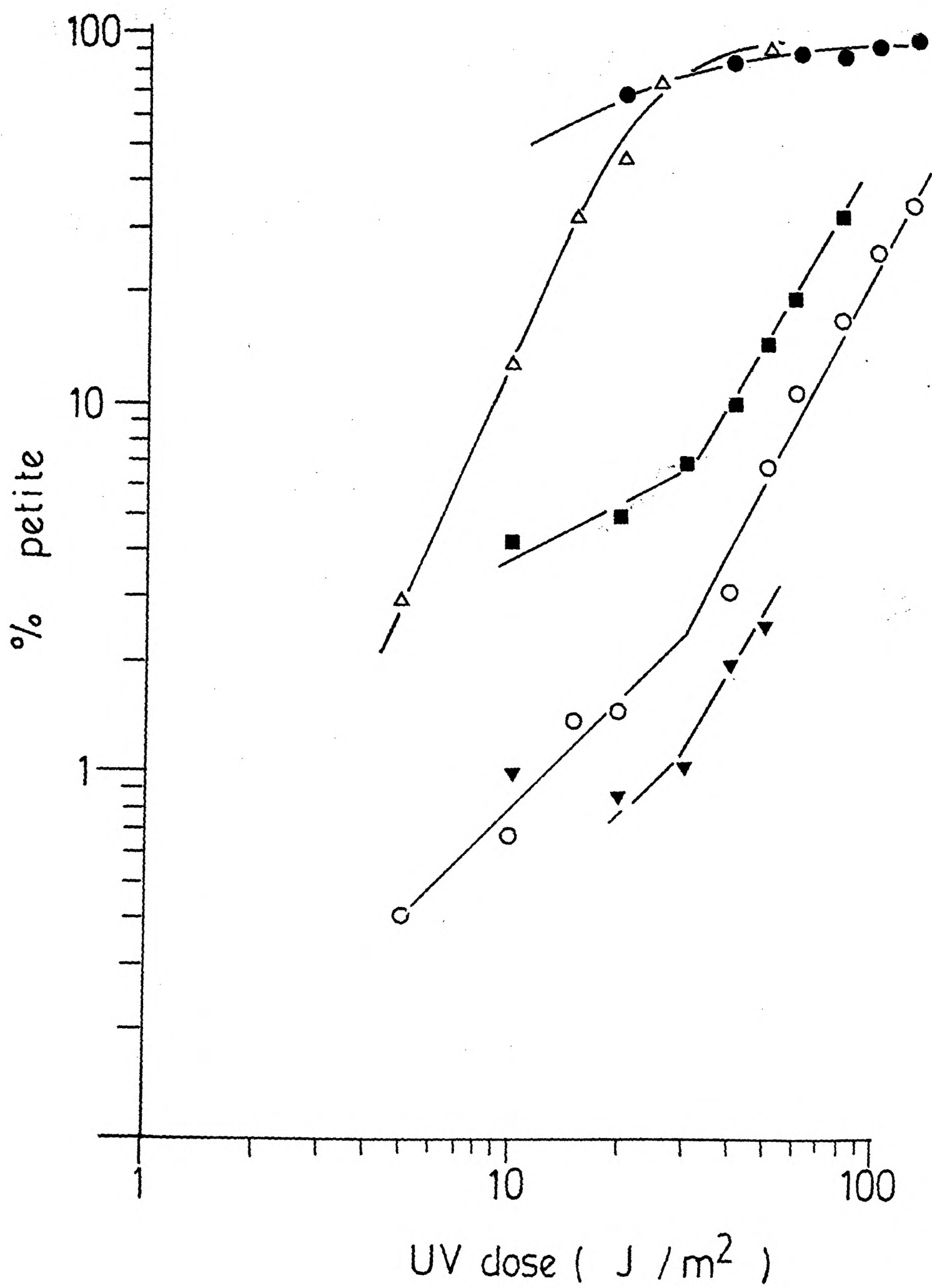


FIGURE 4.20 The frequency of the UV-induced cytoplasmic petite mutation plotted as a function of the UV dose ; strains defective in error-prone repair.

(○)	507-4/2b	RAD ⁺	
(△)	MT196/3a	rev3.1	psi ⁺
(●)	MT195/1b	umr1.1	psi ⁺
(■)	MT209/7b	rad6.1	psi ⁺
(▼)	MT209/7b	rad6.1	psi ⁻



second umr1.1 strain, MT195/2a, indicated that the umr1.1 gene was not responsible for this phenotype. MT195/2a had a 'normal' spontaneous petite mutation frequency (3.0%) and showed a similar response to UV-induction of petites as the RAD⁺ control. Of 9 four-spored tetrads analysed from MT195 all, bar MT195/1b, were stable grande strains. MT195/1b was also one of only three non-killer (K^-) spores derived from MT195, all other spores being either K^+ or K^W . MT195/1b however readily maintained the psi⁺ phenotype.

- iii. rad6; Petite induction in the rad6.1 mutants MT209/7b psi⁺ and MT209/7b psi⁻ was found to approximate to that of the RAD⁺ control (Figure 4.19) although both the spontaneous and induced frequencies were slightly higher in the rad6.1 mutants. Kinetics of induction in both the psi⁺ and psi⁻ were identical to RAD⁺ i.e. were biphasic, although at low doses $n < 1$ was seen. MT209/7b psi⁻ showed a higher spontaneous and induced frequency of petites than the psi⁺ strain.

Moustacchi (1972) found that the rad6.1 mutant used in her study was blocked in petite induction by UV up to the maximum dose used i.e. $25 \text{ J} / \text{m}^2$ (0.01% survival). The possibility that SUQ5, even in a psi⁻ background, suppressed this rad6.1 phenotype cannot be discounted. The rad6.1 psi⁻ parent strain 197/2d had a high spontaneous petite frequency (19.4%).

The unsuppressed rad6.3 strain, JF9-4D, showed a very high spontaneous frequency of petites (29.7%) although petites were apparently induced by UV up to $15 \text{ J} / \text{m}^2$ (Table 4.16). Unfortunately the suppressed rad6.3 strain used in these

studies (MT221/1b) was a petite.

4.3.4 Conclusions

The UV-induced psi⁺ to psi⁻ mutation is enhanced, per unit dose, in mutants defective in excision repair (rad1, rad3) but depressed in mutants blocked in error prone repair (rad6.3, rev1 and umr1). UV-induced forward nuclear mutation shows an identical response in these mutants unlike the cytoplasmic petite mutation. It therefore appears that repair of UV-induced genetic damage of the /psi/ genome is under the control of pathways responsible for the repair and expression of nuclear DNA damage. The repair and expression of mitochondrial DNA damage is not entirely dependent on these pathways.

4.4 Discussion

(a) Photoreactivation

Evidence presented in this chapter indicates that there is an, at least partial, involvement of $\hat{\text{PyPy}}$ in the psi⁺ to psi⁻ mutational event based on the photoreactivability of UV-induced damage leading to the psi⁻ mutation. The substrate for the PRE is the $\hat{\text{PyPy}}$ and competition experiments (Rupert 1964), in which UV-irradiated polynucleotides were allowed to compete with UV-irradiated transforming DNA for PRE, demonstrated that cytosine-cytosine ($\hat{\text{CC}}$) dimers could also act as a substrate for PRE. Such $\hat{\text{CC}}$ dimers could presumably be induced by UV in RNA as well as DNA. Rupert (1964) found that UV-irradiated RNA did not compete with the transforming DNA for PRE but recent evidence has demonstrated that $\hat{\text{PyPy}}$ can be formed in certain RNA viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) (Huang and Gordon 1972) and in the RNA (largely ribosomal) of eggs of certain insects (Jackle and Kalthoff 1978) both in vivo and in vitro. It should be

noted that the $\hat{\text{PyPy}}$ was generated, in both cases, by an abnormal photosensitised reaction. Once $\hat{\text{PyPy}}$ are formed in RNA, they can be recognised and monomerised by PRE, both in vivo (Jackle and Kalthoff 1978) and in vitro (Huang and Gordon 1972; Hurter et al., 1974) with at least partial recovery of biological activity.

The overall process of photorepair of RNA therefore appears to be similar to that of DNA and the reversal of $\hat{\text{PyPy}}$ formation appears to play an important role in the PHR of the biological activities of both DNA and RNA. Consequently, although $\hat{\text{PyPy}}$ formation is involved in the UV-induction of psi⁻ mutants, this could be consistent with either an RNA or DNA genome. The latter is more probable for /psi because the cells were UV-irradiated under non-photosensitising conditions and, to date, there has been no report of the induction of $\hat{\text{PyPy}}$, in cytoplasmic RNA species of yeast, by UV-light.

(b) Repair of UV-induced cytoplasmic genetic damage

It is clear that the repair of UV-induced damage to the /psi genome is under control of nuclear DNA repair pathways. This is not the case for mitochondrial DNA damage, as reported by Moustacchi (1972), and found in this thesis. Mitochondrial DNA appears to be under the control of both specific nuclear and mitochondrial repair processes. UV-induced /psi and nuclear mutation was blocked in two strains (umr1, rev3) which were hypersensitive to petite induction by UV (see Figure 4.19), although it was not, in the case of the umr1 mutant, an effect of the umr1 mutation. These two mutants presumably carried defects in either the repair of UV-induced mitochondrial DNA damage or in the expression of the damage (Moustacchi et al., 1976)

The petite mutation is however unique in that it is characteristically accompanied by gross physical changes in the mitochondrial genome (Nagley et al., 1977). To determine the role nuclear DNA repair processes play in the repair of mitochondrial DNA damage it would therefore be better to look at the UV-induction of point mutations on the mitochondrial genome in rad rev and umr mutants. UV does induce such mutations (Stuart 1970). The psi⁻ mutation appears to be a point mutation rather than physical loss of the /psi genome and it would therefore be more meaningful to compare the results obtained in this chapter with induction of mitochondrial point mutations.

(c) The RAD6 gene

rad6.1 mutants have 4 major phenotypes; x-ray and UV-sensitivity, immutability by UV or chemical mutagens, lack of sporulation in a rad6/rad6 homozygous diploid and sensitivity to trimethoprim (Cox and Parry 1968; Hunnabale 1972; Lawrence et al., 1974). rad6.1 was originally reported as an amber mutant (Lawrence et al., 1974) but data presented in this chapter shows that SUQ5, an ochre suppressor, partially suppresses both the UV-sensitivity and the blocked UV mutagenesis phenotypes. The rad6.1 mutation is therefore suppressible by both ochre and amber suppressors and represents a new class of mutation in yeast.

A second rad6 allele rad6.3 originally isolated by Prakash and Prakash (1977), which shows the normal rad6 phenotypes (Douth-Wright-Fasse et al., 1978), was also studied. SUQ5 only suppressed the UV-sensitivity phenotype; the SUQ5 rad6.3 mutant was still blocked in UV-induced nuclear mutation. A third rad6

allele rad6.4 (Douthwright-Fasse et al., 1978) has normal UV-sensitivity but shows enhanced UV mutagenesis similar to an excision-defective mutant eg. rad1, rad3 (C. Lawrence personal communication to B.S. Cox).

The phenotypes of suppressed and non-suppressed rad6 mutants are summarised in Table 4.18.

Although the RAD6⁺ gene product(s) has not been identified this data suggests that it codes for two gene products, one necessary for the repair of lethal damage (UV-sensitivity), and one required for error-prone repair (UV mutagenesis). These two genes (RAD6⁺-B and RAD6⁺-A respectively) are co-transcribed i.e. $\overrightarrow{AB}$.

rad6.1 would be a polar mutation, suppressible by both ochre and amber suppressors. In a SUP⁺ strain the mutant would be both UV-sensitive and blocked in error-prone repair. The ochre or amber suppressor would relieve this polarity by inserting an amino acid at the site of the rad6.1 (nonsense) mutation and allowing the RAD6⁺ gene to be completely transcribed giving functional A and B gene products.

rad6.3 would also be a polar (nonsense?) mutation suppressible by ochre suppressors (and amber?). The failure of SUQ5 to suppress the phenotype of the A gene product could be due to the amino acid (in this case serine) being unable to restore the in vivo activity of the A gene product, whilst still allowing the RAD6⁺ gene to be transcribed, restoring wild type B function i.e. restoring wild type UV-resistance.

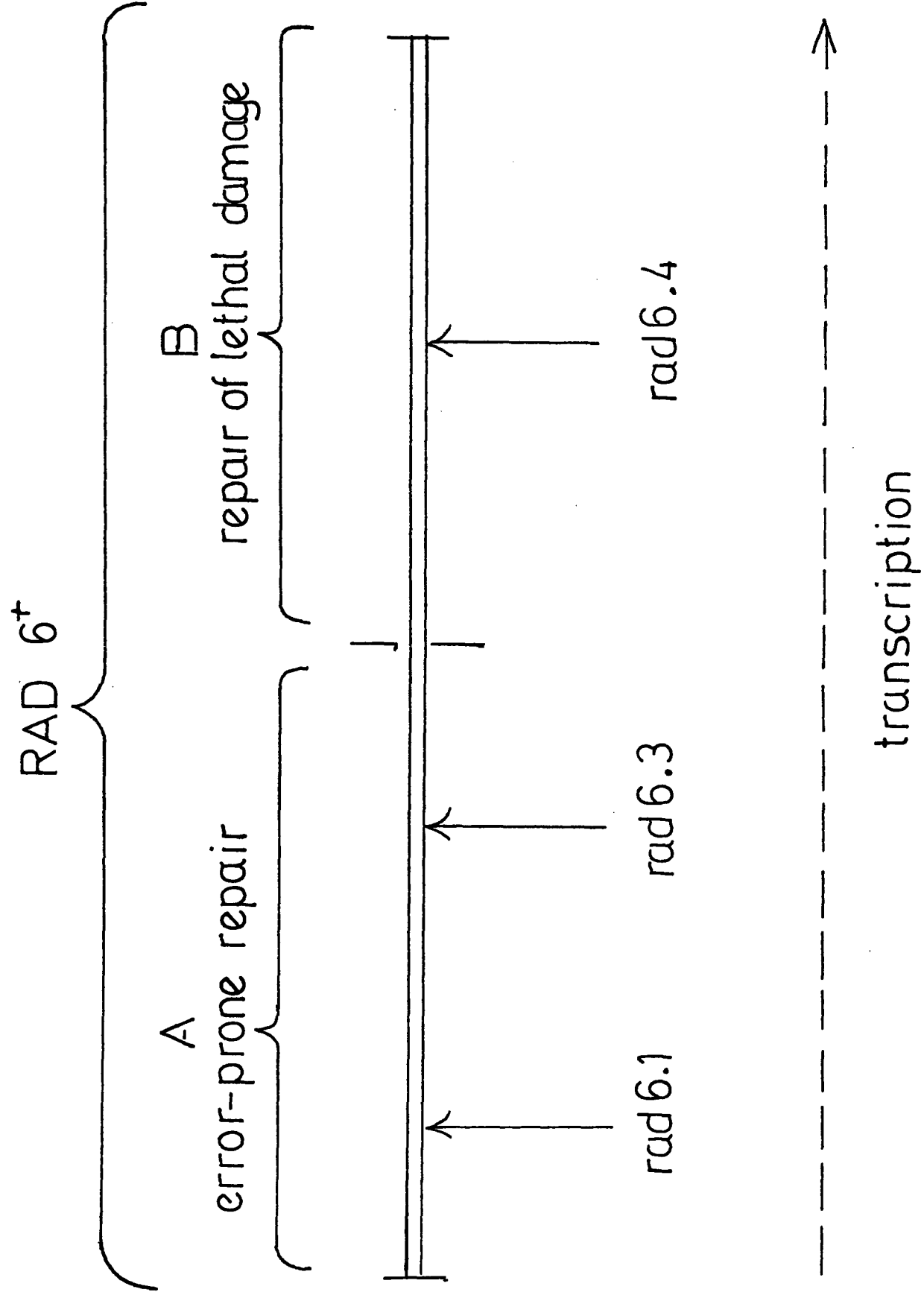
rad6.4 would be a mutation (missense or nonsense?) in the RAD6⁺-B gene and thus have no effect on error-prone repair.

This model is summarised in Figure 4.21.

TABLE 4.18 Phenotypes of rad 6 suppressed and non-suppressed mutants.

MUTANT	rad 6 PHENOTYPES		REFERENCE
	UV-SENSITIVITY	UV-MUTAGENESIS	
RAD6 ⁺	0	++	
rad6.1	+++	0	Cox & Parry (1968) Lawrence <u>et al</u> (1974)
rad6.1 + SUP _{oc}	+	+++	This work
rad6.1 + SUP _{am}	0	++	Lawrence <u>et al</u> (1974)
rad6.3	+++	0	Douthwright-Fasse <u>et al</u> (1978)
rad6.3 + SUP _{oc}	+	0	This work
rad 6.4	+++	+++	C. Lawrence personal communication to B. Cox

FIGURE 4.21 A model for the RAD6⁺ gene.



A detailed study of the effects of nonsense suppression on the phenotypes of rad6.1, rad6.3 and rad6.4^{is required}/and, in particular, how it effects the two other phenotypes not looked into in this study; namely trimethoprim sensitivity and the sporulation defect. Such a study is currently being undertaken in this laboratory.

4.5 Summary

The involvement of nuclear DNA repair systems in the repair of UV-induced damage to the /p̄si/ genome was investigated and compared to the repair of cytoplasmic mitochondrial DNA damage. The evidence obtained demonstrated that repair of the /p̄si/ genome was exclusively under the control of the nuclear DNA repair systems unlike mitochondrial DNA. This suggests that /p̄si/ has a DNA genome. Photoreactivation experiments demonstrated that formation of pyrimidine dimers is involved in the UV-induction of the psi⁻ mutation. On the basis of the effects of nonsense suppression on the phenotypes of different mutant alleles of the RAD6⁺ gene a model, involving the gene coding for two gene products with different functions, was proposed.

CHAPTER 5

PSI MUTAGENESIS : THE EFFECT OF KNOWN CYTOPLASMIC MUTAGENS ON THE PSI⁺ PHENOTYPE

- 5.1 Introduction
- 5.2 Thymidylate starvation
- 5.3 Manganese
- 5.4 5 Fluorouracil
- 5.5 Heat
- 5.6 Cycloheximide
- 5.7 Discussion
- 5.8 Summary

5.1 Introduction

The frequency of mutation of cytoplasmic and chromosomal genes can be raised non-specifically by the same kinds of mutagenic treatment e.g. nitrosoguanidine and UV-light (see Chapter 3). Some cytoplasmic mutants, however, can be induced with a specificity and frequency that are considerably greater than frequencies ever obtained for chromosomal gene mutation e.g. in yeast the treatment of grande cells with the intercalating dye ethidium bromide (EtBr), at non-lethal concentrations, can result in total conversion of cells to petite (Slonimski et al., 1968).

A number of chemical and physical agents have been used to induce cytoplasmic mutation in yeast, the principal agents being listed in Table 5.1. These results provide substantial evidence for differences in response to mutagens of nuclear and cytoplasmic genes in yeast although the molecular basis of these differences are at present not understood.

With the exception of acriflavine, very low levels of 5-fluoruracil (5FU) and Et Br (Young 1969; Cox personal communication all of which proved effective petite inducers in psi⁺ strains with no effect on the /psi phenotype, the effect of the cytoplasmic mutagenic agents on psi⁺ strains is unknown. A survey of the untried agents, for their effects on /psi, was therefore undertaken to see if any were efficient inducers of the psi mutation. Finding a positive inducing effect would be circumstantial evidence for the cytoplasmic location of the /psi genome although it should be noted that, in general, high mutation rates are not characteristic of cytoplasmic genes e.g. mitochondrial drug resistant mutants are found at conventionally low frequencies after mutagenesis (Stuart 1970; Dawes and Carter 1974; Putrament et al., 1973)

TABLE 5.1 Effects of mutagenic agents on nuclear and cytoplasmic genes in yeast.

MUTAGENIC AGENT	EFFECT ON MUTAGENIC AGENTS ON				REFERENCE
	NUCLEAR GENE	CYTOPLASMIC PETITE	CYTOPLASMIC DRUG RESISTANCE	KILLER	
ETHIDIUM BROMIDE	NT?	+	-	-	Sloniski et al (1968) Al-Aidroos et al (1973)
5 . FLUOROURACIL	-	+	NT	+	Moustacchi & Marcovich (1963) Mitchell et al (1976 b)
NITROSOGUANIDINE	+	+	+	NT	Nördstrom (1967) Dawes & Carter (1974)
ETHYL METHANE SULPHONATE	+	+	NT?	+	Wickner (1974 b) Vodkin (1977b)
UV LIGHT	+	+	+	+	Vodkin (1977b) Stuart (1970)
Mn ²⁺	+	+	+	NT	Putrament et al (1973) Baranowska et al (1977)
GUANIDINE HYDROCHLORIDE	NT	+	NT	NT	Juliani et al (1973, 1975 a)
CYCLOHEXIMIDE	-	(+)?	NT	+	Fink & Styles (1972)
GROWTH AT 40°C	+	+	NT	+	Wickner (1974 a) Evans & Parry (1975) Sherman (1959)
HEAT SHOCK AT 52°C	+	+	NT	NT	Sherman (1956, 1959) Evans & Parry (1975)
THYMINELESS DEATH	-	+	+	NT	Barclay & Little (1978)
ACRIFLAVINE	-	+	-	+	Ephrussi et al (1949) Fink & Styles (1972)

5.2 Thymidylate starvation

Incubating thymine-requiring bacterial cells in the absence of thymine, in addition to its lethal effect, causes a variety of the effects associated with damage to DNA; namely mutation, recombination, prophage induction and elimination of some types of plasmid (for a review see Stacey 1976). Thymineless death has recently been shown to occur in a yeast mutant, auxotrophic for deoxythymidine-5'-monophosphate (thymidylate, dTMP), when starved for this compound (Brendel and Langshar 1974). Thymidylate starvation in yeast results in the induction of mutations specifically in the mitochondrial genome in such a mutant, tmp1 (Barclay and Little 1978; Game personal communication).

Game (1976) has reported that the ts cell division cycle (cdc) mutant cdc21, originally isolated by Hartwell (Hartwell 1973), is allelic to tmp1 and confers a ts requirement for dTMP. When held at the non-permissive temperature (37°C) cdc21 mutants undergo thymineless death with a subsequent increase in mitochondrial mutants among the survivors (Game 1976, personal communication). The effect of thymidylate starvation on other cytoplasmic genetic elements of yeast was not investigated in these studies.

(1) Effect on ψ^+ phenotype

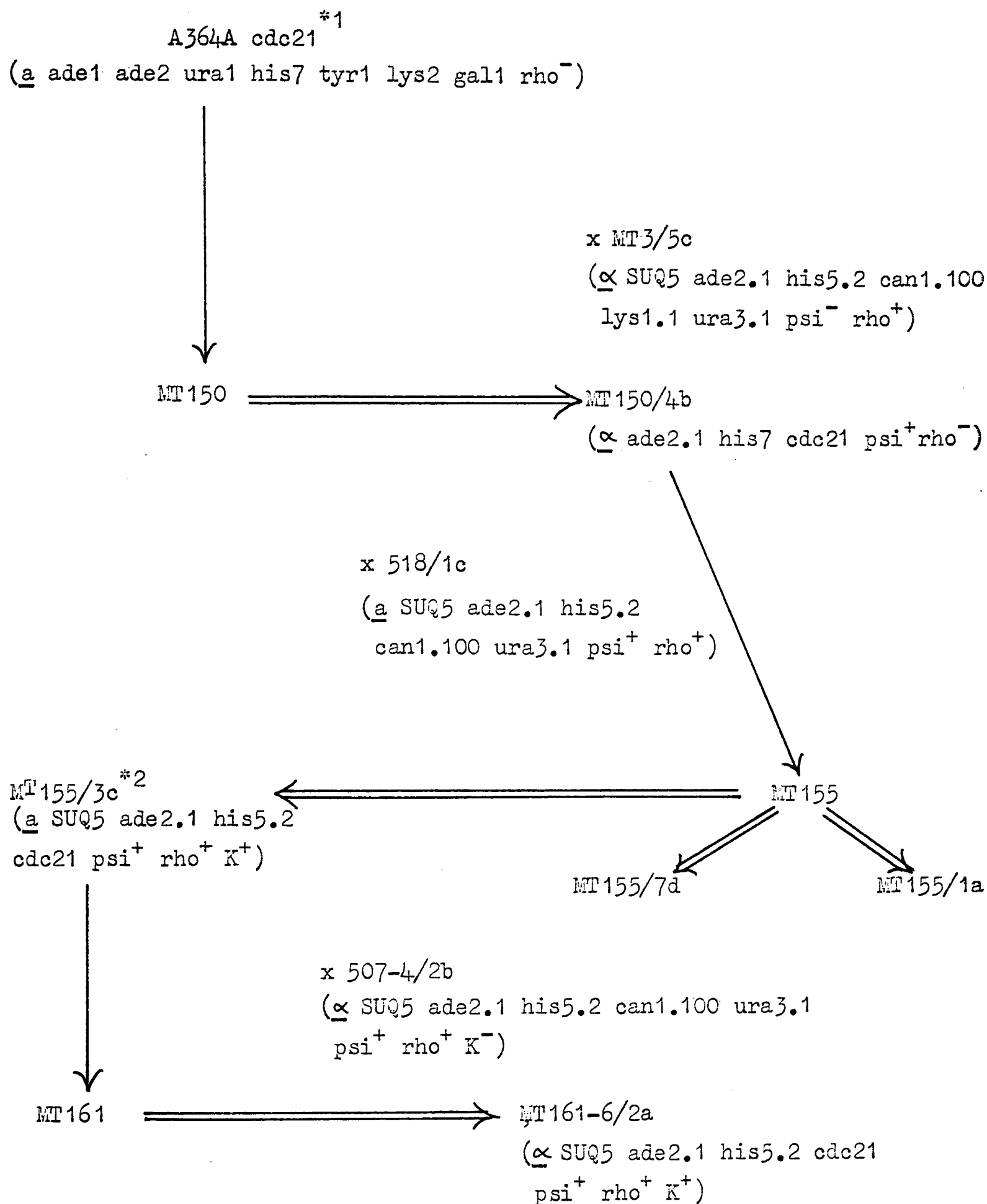
The effects of thymidylate starvation on ψ^- was studied in a ψ^+ K^+ mutant carrying the cdc21 mutation. Maintenance of the ψ^+ phenotype in cdc21 mutants at the permissive temperature (23°C) was also investigated following the recent observation that cdc21 mutants produce petites, at 23°C, at a rate up to X17 that of normal (Newlon et al., 1976). A preliminary study of maintenance of the ψ^+ phenotype in other ts cdc mutants at the permissive temperature, and after incubation at the non-

permissive temperature was also performed. The mutants were cdc4, cdc7 cdc8 and cdc9.

Figure 5.1 outlines how the haploid cdc21 strain MT161-6/2a, used to study the effects of thymidylate starvation on maintenance of the psi⁺ phenotype, was obtained. The strain was grande and was maintained as a grande by growing and recloning on YPGly. When plated onto YC between 23% and 63% of colonies were petite following incubation at 23°C.

MT161-6/2a was confirmed as a ts dTMP auxotroph by plating cells onto YC medium containing dTMP and incubating the plates at 37°C (Game 1976). After 4 days discrete colonies were found on plates containing 20ug/ml or 100ug/ml dTMP whilst none were observed on control plates lacking dTMP (Table 5.2). 49 colonies from the YC + 100ug/ml dTMP plates were retested; all were ts on YC medium and were fully suppressed at 23°C. 46 of the colonies were petite whilst the ts phenotype of all 49 was not overcome when grown at 37°C on YC containing 100ug/ml thymine. "Wild-type" S. cerevisiae lacks the enzyme thymidine kinase (Grivell and Jackson 1968) thus preventing the conversion of thymine to dTMP. It is also impermeable to external dTMP. Colonies arising on dTMP medium at 37°C presumably carry one (or more) tup mutations rendering them permeable to dTMP (Wickner 1975). dTMP had no effect on viability at 23°C.

It was noted that when MT161-6/2a was tested for dTMP auxotrophy, after its initial isolation (Table 5.2(a)), the frequency of colonies arising at 37°C on 100ug/ml dTMP plates was significantly lower than when the strain was retested several months later, after approximately 5 reclonings on YPGly (Table 5.2(b)). Presumably maintenance of cdc21 strains on YPGly selects for tup mutants.

FIGURE 5.1 Isolation of a psi⁺ rho⁺ K⁺ cdc21 mutant strain.

*1 obtained from Dr. L.H. Hartwell via Dr. B.S. Cox. It was psi⁺ because MT150 was suppressed and gave psi⁺ spores.

*2 a fully suppressed rho⁺ derivative of MT155/3c was used in this cross- see text.

→ single cell zygote isolation.

⇒ tetrad dissection.

TABLE 5.2 The ability of dTMP to overcome the ts phenotype of the psi⁺cdc21 strain MT161-6/2a.

MEDIUM	INCUBATION TEMPERATURE				
	23°C		37°C		
	TOTAL CELLS PLATED	NO. OF COLONIES	TOTAL CELLS PLATED *1	NO. OF COLONIES	FREQUENCY COLONIES/ CELLS PLATED
YC	(a) ^{*2}	346	346	7.73 x 10 ⁶	0
	(b)	299	299	8.97x10 ⁵	0
YC + 20µg/ml dTMP	(a)	230	230	NT	NT
	(b)	NT	2.99x10 ³	12	4.01/10 ³
YC + 100µg/ml dTMP	(a)	230	221	9.22x10 ⁶	29
	(b)	398	419	3.79x10 ³	11.82/10 ²

NT= not tested.

*1 estimated from the number of colonies growing on YC at 23°C.

*2 (a) experiment performed after strain isolation on YC (b) experiment performed after recloning on YPGly (see text for details).

The parent cdc21 strain, A364A (a ade1 ade2 ura1 his7 tyr1 lys2 gal1 cdc21), was also checked for ts dTMP auxotrophy with similar findings to those for MT162-6/2a; colonies arose at a frequency of $4.76/10^6$ cells plated at 37°C on YC supplemented with 100ug/ml dTMP and $3.4/10^7$ cells plated on YC + 20ug/ml dTMP.

Diploids constructed by crossing MT161-6/2a with A364A cdc21 were ts showing that MT161-6/2a carried the cdc21 mutation. No revertants of MT161-6/2a, able to grow at 37°C , were found among a total of 8.63×10^6 cells plated (Table 5.2) and MT161-6/2a showed decreased survival at 28°C (6.9%) compared to growth at 23°C (100%). The genotype of MT161-6/2a was confirmed by crossing it to a wild type haploid strain (FL530-1b). The ts phenotype segregated 2:2 in each of 7 tetrads obtained and all 7 gave 3ADE⁺ and 1ade⁻ spores with a similar overall pattern observed for HIS⁺:his⁻ segregation. All 7 red (ade2.1) spores obtained were also ts indicating possible linkage between cdc21 and ade2.1. The linkage between these two markers has recently been confirmed by Game (1976) both being mapped to chromosome IV.

The effect of thymidylate deprivation on MT161-6/2a was examined. A culture was grown overnight at 23°C on a YPGly plate. A saline cell suspension was made and cells plated onto YC to give 100-5000 cells per plate. The plates were then incubated at 37°C for varying lengths of time up to 32 hours and then transferred to an incubator at 23°C . One control experiment using a CDC21⁺ strain (507-4/2b) was also performed. The kinetics of cell death of both the cdc21 and CDC21⁺ strain are shown in Figure 5.2. The number of colonies scored at each incubation time is given in Table 5.3. Biphasic kinetics of cell death were seen

FIGURE 5.2 The effects of holding a cdc21 psi⁺ strain at 37°C on cell viability and the frequency of petite mutants.

- (■) MT161-6/2a cdc21 psi⁺, cell viability
- (●) 507-4/2b CDC⁺ psi⁺, cell viability
- (□) MT161-6/2a cdc21 psi⁺, frequency of petites

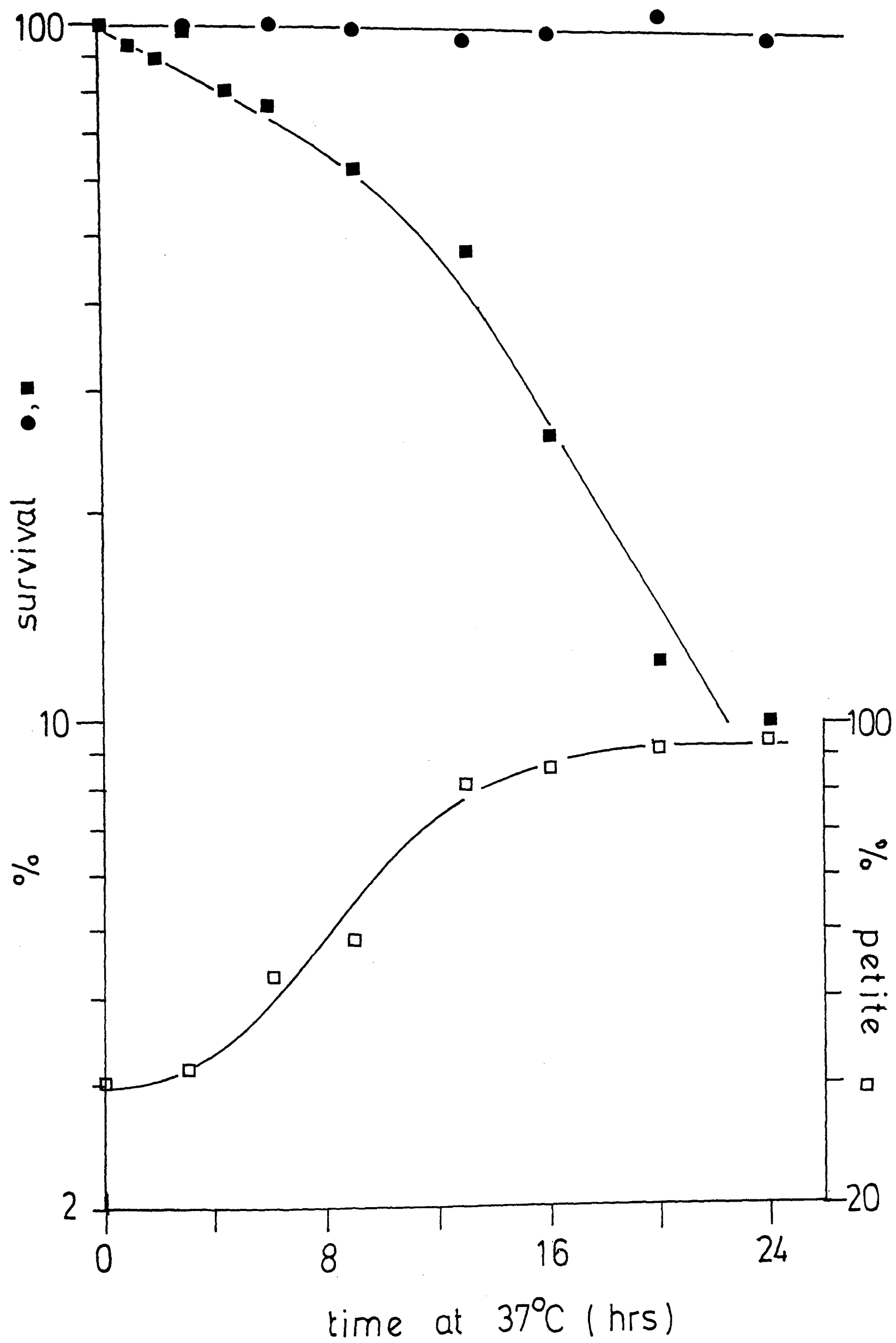


TABLE 5.3 Effect of incubating at 37°C on survival, petite induction and mutation to non-suppression on WT161-6/2a cdc21 and CDC21⁺ control (507-4/2b).

TIME (hours)	NUMBER OF EXPERIMENTS	TOTAL COLONIES SCORED	*1 NON-SUPPRESSED		% SURVIVAL *2	rho ⁻ MUTANTS		CDC ⁺			
			8	1		rho ⁻ COLONIES SCORED	% rho	COLONIES SCORED	MUTANTS WHOLE:SECTORED	% SURVIVAL	
											WHOLE:SECTORED
0	13	8362	8	1	100	1133	30.6	439	0	0	100
1	1	4444	0	0	92.1	NT				NT	
2	2	962	0	0	89.5	NT				NT	
3	3	1875	2	0	97.8	742	34.1	205	0	0	100.5
4.5	2	871	0	0	81.0	NT				NT	
6	3	1652	2	0	77.3	625	42.7	206	0	0	101.0
9	4	1995	1	2	62.6	665	48.4	200	0	0	98.4
13	1	1025	2	0	47.8	655	81.2	150	0	0	95.8
16	2	1604	0	0	25.8	1011	84.9	152	1	0	97.0
20	2	2826	0	0	14.4	1286	92.1	164	0	0	104.7
24	9	14,910	13	1	10.0	1080	95.4	151	0	0	96.4
28	2	128	0	0	2.9	NT		172	0	0	79.4
32	1	46	0	0	0.5	NT		228	0	0	111.8

NT= not tested.

*1 :non-suppressed mutants were isolated in at least 3 of the experiments conducted. The figures do not represent a 'jackpot' event in one experiment.

*2 :is the average value.

for MT161-6/2a, the decrease in survival being slow up to approximately 12 hours, but after 12 hours the decline in survival became more rapid. Holding a CDC21⁺ at 37°C for up to 32 hours had no effect on viability.

Incubation at 37°C did not increase the frequency of non-suppressed cdc21 mutants among the survivors (at time 0, 1.08/10³ survivors; after 24 hours at 37°C, 0.94/10³ survivors). 7 out of 8 non-suppressed cdc21 mutants, from time 0, were psi⁻ (the other being an asu mutant) and all 12 mutants isolated, after incubation at 37°C for either 3, 9, 13 or 24 hours, were psi⁻. The high frequency of spontaneous psi⁻ mutants will be discussed in Chapter 7 (Section 7.3b).

The fraction of survivors that were petite was checked in one experiment with MT161-6/2a and 95% of survivors, following incubation for 24 hours at 37°C, were petite (Figure 5.2, Table 5.3). All the grande colonies scored were very unstable showing many petite sectors.

The effects of dTMP starvation on mitochondrial point mutation induction and maintenance of the /killer7 phenotype in MT161-6/2a was also studied (Table 5.4). A threefold increase in the frequency of mutants, resistant to erythromycin (300µg/ml), was found following incubation at 37°C. The slight increase in K⁻ colonies observed ($\chi^2=1.92$, $p=0.10-0.20$) was probably a result of the elevated temperature per se rather than a result of dTMP deprivation (Wickner 1974a).

Following the recent observation that cyclic thymineless death significantly increased the frequency of R-plasmid elimination in E. coli K12 (Tribe and Pinney 1977) the effect of exposing MT16-6/2a to 3 consecutive cycles of dTMP starvation (24 hours at 37°C) was investigated. An individual colony of

TABLE 5.4 The effect of thymidylate starvation on the K⁺ phenotype and frequency erythromycin resistant mutants in MT161-6/2a.

TIME AT 37°C (hours)	ERYTHROMYCIN RESISTANCE				KILLER		
	TOTAL CELLS PLATED	TOTAL VIABLE CELLS	ery ^r COLONIES	ery ^r /10 ⁵ SURVIVORS	K ⁺ /K ^w	K ⁻	%K ⁻
0	1.25x10 ⁶	1.25x10 ⁶	25	1.99	1044	16	1.51
24	1.25x10 ⁶	2.15x10 ⁵ *1	12	5.57	768	18	2.29

*1 calculated from survival on YC after 24 hours incubation at 37°C.
In this experiment it was 17.1%.

MY161-6/2a, grown on YPGly at 23°C, was suspended in saline, plated onto YC and incubated at 37°C hours before removing to a 23°C incubator (= CYCLE 1). A single surviving colony was then suspended in saline and a second cycle of thymidylate starvation carried out (= CYCLE 2). This was repeated once more (= CYCLE 3). No induction of non-suppressed mutants was observed following any of the cycles. No effect on the K^+ was observed (Table 5.5).

(2) Maintenance of $\bar{\psi}$ in cdc21 strains at the permissive temperature

The ts mutant cdc21 produces petites at abnormal rates at 23°C, the permissive temperature (Newlon et al., 1976). In crosses of the type CDC21⁺ rho⁺ x cdc21 rho⁻ carried out the segregation of the petite and ts phenotype was scored in a total of 164 tetrads in which cdc21 segregated normally. The data obtained (Table 5.6) demonstrated that the ts and petite phenotypes normally co-segregate, although rho⁺ cdc21 spores were obtained. Those cdc21 rho⁺ spores examined (a total of 5) gave very high petite frequencies upon recloning on YC. No stable grande cdc21 strains were found. These findings have recently been confirmed (Newlon et al., 1976). 21 of the tetrads had an excess of petite spores indicating that some of the spontaneous cdc21 petites may be of the suppressive type.

The high spontaneous frequency is not due to a decreased rate in mitochondrial DNA replication, or from a general increase in mutation rate (Newlon and Ludescher 1977), but may be due to a progressive loss of mitochondrial DNA (Barclay and Little 1978). Thus the cdc21 mutation isolated by Hartwell appears to be a 'leaky' allele of the twp1 gene. The twp1-6 mutant used by Barclay and Little (1978), which was shown to be allelic to cdc21 (Game 1976), had a spontaneous petite frequency of 1.5% when

TABLE 5.5 The effect of cyclic thymidylate starvation on the suppressed and K⁺ phenotypes of MT161-6/2a.

	TIME AT 37°C (hours)	COLONIES COUNTED	% SURVIVAL	NON- SUPPRESSED MUTANTS	NO. OF COLONIES	
					K ⁺	K ⁻
CYCLE (1)	0	810	100	0	321	12
	24	3675	9.45	0	NT	
CYCLE (2)	0	238	100	0	151	1
	24	863	13.96	0	286	3
CYCLE (3)	0	694	100	0	109	0
	24	576	7.98	2	197	2

NT = not tested.

TABLE 5.6 Segregation of the ts and petite phenotypes in crosses heterozygous for cdc21.

SEGREGATION IN TETRADES			
PETITE ^{*1}	:	GRANDE	NUMBER OF TETRADES
2cdc21		2CDC ⁺	105
2cdc21 2CDC ⁺		0	5
2cdc21 1CDC ⁺		1CDC ⁺	16
1cdc21		1cdc21 2CDC ⁺	21
0		2cdc21 2CDC ⁺	12
2CDC ⁺		2cdc21	1
1cdc21 1CDC ⁺		1cdc21 1CDC ⁺	4
			164

^{*1} those colonies that were exclusively petite or only showing a few grande papillae on YPGly.

grown on glucose medium (Barclay and Little 1978) compared to a frequency of 23-63% found for MT161-6/2a. In a preliminary cross between 507-4/2b and a strain carrying the ts tmp1 allele tmp1-10 (MB-1076-2B a ade2 adex arg4 his5 leu1 ; obtained from Dr M. Brendel via Dr D.W. Bryant) all the ts spores in 8 tetrads dissected were either petite or showed a high proportion of petites at 23°C.

As mentioned previously MT161-6/2a was also apparently unable to maintain the psi⁺ phenotype in a few mitotic progeny at 23°C with psi⁻ colonies arising at a frequency of about 10⁻³ (Table 5.3). The spontaneous frequency of psi⁻ colonies arising from the CDC21⁺ strain, 507-4/2b, was < 10⁻⁴ (see Chapter 3). Two further strains of the genotype SUQ5 ade2.1 his5.2 cdc21 psi⁺, namely MT155/3c and MT155/7d (see Figure 5.1), were also found to give a high spontaneous frequency of psi⁻ mutants during subcloning on YC although a third strain investigated, MT155/1a, was found to be completely stable for psi⁺ although it still had an unstable grande phenotype. However suppressed clones were obtained from MT155/3c and MT155/7d which proved stable for the psi⁺ phenotype, through many subclonings on YC at room temperature, whilst retaining their ts and petite producing phenotype.

A detailed genetic analysis of the cdc21 strain MT155/3c will be given in Chapter 7. Suffice to say here that the high frequency of spontaneous psi⁻ mutants was not a function of the cdc21 gene but apparently of a nuclear mutation in a psi⁺ maintenance gene. This mutation was also assumed to be present in MT161-6/2a, MT155/3c being one of the parent strains of the MT161 diploid (Figure 5.1).

The K^+ phenotype was stable in strains carrying the cdc21 mutation at the permissive temperature (23°C).

(3) Maintenance of the ψ^+ phenotype in other cdc mutants

The maintenance of the ψ^+ phenotype was examined in 4 other ts cell division cycle (cdc) mutants at the permissive temperature (23°C) and following a period of incubation at the non-permissive temperature (37°C). Table 5.7 summarises the phenotypes of the cdc mutants used in this study.

Strains with the required genotype (i.e. SUQ5 ade2.1 his5.2 ψ^+ cdc) were obtained by crossing Hartwell's original mutants (obtained from Dr J. Game, NIMR, London), which were of a mating type, to 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 ψ^+). Haploid segregants, from the cross, with the required genotype, were detected by crossing all ts ADE^+HIS^+ spores to an SUQ5 ade2.1 PNM1 sal3.5 ψ^- tester (see Materials and Methods). All strains used in this study were K^+ .

(a) cdc : strains carrying the cdc8 mutation/^{give a high frequency} of petite mutants (Newlon and Fangman 1975) and these petites contain deletions of the mitochondrial genome (Newlon and Lubescher 1977). Unlike cdc21, cdc8 mutants do not have a defective thymidylate synthetase (Bisson and Thorner 1977). This was confirmed by growing the cdc8 mutant constructed, MT154/1d, on YC plates supplemented with $100\mu\text{g}/\text{m}$ dTMP at 37°C . Of the 5 colonies found growing at 37°C all were found to be TS^+ on YC alone at 37°C . dTMP therefore did not phenotypically suppress the ts phenotype of the cdc8 mutation, unlike its effect on cdc21 e.g. MT161-6/2a. In tetrads, dissected from MT154, some of the cdc8 spores showed 'leaky' growth at 37°C on YC, but the strain used in the following study (MT154/1d) showed complete inhibition of growth at 37°C .

TABLE 5.7 Phenotypes of cdc mutants used in this study.

cdc	DNA SYNTHESIS AT 37°C (a)		dsRNA (b) PRODUCTION AT 37°C	IMMEDIATE CELL CYCLE (c) EVENT BLOCKED	
	NUCLEAR	MITOCHONDRIAL 2μ			
4	-	+	-	+	spindle plaque separation
7	-	+	-	+	initiation of DNA synthesis
8	-	-	-	-	DNA synthesis-elongation
9	+	+	NT	NT	medial nuclear division (d)
21	-	-	NT	-	DNA synthesis-elongation

(a) Hereford and Hartwell (1974) Newlon and Fangman (1975) Livingston and Kupfer (1977) Cryer et al (1973).

(b) Shalitin and Weiser (1977) : + indicates overproduction, - no overproduction, of dsRNA.

(c) for review see Hartwell et al (1974).

(d) defective in a DNA ligase (L. Johnson, personal communication).

The effect of holding MT154/1d at 37°C, for lengths of time up to 30 hours, prior to transferring to 23°C, was investigated. Experimental protocol was as described above for the cdc21 experiment. Viability declined much more rapidly in the cdc8 mutant (Figure 5.3) compared to the cdc21 strain. There was a 'resistant tail' to the survival curve starting after approximately 14 hours incubation at 37°C. No such 'tail' was seen with the cdc21 mutant (Figure 5.2).

From the data presented in Table 5.8 there seemed to be a significant increase in the frequency of non-suppressed mutants following holding at 37°C.

All 28 non-suppressed mutants were cloned and their genotypes determined by complementation analysis; all 28 complemented as SUQ5 ade2.1 psi⁻ mutants. The possibility that holding the strain at 37°C selects for psi⁻ mutants rather than induces them cannot be ruled out although with the cdc21 psi⁺ strain (MT161-6/2a) this did not seem to be the case the frequency of psi⁻ mutants per survivor being the same, after holding at 37°C for 24 hours, as the control not exposed to the non-permissive temperature (see Table 5.3). An increase in the proportion of survivors that were petite was also observed with increasing time at 37°C, 93.5% of survivors being petite after 24 hours at 37°C. The spontaneous petite frequency was lower than observed for the cdc21 strains used in these studies. (0.1%-2%)

It was concluded that holding a cdc8 psi⁺ mutant at 37°C results in the induction of psi⁻ mutants among survivors with a maximum frequency observed, after 24 hours holding at 37°C of $2.3/10^3$ survivors. The elimination of the grande phenotype was more efficient. The K⁺ phenotype was not tested.

FIGURE 5.3 The effect on cell viability of holding a
cdc8 psi⁺ strain (MT154/1d) at 37°C for
upto 28 hours.

(■) 507-4/2b CDC⁺
(▲) MT154/1d cdc8

----- represents the data for a cdc21 mutant
(taken from Figure 5.2).

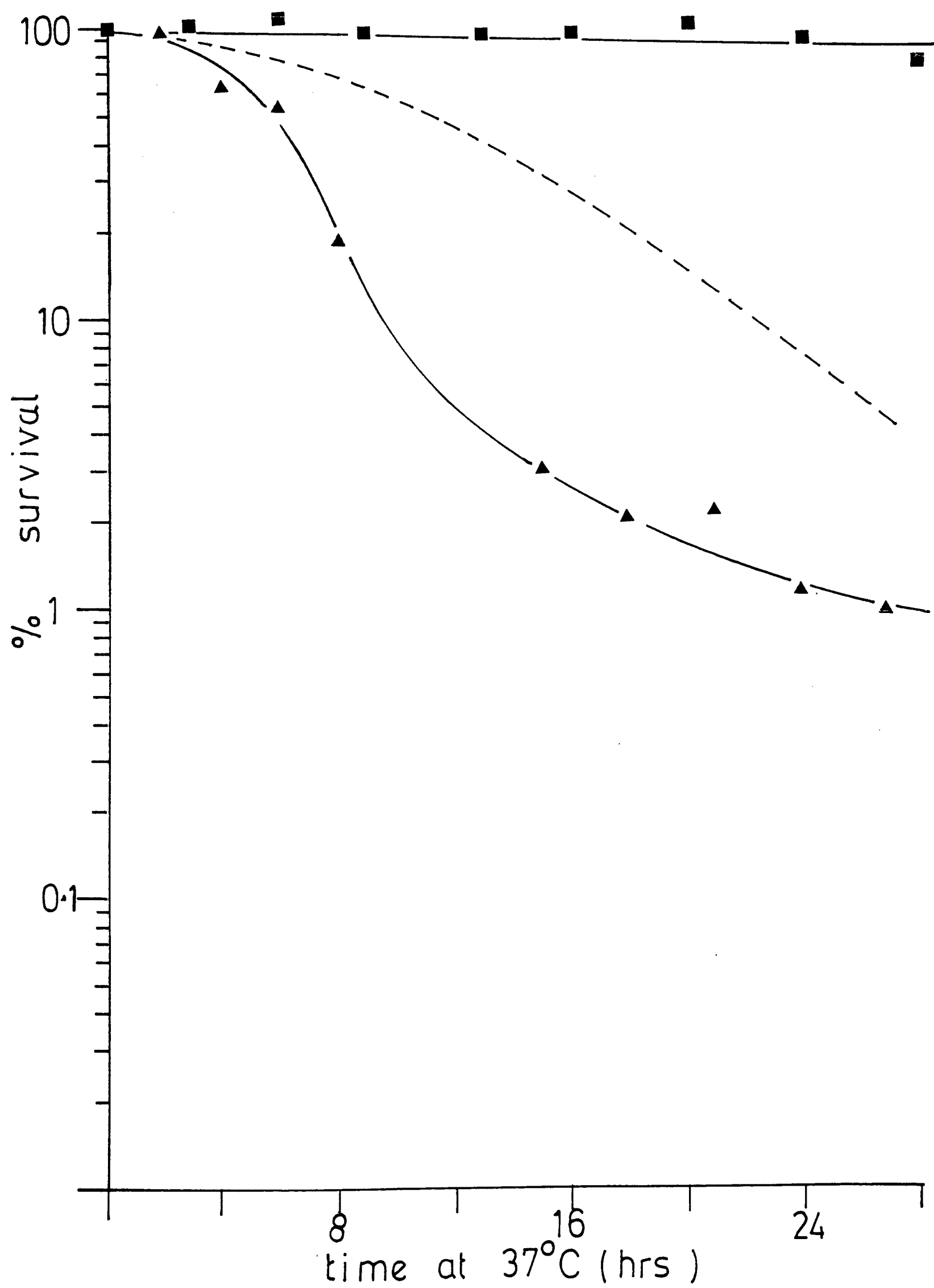


TABLE 5.8 The effect of holding MT154/1d (cdc8) at 37°C on survival and frequency of petite and non-suppressed mutants.

TIME AT 37°C (hours)	NO. OF EXPERIMENTS	COLONIES COUNTED	AVERAGE % SURVIVAL	NON-SUPPRESSED MUTANTS	PETITE	
					COLONIES COUNTED	% p ⁻
0	5	5353	100	1	179	11.2
2	1	1049	96.4	1	NT	
4	2	1436	62.1	0	NT	
6	1	1789	54.8	0	533	44.6
8	2	1471	18.5	1	NT	
15	1	693	3.2	0	NT	
18	1	2247	2.1	0	NT	
21	3	4279	2.2	5	NT	
24	2	6174	1.1	14	1197	93.5
27	1	3312	1.0	6	NT	
30	1	971	0.9	0	92	96.7
48	1	4	0.006	0	NT	

NT = not tested.

For control CDC⁺ see Table 5.3.

(b) cdc4, cdc7, cdc9 : the effect on holding these strains, at 37°C for 24 hours, on the psi⁺ phenotype was investigated using the experimental protocol previously described. The results obtained in two separate experiments are summarised in Table 5.9. All three cdc mutants showed a significant decrease in survival following incubation at 37°C but in no case were any non-suppressed mutants picked up either at the permissive temperature (23°C), or following 24 hours incubation, at the non-permissive temperature. The cdc4 and cdc9 mutants showed no increase in petite frequency after incubation at 37°C although the cdc9 mutant had a high spontaneous petite frequency. With cdc7 however a fivefold increase in petite frequency was observed after such treatment, with the majority of colonies, designated as grande after incubation, showing large numbers of petite sectors compared to stable grande colonies seen in the untreated control. Although only a small number of colonies were screened, incubation at 37°C of all 3 cdc mutants investigated, had no effect on the K⁺ phenotype of the survivors.

(4) Conclusion

Thymidylate starvation is not mutagenic for the /psi genome whilst it does induce both the petite mutation and point mutations on the cytoplasmic mitochondrial genome. Holding a psi⁺ ts cdc8 mutant at the non-permissive temperature for 20-27 hours results in the loss of the psi⁺ phenotype of cells to the petite. The psi⁺ phenotype is stable at both the permissive temperature (23°C) and following incubation at the non-permissive temperature (37°C) in three other cdc mutants, cdc4, cdc7 and cdc9.

5.3 Manganese

Recent work by Putrament and her co-workers (Putrament et al., 1975) has shown that manganese (Mn²⁺) specifically induces

TABLE 5.9 The stability of extrachromosomal phenotypes in cdc4, cdc7 and cdc9 mutants at the permissive temperature (23°C) following a 24 hours incubation at the non-permissive temperature.

cdc MUTANT	STRAIN NUMBER	TIME AT 37°C (hours)	COLONIES COUNTED	AVERAGE % SURVIVAL	NO. OF NON- SUPPRESSED MUTANTS	PETITE (p)		KILLER(K)	
						NO. COLONIES		NO. COLONIES	
						rho ⁺	rho ⁻	K ⁺	K ⁻
							%rho ⁻		% K ⁻
cdc ⁺	MF182/8a	0	586	100	0	227	3	1.30	230
		24	1292	100.2	0	643	13	1.98	656
		0	1916	100	0	296	2	0.67	292
cdc4	MF231/3b	24	1554	12.25	0	183	2	1.08	183
		0	665	100	0	327	16	4.66	129
		24	3858	0.11	0	332	109	24.72	176
cdc7	MF223/2a	0	665	100	0	327	16	4.66	129
		24	3858	0.11	0	332	109	24.72	176
cdc9	MF224/5c	0	724	100	0	349	66	15.90	108
		24	2498	0.22	0	857	97	10.17	169

mitochondrial mutations in yeast (both point mutations e.g. antibiotic resistance, and mutation giving the petite phenotype), whilst being only a very weak nuclear mutagen (Baranowska et al., 1977). Mn^{2+} is believed to act as an error-producing factor during mitochondrial DNA replication. The effect of Mn^{2+} on the psi⁺ phenotype was investigated.

The strain used in this study was 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺), which was grown to stationary phase on solid YPGly, and the cells suspended in saline (4.9×10^6 viable cells/ml). 1ml samples of this suspension were used as inocula for each of two flasks; one had YC with a final concentration of 8.8mM manganese chloride ($MnCl_2$) at a pH 6.0; the second flask was a control with no added $MnCl_2$, but adjusted to pH 6.0 with IN HCl. The two cultures were incubated, with shaking, at 28°C for 7 days. Samples of the culture were taken at various incubation times up to 7 days and plated onto YC plates to determine the viable cell titre (Figure 5.4) and the nature of the cells with respect to their /psi and /rho phenotypes (Table 5.10).

The presence of 8.8mM $MnCl_2$ in the medium resulted in some cell death over the first 24 hours of incubation with the culture reaching a maximum viable cell titre of 4.4×10^6 /ml. The control culture reached a viable cell titre of 2.2×10^8 /ml after 7 days incubation. The fraction of surviving cells that were petite in the Mn^{2+} culture, was significantly higher than in the control culture, reaching almost 100% by 7 days.

A total of 7 non-suppressed (red, ade^- his^- can^r) colonies were obtained, from a total of 8310 colonies screened, following growth in 8.8mM $MnCl_2$. 5 were psi⁻ mutants and two were of the

FIGURE 5.4 The effect on growth and petite induction of MnCl_2 in a liquid YC culture over a 7 day incubation period.

- (□) 507-4/2b, YC , viable cell titre
- (■) 507-4/2b, YC + 8.8 mM MnCl_2 , viable cell titre
- (△) 507-4/2b, YC , frequency of petites
- (▲) 507-4/2b, YC + 8.8 mM MnCl_2 , frequency of petites

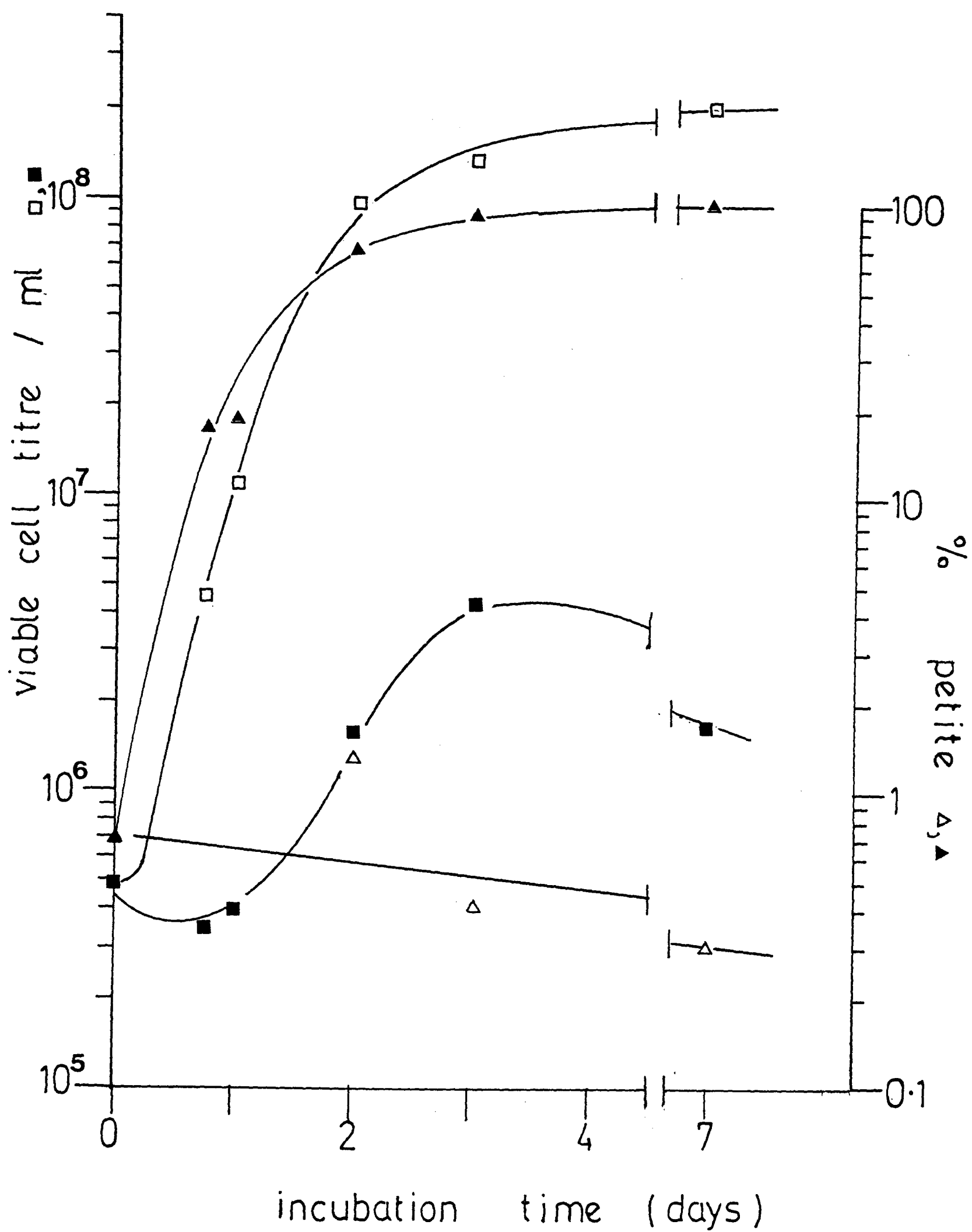


TABLE 5.10 The number of colonies and the frequencies of non-suppressed and petite mutants following growth of 507-4/2b in YC with or without 8.8mM MnCl₂.

INCUBATION TIME (HOURS)	CONTROL (YC)				YC + MnCl ₂		PETITE	
	COLONIES COUNTED	RED COLONIES	PETITE		COLONIES COUNTED	RED COLONIES	NO. COLO- NIES TESTED	% rho
			NO. COLONIES TESTED	% rho				
0	2536	0	904	0.7	(as for control)			
18	1303	0		ND	2141	1	390	17.95
24	306	0		ND	1136	0	153	18.3
48	384	0	384	1.3	319	0	319	67.1
72	3455	0	1457	0.4	3390	4	701	88.2
168	1843	0	1117	0.3	1324	2	945	98.8

ND = not determined.

PNM/ASU class. No mutant colonies were detected out of 9827 colonies screened from the YC control culture.

It was concluded that, whilst being a potent inducer of the petite mutation, Mn^{2+} may also induce the psi⁺ to psi⁻ mutation, but at a very much lower frequency, as has been observed for mitochondrial point mutations (Putrament et al., 1973). The possibility that it was the growth inhibitory effect of Mn^{2+} per se rather than the direct mutagenic action of Mn^{2+} on /psi/ that gave rise to the psi⁻ mutants, could not be ruled out, nor can selection or a "jackpot" event.

5.4 5 Fluorouracil (5FU)

5FU induces the cytoplasmic petite mutation with a high efficiency (Moustacchi and Markovich 1963) and it has recently been suggested that the target for this mutagen is an RNA molecule, synthesised in mitochondria, which may act as a regulatory element in the maintenance of mitochondrial DNA (Oliver and Williamson 1976a,b). 5FU also induces cytoplasmic killer (K^-) mutants in yeast, the mutation always being accompanied by loss of the low molecular weight dsRNA species (P2), and sometimes by the loss of both /killer/-associated dsRNA species (P1 and P2) (Mitchell et al., 1976b). This indicates that 5FU is an efficient cytoplasmic mutagen. There is no reported evidence of 5FU inducing nuclear mutations in yeast although evidence suggests that it is mutagenic in another simple eukaryote Ustilago maydis (Lewis and Tarrant¹⁹⁷¹) and it is a very potent mutagen for RNA viruses (see Drake 1970 for references).

5FU is incorporated into RNA, in the absence of exogenous uracil, making it a potent mutagen towards RNA genomes. Its mutagenic effect on DNA genomes presumably arises indirectly by being incorporated into mRNA resulting in miscoding which, in turn, may result in malfunctioning enzymes concerned with the

replication and stability of DNA (Auerbach 1976).

Young (1969) reported on a preliminary study of the effect of growing a psi⁺ strain in 2µg/ml 5FU with no observable effects on the psi⁺ phenotype. The following is an extension of this study using much higher concentrations of 5FU and different conditions of treatment.

(i) Effect on psi⁺

The strain MT182/8d (α SUQ5 ade2.1 his5.2 leu1 psi⁺ K⁺ rho⁺) was grown to stationary phase on YC. Cells were suspended in liquid YC medium and gave 4.35×10^7 viable cells/ml and 100µl added to each of five 50ml flasks containing 9.9ml of YC liquid medium with either 0, 5, 10, 25 or 50µg/ml 5FU present. 5FU was added from a stock solution of 5mg/ml in 100% DMSO. The cultures were incubated at 28°C with constant shaking. Samples were taken after 3 and 7 days incubation and plated onto YC to give 100-300 colonies per plate. The results are given in Table 5.11.

All concentrations of 5FU were found to inhibit growth of MT182/8d but in no case did it have an effect psi⁺ phenotype of the surviving colonies. 5FU, at both low (5µg/ml) and high (50µg/ml) concentrations, was found to be a potent inducer of the petite mutation after 3 days incubation although further incubation resulted in a decline in the fraction of survivors that were petite at both concentrations. This was presumably due to a selection for mutants, resistant to petite induction by 5FU (Oliver and Williamson 1977), during prolonged growth of the culture. No significant induction of K⁻ mutants was observed after 3 days incubation at low or high 5FU concentrations, but after 7 days growth ⁱⁿ 50µg/ml, 9.1% of the surviving colonies were found to be K⁻. Of the 37K⁻ colonies scored 28 (76%) were

TABLE 5.11 The effect of growing MT182/8d in YC + 5FU over a day period on the suppressed ρ^+ and K^+ phenotypes.

5FU (µg/ml)	INCUBATION TIME (days)	NO. OF COLONIES COUNTED	VIABLE CELL NO./ml	NON-SUPPRESSED MUTANTS	PETITE TOTAL COLONIES SCREENED	% rho ⁺	KILLER	
							TOTAL COLONIES SCREENED	NO. K
0	0	3264	4.35x10 ⁵	0	1108	0.81	NT	
	3	2803	3.50x10 ⁸	0	1419	0.49	NT	
	7	3951	4.45x10 ⁸	0	1439	0.28	859	0
5	3	2456	8.60x10 ⁷	0	371	60.92	371	1
	7	1537	1.71x10 ⁸	0	921	36.48	921	2
10	3	1154	3.53x10 ⁷	0	874	94.39	874	1
	7	NT						
25	3	916	2.76x10 ⁷	0	482	98.96	303	1
	7	NT						
50	3	2980	1.75x10 ⁷	0	1174	99.57	368	0
	7	1682	1.50x10 ⁷	0	408	44.36	408	37
							201	

grande and 9 (24%) were petite. 5FU can therefore eliminate the K^+ phenotype whilst having no effect on the rho⁺ or psi⁺ phenotypes. No significant increase in the frequency of K^- survivors was observed following 7 days incubation of MT182/8d in 5µg/ml 5FU.

This experiment showed that, although a potent inducer of the petite mutation and, at high concentrations, of K^- mutants, 5FU does not mutate psi⁺ to psi⁻ at a high frequency in complete growth medium.

A second set of experiments were conducted using the uracil-requiring psi⁺ strain 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺) to determine whether 5FU could be used by this strain, instead of uracil, in defined media thus ensuring that 5FU, rather than uracil, became incorporated into the cell's RNA. Preliminary experiments showed that 507-4/2b would not grow in defined media (YNB + ade + his) containing 10µg/ml or 50µg/ml 5FU. 507-4/2b was therefore grown in such defined media but with a low concentration of exogenous uracil added (Table 5.12).

507-4/2b was grown to stationary phase in 10ml of YNB + ade + his + ura and 100µl of this culture used to inoculate each of 6, 50ml flasks containing 9.9ml of the appropriate media (Table 5.12). After 5 days growth at 28°C, with constant agitation, cells were plated onto YC. The results are given in Table 5.12. Growth of 507-4/2b was severely inhibited by 5FU at 50µg/ml in complete media whilst in defined medium the viable cell titre fell from 4.51×10^5 /ml to 2.66×10^4 /ml. 25µg/ml 5FU also inhibited growth of 507-4/2b in both types of media used compared to the control without 5FU.

Growth of 507-4/2b, in the presence of either 25µg/ml or 50µg/ml 5FU, had no effect on the psi⁺ phenotype of cells surviving in defined medium or under conditions of limited exogenous uracil

TABLE 5.12 The effect of growing a uracil-requiring psi⁺ strain (507-4/2b) for 6 days in complete or defined media containing 5FU on the rho⁺ and suppressed phenotype.*2

5FU μg/ml	GROWTH MEDIUM	COLONIES COUNTED (VIABLE CELLS/ml) *1	NON-SUPPRESSED MUTANTS	PETITE	
				TOTAL COLONIES SCREENED	% rho ⁻
0	YC	3113 (1.56x10 ⁸)	0	1584	0.06
25	YC	2990 (5.98x10 ⁷)	0	1273	20.82
50	YC	56 (5.60x10 ⁵)	0	56	89.28
0	YNB + ade +his + ura (10μg/ml)	1516 (7.58x10 ⁷)	0		NT
25	YNB + ade +his + ura (5μg/ml)	2493 (8.95x10 ⁶)	0		NT
50	YNB + ade +his + ura (1μg/ml)	875 (2.66x10 ⁴)	0		NT

NT = not tested.

*1 after 5 days growth at 28°C in liquid medium.

*2 all cultures started at 4.51x10⁷/ml (viable cells).

in defined medium.

(ii) Terminal Phenotype

During these experiments microscopic observation showed that, following incubation in 5FU, the majority of cells were present as "dumbbells". After 7 days growth of MT182/8d in YC (see Table 5.11) the control culture with no 5FU had 7 out of 128 (5.5%) cells as 'dumbbells'; with 5 μ g/ml 5FU, 54/173 (31.2%) and with 50 μ g/ml 5FU 34/47 (72.3%) of the cells were present as "dumbbells". Such a "dumbbell" phenotype is suggestive of a block in DNA synthesis (Hartwell 1973). Similar results were seen for 507-4/2b.

(iii) Phenotypic Suppression

Preliminary studies with non-suppressed mutants of the general genotypes SUQ5⁺ ade2.1 his5.2 ura3.1 psi⁺ (e.g. 465/2a, 465/4d and MT91/3b) or SUQ5 ade2.1 his5.2 ura3.1 psi⁻ (e.g. 468/1a, 507Me3 psi⁻) indicated that 5FU may cause phenotypic suppression of the nonsense mutation ade2.1 i.e. when plated onto YC + 5-20 μ g/ml 5FU (concentrations of 5FU which did not affect viability of any of these strains) the colonies that grew were white in colour. When picked and recloned on YC alone these colonies were found to be still red and ade⁻ his⁻. Phenotypic suppression by 5FU has been reported in bacteria (Rosen et al., 1969). In an attempt to confirm that 5FU was causing phenotypic suppression two follow up experiments were conducted:

(1) cell suspensions (c. 5×10^6 /ml) of 465/2a) and of 507Me psi⁻ were prepared and 100 μ l spread onto plates with the following media:

A ;	YNB	+	ura (10 μ g/ml)
or B ;	YNB	+	ura (10 μ g/ml) + 5FU (20 μ g/ml)
or C ;	YNB	+	ura (1 μ g/ml) + 5FU (20 μ g/ml)
or D ;	YNB	+	ura (1 μ g/ml) + 5FU (50 μ g/ml)

Following incubation for 14 days at 28°C no significant growth of either strain was observed on any of the plates, apart from a number of ADE⁺ revertants on plate A, which were confirmed as such by restreaking onto YC where they gave white colonies.

(2) 5FU at concentrations between 50ug/ml and 500ug/ml (dissolved in 100% DMSO) was added to 1.4cm "wells" bored in the middle of plates of YNB + ura (10ug/ml) onto which 100ul of the above suspensions of 465/2a and 507Me3 psi⁻ had been spread. After 14 days growth at 28°C no ring of growth, which would indicate phenotypic suppression, was observed for either strain.

Similar experiments were conducted using 2 thio-uracil (2TU) which is also known to suppress ochre mutations in Salmonella typhimurium phenotypically (Beck and Howlett 1977). 2TU, at concentrations up to 200ug/ml, gave no phenotypic suppression of the ade2.1 and his5.2 mutations in psi⁺ or psi⁻ strains.

It was concluded that neither 5FU nor 2TU can permit growth of a strain carrying the ade2.1 and his5.2 mutations on defined media in the absence of exogenously added adenine and histidine irrespective of whether the strain carried SUQ5 or was psi⁺ or psi⁻. It was also concluded that 5FU does not cause a high frequency of loss of the psi⁺ phenotype.

5.5 Heat

Incubating yeast at 52°C causes cell death and is mutagenic for both the nuclear and mitochondrial genome of yeast (Sherman 1956, 1959; Schenberg-Frascino and Moustacchi 1972; Evans and Parry 1975). The molecular basis of heat mutagenesis is unclear, but is believed to arise from perturbations of enzymatic reactions involved in DNA synthesis rather than from direct effects upon DNA (Drake and Baltz 1976). This gives rise to GC → AT trans-

versions in the phage T4. It has also been reported that growth at elevated temperatures (37-40°C) causes loss or mutation of at least part of the non-chromosomal /killer/ genome of yeast (Wickner 1974b).

The effect of growing cells at elevated temperatures or holding cells at 52°C, on the psi⁺ phenotype, was investigated.

(i) GROWTH AT ELEVATED TEMPERATURES

A saline suspension of an exponential culture (40% budded cells) of 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺) was plated onto YC to give 100-200 colonies per plate. These plates were then incubated at either 28°, 37° or 39°C for 5 days. The results given in Table 5.13 summarise data obtained in 3 independently conducted experiments.

Growth at 37°C did not result in the loss of the psi⁺ phenotype, but did result in a 2-fold increase in the frequency of petites. 507-4/2b is a non-killer (K⁻) strain. Growth of a psi⁺ K⁺ strain (MT182/8d see Table 5.9) at 37°C gave essentially similar results. A number of other strains of the general genotype SUQ5 ade2.1 psi⁺ were grown at 37°C during the course of this research project and in no case was an effect on psi⁺ phenotype observed. 507-4/2b and, at least 3 other psi⁺ strains tested, failed to grow at 39°C.

(ii) HEAT SHOCK (52°C)

The effect of holding psi⁺ cells at 52°C was investigated using 2 different strains, 465/1b (SUQ5 ade2.1 his5.2 ura3.1 psi⁺ K⁺) and 507-4/2b. Heat shock was performed by exposing either log or stationary phase cells, at a titre of approximately 10⁷/ml in saline, for periods of up to 80 minutes in a water bath maintained at 52 ± 0.5°C. The cell suspension was only agitated prior

TABLE 5.13 The effect of incubating a psi⁺ strain at 28°C, 37°C or 39°C on its suppressed phenotype and the frequency of petite mutants.

TEMPERATURE °C	COLONIES SCORED	% SURVIVAL	RED COLONIES	PETITE	
				TOTAL COLONIES SCORED	% rho
28	3566	100	0	1084	6.1
37	1745	100	0	1745	13.8
39	NG	0	-	-	-

NG = no growth.

to sampling. The sample (1ml) was diluted appropriately in order to give 50-300 colonies per plate upon YC. Plates with treated cells were incubated at 28°C.

Figure 5.5 demonstrates the effect of 52°C heat shock on cell viability for log phase cells of both strains and for stationary phase cells of 465/1b. Cell viability of log phase cells of both strains declined rapidly following heat shock whilst the stationary phase cells of 465/1b were much more resistant to the lethal effects of heat shock. The kinetics of cell death for the log phase cells of the two strains were slightly different with 465/1b being slightly more resistant, following short incubation periods at 52°C (up to 30 minutes), but more sensitive following incubation periods longer than 30 minutes (Figure 5.5).

For log phase cells of 465/1b holding at 52°C resulted in the appearance of a number of non-suppressed mutants among survivors (Table 5.14). 22 of the 24 mutants were recloned and subjected to complementation analysis. 18 were psi⁻ (including the mutant isolated in the control), 3 were asu and one either a PNM or ASU mutant. 21 of the mutants were K⁺ rho⁺ whilst the other mutant was K^W rho⁻ and psi⁻. Psi⁺ could therefore be mutated to psi⁻ by heat shock at 52°C whilst the cell remained grande and K⁺. An attempt to confirm this observation with a second psi⁺ strain (507-4/2b) resulted in only one non-suppressed mutant being obtained after heat shock (Table 5.14). This mutant was psi⁻. It was possible that 465/1b was more susceptible to cytoplasmic genetic damage, induced by heat shock, because petite mutants were induced much more readily in this strain as compared

FIGURE 5.5 The effect on cell viability of heat shock at
52°C in two strains , 465/1b and 507-4/2b .

- (□) 465/1b , log phase cells
- (△) 507-4/2b , log phase cells
- (■) 465/1b , stationary phase cells

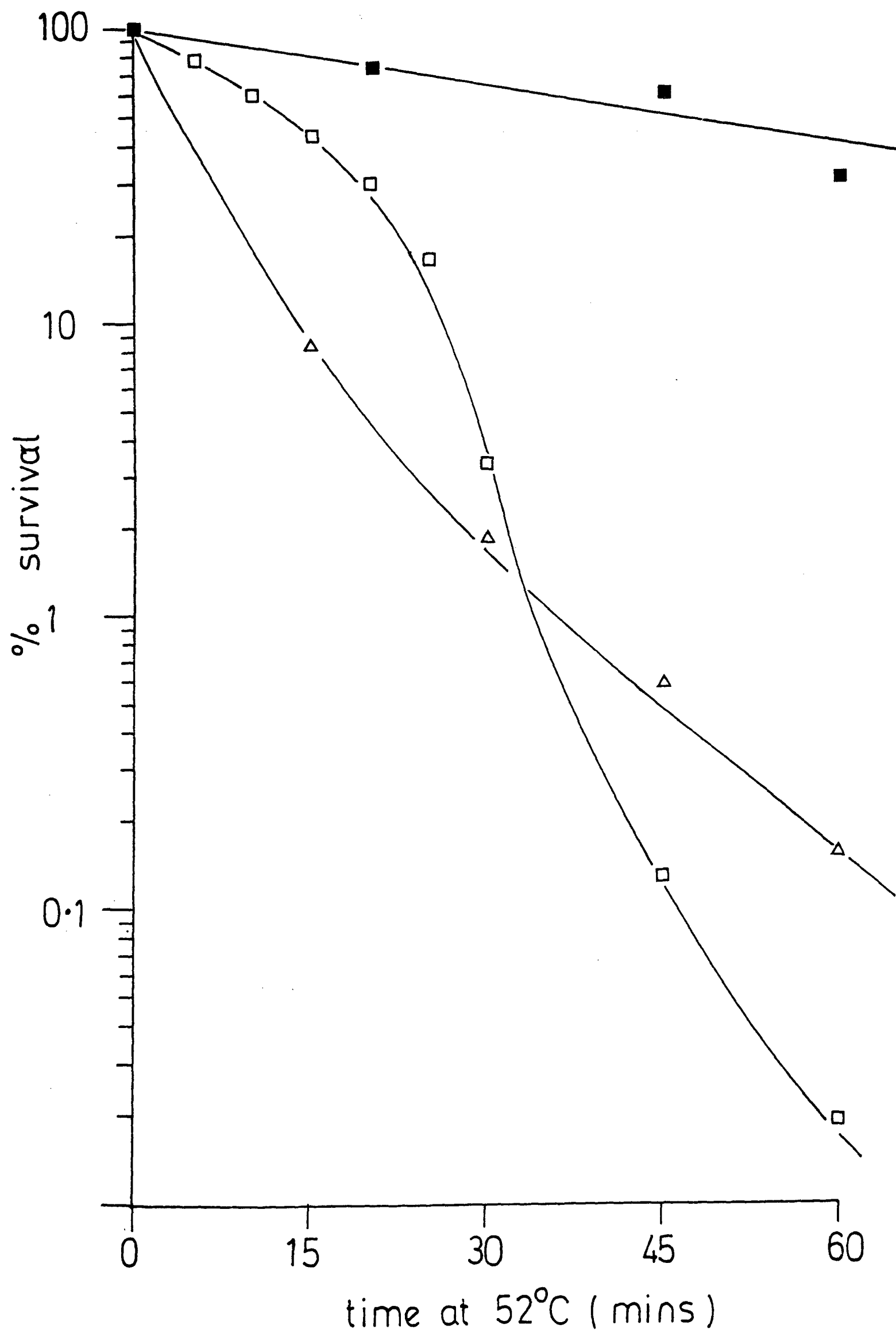


TABLE 5.14 The effect of heat shock (52°C) on cell viability and the frequency of non-suppressed mutants and petite mutants in two psi⁺ strains 507-4/2b and 465/1b.*¹

TIME AT 52°C (min)	465/1b STATIONARY PHASE				465/1b LOG PHASE				507-4/2b LOG PHASE			
	TOTAL COLONIES	SURVIVAL %	RED COLONIES	TOTAL COLONIES	SURVIVAL %	RED COLS.	% rho	% rho	TOTAL COLONIES	SURVIVAL %	RED COLS.	% rho
0	840	100	2	3 421	100	1	6.5		899	100	0	0.6
5		-		3 214	77.8	2	-			-		
10		-		2 817	62.4	1	10.9			-		
15		-		3 105	44.6	0	-		152	8.5	0	1.0
20	633	75.4	0	5 834	29.8	4	17.4			-		
25		-		1 205	16.7	1	-			-		
30		-		2 923	3.4	0	-		229	1.9	0	1.2
45	364	65.6	0	3 401	0.13	15	46.1		1 776	0.6	1	2.2
60	1 407	33.5	1	715	0.02	0	33.4		3 448	0.2	0	3.8
80	1 108	26.4	1		-					-		

- implies not tested.

*¹ the data for 465/1b is from 3 independant experiments, the remaining data is from a single experiment.

*² at least 150 colonies tested.

to 507-4/2b (Table 5.14). The fact that petite strains are more thermosensitive than grande strains (Sherman 1956; Schenberg-Frascino and Moustacchi 1972) could explain the decrease in the % of petites, among survivors in 465/1b, after reaching a maximum frequency after 45 minutes incubation at 52°C. The heat-sensitivity of petite and grande strains of 465/1b were not checked. 507-4/2b showed only a low level of induction of petites among survivors with no decline in frequency at low cell survival levels.

To determine whether psi⁻ mutants of 465/1b were more thermo-resistant than the psi⁺ parent the survival of 465/1b, and one of the psi⁻ mutants obtained in these experiments, was determined following a 30 minute exposure in saline to 52°C heat shock. Both were in log phase. The % survival was; psi⁺ 2.02%, psi⁻ 0.09%; that is the psi⁻ mutant was significantly more thermosensitive than the psi⁺ parent. This suggests that heat shock does not select for psi⁻ mutants.

It was concluded that heat shock at 52°C was probably inducing psi⁻ mutants of 465/1b (in log phase cells) but that 465/1b may be more susceptible to heat-induced cytoplasmic genetic damage than 507-4/2b.

5.6. Cycloheximide

Treatment of K⁺ strains of yeast, with sub-lethal concentrations of the protein synthesis inhibitor cycloheximide, converts them to K⁻ strains at a very high frequency (Fink and Styles 1972). They also found that such treatment gave an increase in the frequency of petite mutants (Fink and Styles 1972). Recent evidence however, indicates that petite mutants, both in vivo and in vitro are more resistant to cycloheximide than the corresponding grande

parent strain (Bilinski and Jachymezyk 1973). Growth on cycloheximide-containing medium would presumably select for the petite mutants in the population.

The effect on the psi⁺ phenotype, of growing strains on solid YC medium containing lethal concentrations of cycloheximide, is described in Chapter 8. The effect of sublethal concentrations of cycloheximide on the growth and psi⁺ phenotype of 507-4/2b, in liquid culture, are described here.

A saline suspension of log phase 507-4/2b cells (5.8×10^6 viable cells / ml) was used to inoculate 6 YC cultures containing 0, 0.2, 0.4, 0.6, 0.8 and 1.0 $\mu\text{g/ml}$ cycloheximide. Following inoculation the cultures were incubated at 28°C in a shaking water bath. 1ml samples were withdrawn from the cultures after 24 and 72 hours incubation, with a further sample being taken from the culture with 1 $\mu\text{g/ml}$ cycloheximide, after 7 days incubation. Cells were plated onto YC and colonies scored after incubation at 28°C for 5 days (Table 5.15).

5 non-suppressed mutants were obtained from a total of 10,754 colonies screened following "growth" in the presence of several different concentrations of cycloheximide (Table 5.15). Three were asu mutants, one was an SUQ5⁺ mutant and one a psi⁻ mutant. The effect of these treatments/killer on could not be determined because 507-4/2b was K⁻. The number of petite mutants among the survivors of the control culture and that containing 0.6 $\mu\text{g/ml}$ cycloheximide was counted with the following result:-

cyclohexi- imide $\mu\text{g/ml}$	INCUBATION			
	24 hours		72 hours	
	colonies	<u>petite</u> (% <u>petite</u>)	colonies	<u>petite</u> (% <u>petite</u>)
0	18	(3.7%)	3	(0.6%)
0.6	3	(1.1%)	7	(4.9%)

TABLE 5.15 Growth of a psi⁺ strain (507-4/2b) in liquid culture with cycloheximide and its effect on the frequency of non-suppressed mutants.

INCUBATION TIME--		24 HOURS			72 HOURS			7 DAYS		
CHX µg/ml	COLONIES COUNTED	RED COLONIES	VIALE CELLS/ml x 10 ⁶		COLONIES COUNTED	RED COLONIES	VIALE CELLS/ml x10 ⁶	COLONIES COUNTED	RED COLONIES	VIALE CELLS /ml x 10 ⁶
0	486	0	60.75		462	0	116.0		NT	
0.2	682	0	1.36		628	1	37.7		NT	
0.4	537	1	1.07		714	0	7.14		NT	
0.6	284	0	0.57		237	0	0.24		NT	
0.8	705	0	0.56		196	0	0.20		NT	
1.0	1926	0	0.48		4389	3	0.12	456	0	13.4

NT = not tested.

During the period of incubation no growth (i.e. no increase in viable cell number), in the culture containing 0.6 μ g/ml cycloheximide, was observed although the frequency of survivors that were petite increased fivefold. A decline in petite frequency was seen in the control flask during the same incubation period.

It was concluded that growth of a psi⁺ strain in sublethal and lethal concentrations of cycloheximide does not result in the high frequency loss of the psi⁺ phenotype, unlike its effect on the K⁺ phenotype (Fink and Styles 1972).

5.7 Discussion

A number of chemical and physical treatments known to have an extremely marked mutagenic effect on extranuclear, rather than nuclear, genes in yeast were tested for their ability to induce the psi⁺ to psi⁻ mutation. Thymidylate starvation, growth in the presence of 5FU or MnCl₂ induced the cytoplasmic petite mutation at very high frequencies (up to 100%), but in no case was such a high induction of psi⁻ mutants observed. This indicates that the loss of the grande phenotype is an event independent of loss of the psi⁺ phenotype confirming the non-identity of the /rho and /psi genomes. The results obtained with 5FU similarly indicate the non-identity of the /rho, /psi and /killer genomes because loss of the K⁺ phenotype was an event independent of loss of both the psi⁺ and rho⁺ phenotype. Any one of these three cytoplasmic genetic elements are capable, therefore, of propagating in the absence of either, or both, of the other two functional cytoplasmic genomes.

Two treatments did seem to induce significant numbers of psi⁻ mutants; holding a psi⁺ cdc8 mutant at the non-permissive temperature and heat shock of a psi⁺ strain at 52°C.

The CDC8⁺ gene product is not known, but it appears to be essential for nuclear, mitochondrial and 2μ DNA replication (Newlon and Fangman 1975; Livingston and Kup^fer 1977). It has recently been suggested that the CDC8⁺ gene does not code for an enzyme directly involved in DNA metabolism but rather affects the level of a protein binding to single-stranded DNA (F. Fabre personal communication). Loss of the psi⁺ phenotype cannot be a direct consequence of inhibiting DNA replication because no effect was seen when either a cdc21 psi⁺ or cdc7 psi⁺ strain were held at the non-permissive temperature. Nor does holding a CDC⁺ strain at 37°C have an affect on the psi⁺ phenotype. These experiments also demonstrated that holding a cdc8 mutant at the non-permissive temperature resulted in almost total loss of the rho⁺ phenotype. Until the molecular basis of the cdc8 mutation is known it is difficult to postulate why holding such a mutant at the non-permissive temperature should cause a loss of the psi⁺ or rho⁺ phenotypes. To date no effect on nuclear gene mutation by such treatment has been reported.

Exposure of the psi⁺ strain 465/1b, to heat shock, resulted in the isolation of a number of psi⁻ mutants with a maximum frequency per survivor of 4 in 10³ following 45 minutes incubation at 52°C. This compares to 46.1% of survivors being petite after the same treatment. The failure to induce a significant number of psi⁻ mutants or petite mutants in a second psi⁺ strain (507-4/2b) indicates that certain strains may be more susceptible than others to heat shock-induced cytoplasmic genetic damage.

Overall these experiments with characterised "cytoplasmic mutagens" demonstrate that the psi⁺ phenotype is not curable (i.e. is not lost at a very high frequency) by agents that "cure" both

the K^+ and grande phenotypes. This does not rule out that the $\bar{\psi}$ genome is cytoplasmically located because loss of the K^+ and grande phenotypes are generally accompanied by physical loss of part, or all, of the cytoplasmic genome. The ψ^+ to ψ^- mutation may not be a physical loss of the genome but rather a point mutation in the same class as small nucleotide substitutions, additions or deletions. Such cytoplasmic point mutations e.g. mitochondrially-coded erythromycin resistance, are induced by a number of "cytoplasmic mutagens", but at a frequency substantially lower than they induce the petite mutation (Putrament et al., 1973; Barclay and Little 1978). The limitations of the direct screening method for ψ^- mutants would prevent detection of such low, but significant levels of induction. It cannot be ruled out therefore that, for example, $MnCl_2$ is indeed inducing point mutations on the $\bar{\psi}$ genome rather than causing physical loss of the $\bar{\psi}$ genome per se.

The finding of chemical and physical agents that are not nuclear mutagens, but induce a high frequency of loss of the ψ^+ phenotype (i.e. "cure" ψ^+), would argue against the induction of only point mutations on the $\bar{\psi}$ genome being responsible for the ψ^- phenotype. During the course of this research several chemical agents were found with this ψ^+ "curing" property and are reported on in the next chapter.

5.8 Summary

A number of treatments known to be mutagenic for the cytoplasmic $\bar{\rho}$ and $\bar{k}$ determinants had no effect on the ψ^+ phenotype. Those tried were thymidylate starvation and growth in the presence of either $MnCl_2$, 5FU or sublethal concentrations of

cycloheximide. Heat shock at 52°C induced psi⁻ mutants in one of the two psi⁺ strains tested. Holding a cdc8 psi⁺ mutant at the non-permissive temperature for up to 30 hours, before returning to the permissive temperature, gave rise to a high frequency of psi⁻ mutants. Holding three other ts cell division cycle mutants, cdc4, cdc7 and cdc9, at the non-permissive temperature for 24 hours, had no effect on the psi⁺ phenotype.

CHAPTER 6

/P̄si/ MUTAGENESIS : CURING OF THE psi⁺ PHENOTYPE

6.1 Introduction

6.2 Guanidine Hydrochloride

6.3 Dimethyl Sulphoxide

6.4 Miscellaneous treatments that eliminate the psi⁺
phenotype at a high frequency

6.5 Discussion

6.6 Summary

6.1 Introduction

As discussed in Chapter 5 a number of chemical and physical agents can induce cytoplasmic mutations at very high frequencies (up to 100%). The maximum mutation induction frequency of any nuclear gene is typically of the order of 10^{-4} and this is usually obtained at mutagen concentrations giving 1-5% survival.

In yeast the cytoplasmic petite mutation can be induced at frequencies of 100% by non-lethal concentrations of specific mutagens including the acridine dye acriflavine (Ephrussi et al., 1949), the DNA-intercalating agent ethidium bromide (Slonimski et al., 1968), the trypanocidal drug berenil (Mahler and Perlman 1973) and the protein denaturing agent, guanidine hydrochloride (Juliani et al., 1973). The 'mutation' is accompanied by either a gross physical change in the mitochondrial DNA, the mutation being designated ρ^- or ρ^- , or complete loss of the mitochondrial DNA when it is called ρ^0 or ρ^0 (see Nagley et al., 1977 for a review). High frequencies of induction of cytoplasmic non-killer mutants (K^-) of yeast can be achieved with a number of chemical and physical agents (Fink and Styles 1972; Wickner 1974a; Naumova and Naumov 1974; Mitchell et al., 1976b). These mutants always show loss of the /killer/-associated P2 dsRNA and sometimes loss of both the P2 and P1 /killer/ - associated dsRNA's (Mitchell et al., 1976b; Sweeney et al., 1976).

Curing of a phenotypic (which will be defined here as 'induced, high frequency loss of a cytoplasmically inherited phenotype') is a characteristic of extrachromosomal elements of yeast and is usually accompanied by physical loss of part or all of the cytoplasmic genome. Curing of extrachromosomal elements

is also known in bacteria. Acriflavine orange (Hirota 1960) sodium dodecyl sulphate (Tomoeda et al., 1968) and thymine starvation (Tribe and Pinney 1977) all eliminate F or R plasmids from E. coli.

To date no effective curing agent has been reported for the psi⁺ phenotype. Several agents known to induce petite or K⁻ mutants at a high frequency do not affect the psi⁺ phenotype (see Chapter 5). Finding an agent that efficiently cured cells of their psi⁺ phenotype would greatly assist in the search for an extrachromosomal nucleic acid species associated with the /psi genetic determinant and would be further evidence for the extrachromosomal location of /psi.

This chapter describes several chemical agents that were found to eliminate the psi⁺ phenotype at high frequencies. In order to determine whether these agents were inducing the loss of the psi⁺ phenotype, rather than selecting for the mutant psi⁻, three criteria, indicative of induction, were looked for:

i) selection against the mutant phenotype. An agent which selectively inhibited the growth of the non-suppressed phenotype would be unable to select for that phenotype in a cell population.

(ii) the occurrence of sectored mutant colonies after exposure to the drug. It is difficult to envisage how cells giving rise to clones with a mutant sector could be selected for.

(iii) mutants in media which they cannot proliferate i.e. under conditions where only the suppressed non-mutant cells would grow.

6.2 Guanidine Hydrochloride

Guanidine hydrochloride (GuHCl) is a protein denaturing agent that is a potent inducer of cytoplasmic petites of the suppressive type in yeast and belongs to the class of agents that are effective petite inducers in both growing and non-growing cells (Juliani et al., 1973, 1975a). It also eliminates the penicillinase plasmid of Staphylococcus aureus (unpublished data cited in Giuliani et al., 1975b) and thus belongs to the class of petite - inducing agents that also eliminate extra-chromosomal elements from bacteria. Other such agents include ethidium bromide (Bouandchaud et al., 1968) and acriflavine (Hirota 1960).

An effect of GuHCl on the psi⁺ phenotype was originally observed during an extensive search for psi⁺ curing agents using a modified well test (see Materials and Methods). Stationary phase cells of 507-4/2b (α SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺ K⁻) were suspended in saline, diluted appropriately, and plated onto YC to give approximately 300-500 colonies/plate. 100ul of the chemical under test, in this case 0.1M and 0.5M GuHCl, was added to a 1.2cm diameter well bored in the centre of the plate and the plate incubated for 5 days. If any effect on the suppressed phenotype of the colonies near the well was observed the colonies were replica-plated onto fresh YC plates to determine whether the effect was phenotypic or was inherited.

GuHCl was dissolved in water and filter sterilised rather than autoclaved.

GuHCl, 0.1M gave no zone of killing but colonies up to 1.7cm from the well centre were red. 0.5M GuHCl gave a small zone of

killing (2.3cm diameter) with all colonies up to 3.2cm from the well centre being red. When replica plated onto YC the red colonies were found to be mosaic; they grew with large numbers of alternating red and white sectors. 11 such colonies were cloned on YC and a stable red mutant colony from each examined. All were ade^- his^- can^r and complemented as psi⁻ mutants. 2 of the 11 were petite. No red colonies were present amongst 471 colonies on the control plate even after replica plating.

Identical "well test" results were obtained using the unrelated SUQ5 ade2.1 his5.2 leu1 psi⁺ K⁺ strain MT182/8d showing that the effect was not specific to 507-4/2b.

i. Growth of Psi⁺ strains on YC agar + GuHCl medium

Saline suspensions of 507-4/2b and MT182/8d stationary phase cells were prepared and cells plated onto solid YC containing several concentrations of GuHCl to give 100-300 colonies per plate. After incubating for 5 days the plates were examined with the results given in Table 6.1. Figure 6.1 illustrates the colony phenotypes present on the plates.

In both strains growth on YC + > 1mM GuHCl resulted in a significant loss of the suppressed phenotype. Some of the mosaic and whole red colonies were replica plated onto fresh YC plates with no change in colony phenotype. At 1mM GuHCl almost 100% of the colonies arising in both strains were mosaic with the white sectors growing faster overall than the red sectors in the colony (Figure 6.1). This suggests that red non-suppressed cells had no selective advantage over suppressed cells on GuHCl medium.

TABLE 6.1 Survival of 507-4/2b and MT182/8d plated on solid YC + GuHCl medium and the phenotypes of colonies that grew.

507-4/2b										MT82/8d			
GuHCl mM	Colonies Counted	% Survival	PETITE			Phenotype of Colonies	Colonies Counted	% Survival	Total Colonies Scored	PETITE			Phenotype of Colonies
			% Survival	Colonies Scored	%rho					%rho	Colonies Scored	%rho	
0	2051*	100		1281	0.5	ALL WHITE	856*	100	239	2.1			ALL WHITE
0.5	545	94.0		340	0.3	ALL WHITE	NT		NT				-
1	1755*	95.4		1378	0.7	1751 MOSAIC 4 WHITE	351	94.6	351	2.6			ALL MOSAIC
2.5	681	97.2		681	2.1	ALL RED	527	85.2	527	6.8			526 RED 1 MOSAIC
5	548	78.2		352	38.9	ALL RED	528	85.4	349	7.2			ALL RED
7.5	NT				NT		494	79.9	332	13.6			ALL RED
10	2	0.7		2	100	BOTH RED	3	0.5	3	100			ALL RED
50	0	0.001					NT					NT	

NT = not tested.

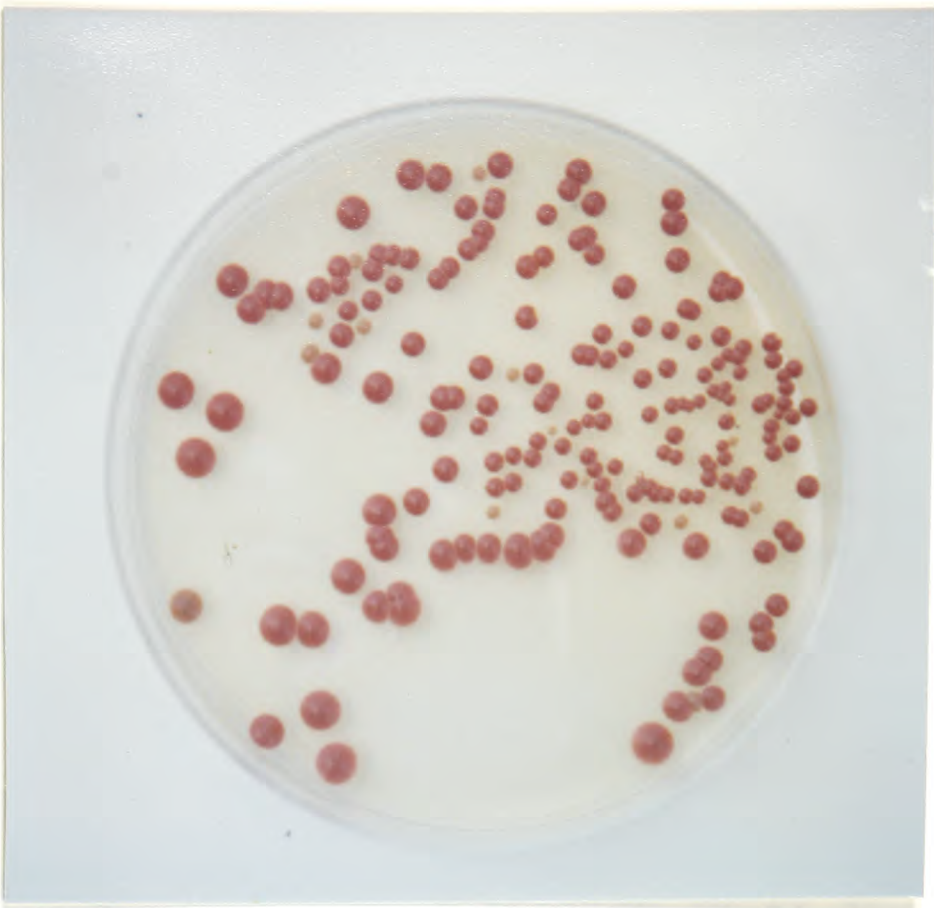
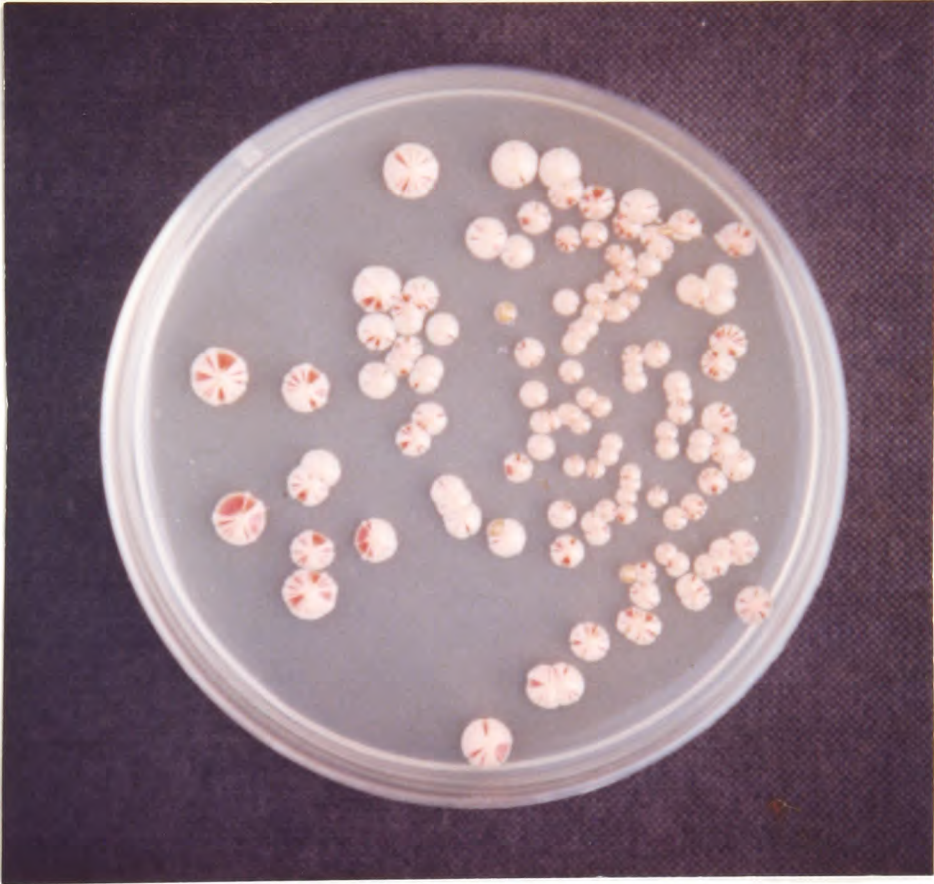
* total from 2 different experiments.

FIGURE 6.1 Colony phenotypes of MT182/8a grown on YC plates with or without GuHCl .

(b) YC + 1mM GuHCl



(a) YC



(c) YC + 5mM GuHCl

Growth of both strains was severely inhibited at concentrations $\gg$ 10mM GuHCl. At GuHCl concentrations that resulted in all colonies losing the suppressed phenotype e.g. 2.5mM, no significant induction of petite mutants was observed (Table 6.1) although at higher concentrations petite mutants were induced. At concentrations $>$ 5mM all colonies showed large numbers of petite sectors. The effect GuHCl had on the killer (K^+) phenotype of MT182/8d was measured; no K^- colonies were observed among 110 colonies arising on YC or among 196 colonies arising on YC + 5mM GuHCl. 2 K^- mutant colonies were present among 332 colonies growing on YC + 7.5mM GuHCl.

The 4 wholly white 507-4/2b colonies arising on the 1mM GuHCl plates (Table 6.1) were each suspended in saline and plated onto YC, YC + 1mM and YC + 2.5mM GuHCl. All 4 gave exclusively white colonies on YC, mosaic colonies on YC + 1mM GuHCl and wholly red colonies on YC + 2.5mM GuHCl. These were not therefore mutants resistant to loss of the suppressed phenotype in the presence of GuHCl.

12 wholly red colonies of each strain growing on YC + 2.5mM GuHCl were picked and analysed. All were ade^- his^- and complemented as psi⁻. Also 3 of the mosaic colonies of 507-4/2b from YC + 1mM GuHCl were suspended in saline, sonicated and replated onto YC. The phenotypes of colonies arising indicated (Table 6.2) that the mosaic colonies were a mixture of stable red and white clones. GuHCl does not induce a self-replicating unstable state. In all 3 mosaic colonies at least 95% of the cells gave rise to pure mutant clones with the red colonies being in excess of the pure white colonies. 5 red colonies from each mosaic were shown, by complementation analysis, to be

TABLE 6.2 The number of colonies of different phenotypes arising from mosaic colonies of 507-4/2b, taken from plates of YC + 1mM GuHCl, replated on YC. (Figures in brackets are the percentage in each class.)

MOSAIC COLONY	PHENOTYPES OF MUTANT COLONIES ON YC				
	RED	WHITE	SECTORED RED/WHITE	MOSAIC	TOTAL
M1	100	59	4	1	152
	(57.9)	(38.8)	(2.6)	(0.6)	(100)
M2	69	53	3	2	127
	(54.3)	(41.7)	(2.4)	(1.6)	(100)
M3	201	163	14	2	380
	(52.9)	(42.9)	(3.7)	(0.5)	(100)

psi⁻. 5 white colonies from each replating were crossed to 584/1d (SUQ5 sal3.5 PNM2 psi⁻). All showed strong complementation. Upon restreaking onto YC + 2.5mM GuHCl all gave red colonies. Both these tests indicate that they were psi⁺. The sectorred red/white colonies had either $\frac{1}{4}$ or $\frac{1}{2}$ red sectors present.

A series of standard well tests (see Materials and Methods) were conducted with 507-4/2b or a methanol-induced psi⁻ strain 507 Me3psi⁻ to determine whether there was a significant difference in response between isogenic psi⁺ and psi⁻ strains to growth inhibition by GuHCl. A concentration range of 5-500mM was used but no significant difference was observed (Figure 6.2). This confirms that psi⁻ strains have no selective advantage over psi⁺ strains in the presence of GuHCl on solid growth medium.

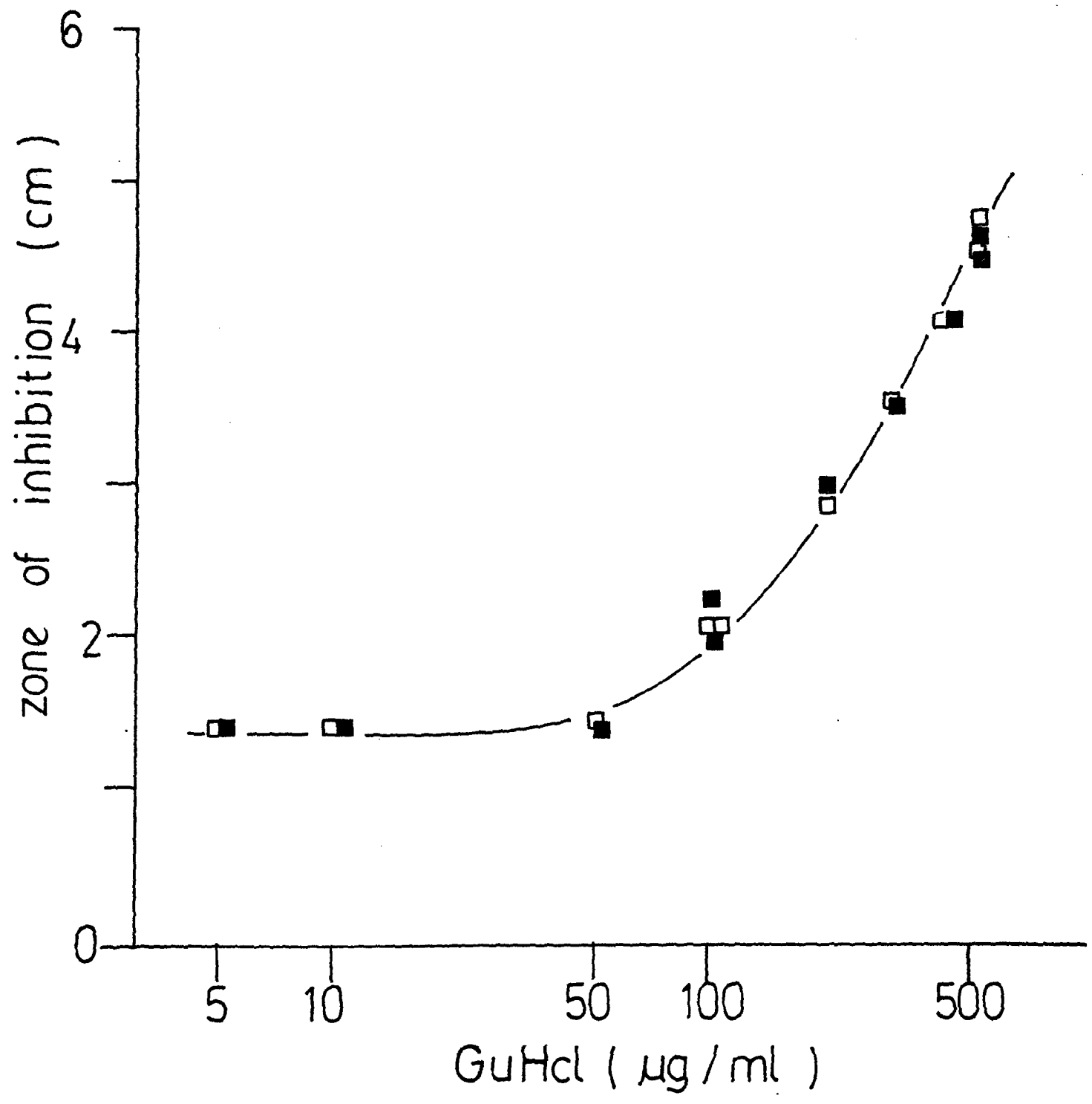
Stationary phase cells of the SUQ5⁺ ade2.1 psi⁺ strain 465/2a were plated onto YC and YC + 1mM GuHCl to determine whether the psi⁺ phenotype could be lost in strains that are not suppressed. 16 of the 214 colonies arising on YC + 1mM GuHCl (91.1% survival) were crossed to two different α SUQ5 ade2.1 psi⁻ strains (468/1d, 483/1d). Only one complemented these two strains indicating that this was psi⁺ and the remaining 15 colonies were psi⁻. 10 colonies taken from the YC control plate complemented both testers. Thus the psi⁺ phenotype can be cured by growth on YC + 1mM GuHCl in strains that do not carry a suppressor.

ii. Growth of Psi⁺ strains in liquid YC + GuHCl cultures

The ability of GuHCl to cure 507-4/2b of the psi⁺ phenotype during growth in liquid culture was investigated. 10ml YC cultures, inoculated with stationary phase cells to a final titre

FIGURE 6.2 Results of a well test comparing the growth inhibition by GuHcl of isogenic psi⁺ and psi⁻ strains (507-4/2b and 507Me3).

(□) 507-4/2b psi⁺
(■) 507Me3 psi⁻



of 1×10^5 cells/ml, and containing either 0mM, 5mM or 50mM GuHCl, were incubated for 5 days. Cells were then plated onto YC to determine the viable cell titre and the frequency of non-suppressed mutants. The petite frequency was also estimated. Results are given in Table 6.3.

5mM GuHCl had only a slight inhibitory effect on growth whilst 50mM GuHCl caused a $\times 10^5$ reduction in the viable cell titre compared to the YC control culture. The frequency of non-suppressed mutants was significantly higher in the 50mM culture than in the 5mM culture. Both concentrations of GuHCl gave mosaic and sectored mutant colonies with the proportion of cells, giving wholly mutant colonies, being less in the 50mM culture (41.8%) than in the 5mM culture (83.9%). The frequency of petite colonies (2.7%) was approximately the same as the frequency of non-suppressed mutant colonies arising from the 5mM culture (2.1%) whilst in the 50mM culture the frequency of petites (6.5%) was half that for non-suppressed mutant colonies (12.5%). The curing of the psi⁺ phenotype was therefore more efficient than curing of the grande phenotype.

25 of the whole mutant colonies were analysed, all were ade⁻ his⁻ can^r and complemented as psi⁻. 4 were petite. The suppressed sectors of $5 \frac{1}{2}$ red, $\frac{1}{2}$ white sectored colonies were each recloned, on YC, and found to be stable for the suppressed phenotype upon this, and at least 2 subsequent, reclonings. The $\frac{1}{2}$ sectored colonies were therefore not mutant suppressed colonies with a tendency to segregate psi⁻ sectors at a high frequency.

iii. Experiments to determine whether GuHCl induces or selects psi⁻ mutations

The evidence so far presented indicates that there is true induction of psi⁻ mutants; namely the occurrence of sectored

TABLE 6.3 The number of colonies scored and the number of non-suppressed colonies found after growth of 507-4/2b in YC with or without GuHCl.

GuHCl mM	COLONIES SCORED	VIABLE CELL TITRE	NO. NON-SUPPRESSED COLONIES				% MUTANT COLONIES	TOTAL COLONIES SCORED	% rho
			WHOLE SECTORED			MOSAIC			
			RED	RED/WHITE					
CONTROL 0	2748	2.29x10 ⁸ /ml	0	1		0	0.04	1343	0.1
5mM	2537	1.27x10 ⁸ /ml	47	1		8	2.21	930	2.7
50mM	1266	6.10x10 ³ /ml	66	45		47	12.48	1208	6.5

mutant clones and the lack of selective advantage of psi⁻ mutants. To confirm that psi⁻ mutants are induced by GuHCl rather than selected by it MT182/8d (SUQ5 ade2.1 his5.2 leu1 psi⁺) was exposed to GuHCl in liquid YNB + leu. This medium only supports the growth of fully suppressed strains. A psi⁻ or any other non-suppressed mutant would not grow because of their requirement for exogenous ade and his.

MT182/8d was grown to stationary phase in a 10ml YNB + leu liquid culture. The viable cell titre was 2.5×10^6 cells/ml. 100 μ l aliquots of this culture were used to inoculate flasks containing 10ml of YNB + leu liquid medium containing either 0, 1mM or 2.5mM GuHCl, flasks containing 10ml of YNB + ade + his + leu with either 0, 1mM or 2.5mM GuHCl and flasks containing 10ml of liquid YC with either 0, 1mM or 2.5mM GuHCl. The cultures were incubated at 28°C, with constant shaking, for 7 days. Samples were plated onto YC to determine the viable cell titre and frequency of non-suppressed mutants. The frequency of petite and K⁻ mutants in each culture was also determined. The results are shown in Table 6.4.

Several conclusions can be drawn from the data obtained:

(a) under conditions which do not allow a non-suppressed revertant to grow 2.5mM GuHCl in the medium resulted in 3% of the cell population being non-suppressed mutants. 10 of the wholly non-suppressed colonies from YNB + leu + 2.5mM GuHCl were analysed and all complemented as psi⁻ mutants,

(b) compared to 507-4/2b (see Table 6.3) MT182/8d appeared to be more susceptible to loss of the psi⁺ phenotype in liquid medium + GuHCl and similarly more susceptible to petite induction. Growth of MT182/8d in the presence of GuHCl was also inhibited to a far greater extent than for 507-4/2b in liquid medium but

TABLE 6.4 The number of colonies scored and the number non-suppressed colonies found after growth of 507-4/2b in defined media with or without GuHCl.

MEDIUM+GuHCl (mM)	COLONIES SCORED	VIABLE CELL TITRE/ml	NO. NON-SUPPRESSED COLONIES					% MUTANT COLONIES	PETITE		KILLER	
			SECTORED						TOTAL COLONIES SCORED	% rho ⁻	TOTAL COLONIES SCORED	NO. K ⁻
			WHOLE RED	RED/ WHITE	MOSAIC							
YC	3799	1.80x10 ⁸	0	0	0		0.03	1494	0.3	705	0	
YC + 1mM	1755	5.06x10 ⁷	1230	3	0		70.3	418	22.7	418	0	
YC + 2.5mM	23	42	1	0	0		4.3		NT		NT	
YNB + leu	1963	3.08x10 ⁶	0	0	0		0.05	735	0.4	309	1	
YNB + leu + 1mM	3330	5.64x10 ⁶	3	0	0		0.09	345	0.6	245	0	
YNB + leu + 2.5mM	1992	1.99x10 ⁶	43	18	0		3.1	425	28.2	425	14	
YNB + ade + his + leu + 1mM	593	1.07x10 ⁶	1	0	0		0.2	200	0.5	280	0	
YNB + ade + his + leu + 2.5mM	681	1.36x10 ⁶	104	15	2		17.7	412	2.4	273	0	

the two strains were equally sensitive to GuHCl on solid medium (see Table 6.1).

(c) the proportion of mosaic colonies among the non-suppressed mutant colonies of MT182/8d (0.14%) was significantly less than observed following growth of 507-4/2b in complete medium with GuHCl (25.6%). The GuHCl concentration was higher for the 507-4/2b experiments. MT182/8d is a non-clumping strain whilst 507-4/2b tends to form 10-50/^{cell} aggregates during growth in liquid media. As no sonication was employed prior to plating either of these strains in these experiments, the mosaics found for 507-4/2b were probably a consequence of mutant - non-mutant cell adhesion. This appears to be confirmed by the results presented in Table 6.2 where the mosaic colonies were sonicated prior to replating on YC.

(d) GuHCl inhibited growth of MT182/8d to a greater extent in YC than in a defined medium. The YNB + leu + GuHCl cultures gave higher viable cell titres after 7 days growth than the YNB + ade + his + leu + GuHCl cultures. The viable cell titre of MT182/8d, in YNB + leu with no GuHCl, was significantly less than for the YC culture.

(e) a higher frequency of K⁻ mutants were present in the YNB + leu + 2.5mM culture ($\frac{14}{425} = 33\%$) than in the YNB + leu or YC cultures without GuHCl ($\frac{1}{1014} = 0.1\%$). The frequency of petite mutants in the YNB + leu + 2.5mM GuHCl culture (28.2%) was also significantly higher than in the two cultures without GuHCl (0.4%).

15 wholly mutant colonies from YC + 1mM GuHCl all complemented as psi⁻ mutants. Of the 36 sectored colonies obtained in these experiments 4 were red sectors, 19 were $\frac{1}{2}$ red sectors, 8 were $\frac{3}{4}$ red sectors and 5 were "hairline" red sectors (i.e. $< \frac{1}{4}$ red).

Figure 6.3 illustrates the different classes of sectored colonies obtained following growth of MT182/8d in YNB + ade + leu + 2.5mM GuHCl. 8 of the $\frac{1}{2}$ red, $\frac{1}{2}$ white sectored colonies were recloned on YC and both the red and white sectors examined. The red sectors of all 8 mutant colonies were psi⁻ whilst the corresponding white sectors all complemented the standard SUQ5 ade2.1 sal3.5 PNM2 psi⁻ strain 584/1d and gave red colonies on sub-cloning on YC + 2.5mM GuHCl plates. These tests indicate they were psi⁺.

Of 1230 presumptive psi⁻ mutants, obtained from the YC + 1mM GuHCl culture, 569 (46.3%) were petite although in the overall population the petite frequency was 22.7%. This suggests that, at least in YC, if a cell becomes psi⁻ it has a greater chance of becoming petite or vice versa.

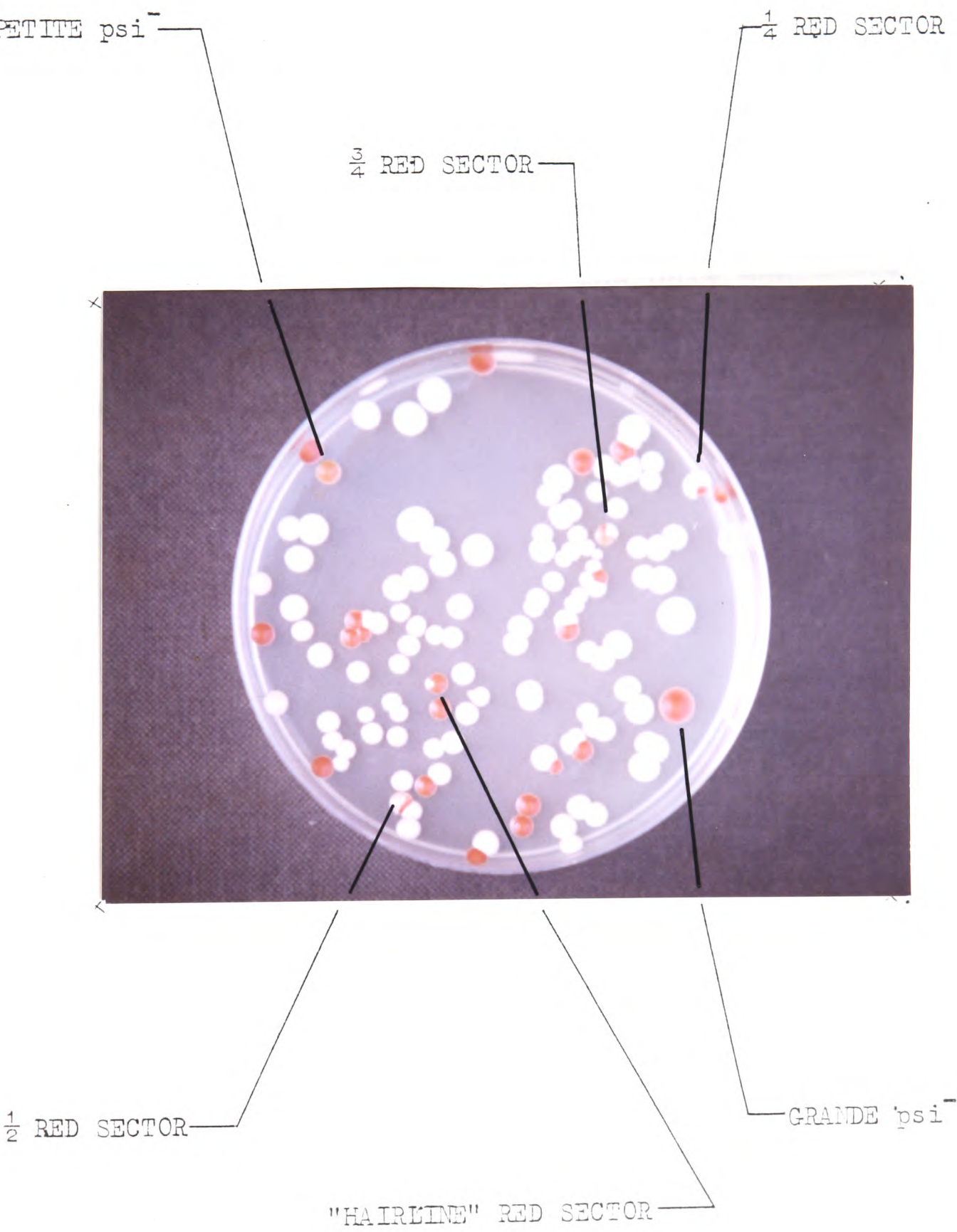
425 colonies arising from cells grown in the YNB + leu + 2.5mM GuHCl medium were classified with respect to their cytoplasmic phenotypes. The following classes and numbers were found:

<u>CLASS</u>	<u>NUMBER OBSERVED</u>
K ⁺ rho ⁺ psi ⁺	293
K ⁺ rho ⁻ psi ⁺	111
K ⁺ rho ⁻ psi ⁻	2
K ⁺ rho ⁺ psi ⁻	5
K ⁻ rho ⁺ psi ⁺	7
K ⁻ rho ⁻ psi ⁺	7

This is further circumstantial evidence for the non-identity of these 3 cytoplasmic genomes. None of the 425 colonies were K⁻ rho⁻ psi⁻.

To summarise, GuHCl does induce psi⁻ mutants in both defined or complete medium and is independent of loss of the K⁺ or grande phenotypes.

FIGURE 6.3 Sectored colonies obtained by growing MT182/8d in defined medium with 2.5mM GuHCl.



iv. Genetic analysis of GuHCl-induced psi^- mutants

11 non-suppressed mutants of MT182/8d, 6 isolated as whole mutant colonies and 5 as sectored mutant colonies obtained from the cell population grown in YNB + leu + 2.5mM GuHCl, were subjected to standard complementation analysis. All were psi^- . To confirm the complementation data each mutant was crossed to 465/2a ($\text{SUQ5}^+ \text{ade2.1 psi}^+$), zygotes isolated, and tetrads dissected from the resulting diploids. Suppressed diploids were obtained for each mutant and tetrad analysis (Table 6.5) demonstrated that all 11 mutants were truly psi^- . As described in Chapter 3 psi^- mutants are the only non-suppressed mutants of MT182/8d that would give rise to a 2 suppressed: 2 non-suppressed spore segregation when crossed to 465/2a, although 4 of them (G1, G2, G5 and G8), gave at least 1 tetrad with 3 non-suppressed spores.

v. Effect of GuHCl on the psi^+ phenotype of MT182/8d in non-growth conditions

A 10ml saline suspension of stationary phase cells of MT182/8d was made 2.5mM for GuHCl. The suspension was incubated at room temperature with samples being taken at 3 and 7 days and plated onto YC (Table 6.6). No high frequency of psi^- mutants was observed although only a small number of colonies were scored because of the low viable cell titres. In YNB + leu + 2.5mM GuHCl (see Table 6.4) 3% of the colonies obtained after 7 days incubation were psi^- . It was tentatively concluded that growth of a psi^- strain is required for GuHCl to induce psi^- mutants. Some petite mutants were found after the incubation in saline. It has been previously reported that GuHCl can induce

TABLE 6.5 Genetic analysis of GuHCl-induced ψ^- revertants of MT182/8d x 465/2a.

ψ^- MUTANT NO.	TETRAD ANALYSIS			SUPPRESSED:NON-SUPPRESSED			SPORE* TOTALS S:NS
	NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRAD	3:1	2:2	1:3	
WHOLE	G1	100	10	0	9	1	19:21
	G2	97.5	10	0	7	2	18:21
	G3	92.5	10	0	7	0	20:17
	G4	100	9	0	9	0	18:18
	G5	90.6	8	0	5	1	16:13
	G6	100	6	0	6	0	12:12
SECTORED	G7	100	7	0	7	0	14:14
	G8	88.5	13	0	8	1	22:24
	G9	100	6	0	6	0	12:12
	G10	100	12	0	7	0	14:14
	G11	87.5	12	0	8	0	22:20

TABLE 6.6 The effect of 2.5mM GuHCl on the psi⁺ and grande phenotypes of MT182/8d held in saline for up to 7 days.

INCUBATION TIME (days)	NO. OF COLONIES SCORED	VIABLE CELL TITRE/ml	RED COLONIES	PETITE	
				TOTAL COLONIES SCORED	%rho ⁻
0	367	2.2x10 ⁵	0	229	1.3
3	530	2.27x10 ³	0	452	4.9
7	263	4.38x10 ²	0	NT	

petites in non-growing cells (Juliani et al., 1973).

(vi) Effect of GuHCl on other suppressed strains

To check that GuHCl only affected the suppressed phenotype of SUQ5 ade2.1 psi⁺ strains and not other psi⁻, suppressed strains, saline suspensions were prepared for each of three different SUQ5 ade2.1 psi⁺ strains (MT182/8d, 466/4a and MT193/3a), 562/2a (SUP2.1 ade2.1 lys1.1 ura3.1 psi⁻), X2315-14C (SUP3.1 ade2.1 his5.2 lys1.1 trp5.48 can1.100 ura3.1 psi⁻) and five strains each of which carried a different sal mutation (Cox 1977b) in an SUQ5 ade2.1 psi⁻ background. 10ul aliquots of each suspension were spotted onto YC and YC + 1, 2.5, 5, 7.5 or 10mM GuHCl and the plates incubated for 5 days. At all concentrations of GuHCl the SUQ5 ade2.1 psi⁺ strains grew as red colonies on the YC + GuHCl medium. The sal, SUP2 and SUP3 strains retained their white suppressed phenotypes on all six media. Similar spot tests were carried out with MT35 (an α/a SUQ5/SUQ5 ade2.1/ade2.1 psi⁺ diploid) and an α/α (HM2) and a/a (HM3) mutant of MT35 (see Chapter 3) and four diploids with the genotype α/a SUQ5/SUQ5 ade2.1/ade2.1 psi⁻ salx/salx (revertans of M7, see Chapter 7). Only the psi⁺ diploids were red on YC + GuHCl medium and the non-suppressed phenotype was maintained on replating onto YC. This suggests that GuHCl can also revert diploid psi⁺ strains to psi⁻.

(vii) Other protein-denaturing agents

The detergents sodium dodecyl sulphate (SDS) and sodium-N-palmitoyl sarcosinate (SARKOSYL) are two other protein-denaturing agents which induce petite mutations (unpublished data cited in Giuliani et al., 1975b). In the modified well test system described, no effects on the psi⁺ phenotype of 507-4/2b were observed when either 20% (W/V) SDS or 35% (W/V) SARKOSYL sol-

utions were present in the well although both gave a zone of killing (4.3cm and 4.2cm respectively).

(viii) Summary

The protein denaturing agent guanidine hydrochloride was found to revert strains from psi⁺ to psi⁻ at very high frequencies, both in liquid medium (complete or defined) and on solid medium (complete). This involved induction rather than selection of psi⁻ mutants and required growth of the psi⁺ strain. It was observed that cells obtained from populations in liquid medium could give rise to both whole psi⁻ mutant colonies or part psi⁺, part psi⁻ sectored mutant colonies.

6.3 Dimethyl Sulphoxide

Dimethyl sulphoxide (DMSO) is commonly used in the laboratory as a cryoprotective agent and as an organic solvent. No effect of DMSO on cytoplasmic or nuclear mutation in any organism has been reported. It is known to protect S. cerevisiae from UV-induced death and mutation (Singh et al., 1976).

During an analysis of the sensitivity of the psi⁺ strain 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺) to various ribosomal drugs a control was run, using the modified well test previously described, with 100% DMSO, a solvent used for one of these drugs, in the well. A 2.7cm diameter zone of inhibition was obtained. When the plate was replica-plated onto a fresh YC plate it was found that all viable clones, within the zone of inhibition, were non-suppressed. 7 such colonies were picked, recloned on YC and tested; all 7 were ade⁻ his⁻ can^r rho⁻ and complemented as psi⁻. The effects of DMSO on the psi⁺ phenotype were therefore investigated.

(1) Growth of psi⁺ strains in liquid YC + DMSO cultures

Three strains of the general phenotype SUQ5 ade2.1 psi⁺

(507-4/2b, MT182/8d, MT141-4/6d), and an ade2.1 psi⁻ strain carrying the /psi⁻ - independent ochre suppressor SUP2.1 (562/2a), were each grown in 10ml YC cultures containing 5%-20% (V/V) DMSO. Stationary phase cells were used to inoculate the cultures with a starting cell titre of approximately 2×10^5 viable cells/ml. After 6 days incubation samples were plated onto YC and the colonies that arose scored for their suppressed phenotype (Table 6.7).

Growth of two of the SUQ5 ade2.1 psi⁺ strains (MT182/8d and 507-4/2b), in YC+DMSO, resulted in the appearance of a high frequency of non-suppressed mutant clones, even at DMSO concentrations that had very little effect on growth. The suppressed phenotype of MT141-4/6d was significantly more stable during growth in YC + 5% DMSO than the other two SUQ5 ade2.1 psi⁺ strains. When approximately 10,000 colonies per plate were grown from this population the frequency of red colonies was approximately 10^{-3} . The SUP2.1 strain 562/2a was completely stable with no non-suppressed colonies being observed even after plating cells at a density of 10^4 per plate.

25 mutant colonies were picked from YC plates following growth in YC + 20% DMSO, together with 24 mutant colonies of MT182/8d following growth in YC + 5% DMSO, were characterised. All were ade⁻ his⁻ and complemented as psi⁻. Of the 24 MT182/8d mutants, 3 were petite, but all remained K⁺. 20 mutant colonies of 507-4/2b were also picked following growth in YC + 5% DMSO; 15 were psi⁻ with the remaining 5 being asu mutants. No mosaic clones arose following growth of 507-4/2b or MT182/8d in YC + DMSO.

It is not clear from these results whether DMSO is selecting against the psi⁺ phenotype, against the suppressed phenotype or

TABLE 6.7 The viable cell titre and frequency of non-suppressed mutant colonies, obtained after 6 days growth in YC + DMSO liquid medium, of 4 different suppressed strains.

STRAIN	%(^v / _v) DMSO	COLONIES COUNTED	VIABLE CELL TITRE /ml	NO. MUTANT COLONIES		% MUTANT COLONIES
				WHOLE	SECTORED	
507-4/2b	0	701	5.6x10 ⁷	0	0	0.14
	5	187	3.7x10 ⁷	20	1	11.2
	10	478	6.2x10 ³	157	8	34.5
	20	362	3.5x10 ³	256	4	71.8
MT182/8d	0	1371	1.9x10 ⁸	0	0	0.07
	5	782	1.6x10 ⁸	65	0	8.3
MT141-4/6d	5	1291	7.8x10 ⁷	0	0	0.08
562/2a (SUP2)	0	1210	2.4x10 ⁸	0	0	0.08
	5	1410	1.4x10 ⁸	0	0	0.07

is inducing psi⁻ mutants at a high frequency. The failure to obtain non-suppressed revertants of the SUP2 strain suggests that it is not selecting against the suppressed phenotype per se.

(ii) Growth of psi⁺ strains on YC agar + DMSO medium

Stationary phase cells of 507-4/2b were plated onto freshly prepared YC + DMSO plates at a cell density of between 10^3 and 10^5 viable cells per plate. At DMSO concentrations up to 4% (V/V) 100% survival was seen whilst at concentrations $> 7.5\%$ no colonies were obtained from the 8.4×10^5 plated. Up to five independent experiments were carried out with DMSO concentrations between 5% and 7%. The frequency of non-suppressed mutants arising at these concentrations are given in Table 6.8. The control platings, on YC to estimate the number of viable cells, gave a total of 10,148 colonies of which one was an SUQ5⁺ mutant.

At DMSO concentrations $> 5.5\%$ significantly more non-suppressed mutant colonies per 10^4 viable cells plated were scored compared to the spontaneous frequency ($< 1/10^4$). The maximum average frequency, $38.6 / 10^4$ cells plated, was obtained on medium containing 6.5% DMSO. 75 individual mutant colonies were picked from YC + 6% DMSO plates in three different experiments and classified. All were ade⁻ his⁻ can^r and complemented as psi⁻ mutants. Four were petite.

At concentrations $\geq 6\%$ DMSO non-suppressed mutants occurred as individual colonies with no visible background growth. Microscopic examination revealed that all the non-visible cells were present as single or budded cells with no microcolonies present. However the observation of faster growing papillae on

TABLE 6.8 The frequency of non-suppressed mutant colonies of 507-4/2b arising on YC + DMSO plates.

RED MUTANT COLONIES /10 ⁴ VIABLE CELLS PLATED * 1						
%(^V / _V)	EXPERIMENT NUMBER					COMMENTS
	I	II	III	IV	V	
5.0	1.3 (5)	-	-	-	-	1.3 non-suppressed colonies were obtained as red papillae on a confluent background of suppressed colonies.
5.5	10.4 (41)	21.4 (1196)	-	3.2 (128)	11.7	11.7 similar to 5.0% except that the background lawn was weaker.
6.0	10.2 (401)	49.9 (2783)	54.8 (18)	11.7 (46)	50.4 (133)	35.4 there was no background growth. In addition some white colonies were obtained.
6.5	-	-	32.3 (10)	-	44.8 (1170)	38.6 Similar to the growth on 6%.
7.0	59.0 (23)	-	6.4 (2)	9.5 (37)	38.7 (304)	28.4 Similar to the growth on 6%.

*₁ The figure in parentheses is the number of red mutant colonies scored.

the YC + 5.5% DMSO media indicated that the non-suppressed mutants had a growth advantage over suppressed psi⁺ strains on DMSO medium. This was tested by taking an isogenic series of adenine-requiring mutants of 507-4/2b induced by UV-irradiation (see Chapter 3) and plating cells from a saline suspension of each onto YC, YC + 5% DMSO, YC + 5.5% DMSO or YC + 6% DMSO to give approximately 500 colonies per plate. Growth was scored after 3 and 7 days incubation at 28°C and the results, presented in Table 6.9, demonstrated that the three psi⁻ mutants grew much better on the DMSO media than the psi⁺ adenine-requiring mutants. It is clear that psi⁺ strains are more sensitive to DMSO than psi⁻ strains.

None of the 75 non-suppressed mutants of 507-4/2b obtained after growth on DMSO-containing plates had a nuclear gene defect resulting in a psi⁻ phenotype, that is none were PNM⁻ or pnm⁻. All were cytoplasmic psi⁻ mutants. Thus, either DMSO may be both inducing cytoplasmic psi⁻ mutants and selecting for them, or it is only selecting mutants with a psi⁻ phenotype from the population and the spontaneous frequency of the psi⁺ to psi⁻ mutation was significantly higher than that of nuclear mutations resulting in the strain being psi⁻. Of 12 spontaneous adenine-requiring mutants of 507-4/2b, analysed during the course of this thesis, 4 were psi⁻ mutants. Although the numbers are small it does suggest the spontaneous frequency of the psi⁺ to psi⁻ mutation is not significantly higher than for forward nuclear mutation. Induction is suggested by the fact that the average frequency of red colonies obtained was higher on the YC + 6% DMSO, which allowed no cell division on the plate, than on the 5.5% DMSO plate on which there was background growth.

TABLE 6.9 The growth of 507-4/2b and an isogenic series of adenine-requiring mutants derived from it on YC + DMSO.

GENOTYPE OF THE 507-4/2b DERIVATIVE	GROWTH AT 3 DAYS*1					GROWTH AT 7 DAYS*1				
	5.0%		5.5%		6.0% DMSO	5.0%		5.5%		6.0% DMSO
	0	DMSO	0	DMSO		0	DMSO	0	DMSO	
507-4/2b psi ⁺	+	+	+	+	+	+	+	+	+	+
ade1 psi ⁺	+	-	-	-	-	+	+	+	+	-
ade2.1.x psi ⁺	+	+	-	+	-	+	+	+	+	-
psi ⁻	+	+	+	+	+	+	+	+	+	+
PNM psi ⁻	+	+	+	+	+	+	+	+	+	+
prnm psi ⁻	+	+	+	+	+	+	+	+	+	+
asu psi ⁺	+	-	-	-	-	+	+	+	+	-
SUQ5 ⁺ psi ⁺	+	-	-	-	-	+	-	-	-	-

*1 + = good + = poor + = very poor - no growth (estimated by colony number and colony size).

(iii) Experiments to determine whether DMSO induces or selects psi^- mutations

The ability of both 5% and 10% DMSO to induce psi^- mutants in a medium unable to support the growth of non-suppressed mutants was investigated. The experimental protocol used was identical to that used for the experiments with GuHCl . The strain used was again MT182/8d. The results obtained (Table 6.10) clearly demonstrated that both 5% and 10% DMSO, in YNB+ leu medium, induced a high frequency of non-suppressed mutants. Fifteen whole mutant colonies, arising from cells grown in these media, were analysed and found to be psi^- . Ten non-suppressed mutants from the YC + 5% DMSO culture all complemented as psi^- .

As was observed in the experiments with GuHCl (Table 6.4), sectored mutant colonies were found. Of the 25 colonies with red sectors 4 were $\frac{1}{4}$ sectors, 11 were $\frac{1}{2}$ sectors, 8 were $\frac{3}{4}$ sectors and 2 were "hairline" sectors. Four of the red $\frac{1}{2}$ sectors were analysed and found to be psi^- whilst the corresponding half sector was psi^+ i.e. complemented the SUQ5 sal3.5 ade2.1 PNM2 psi^- strain 584/1d and gave red colonies on YC + 2.5mM GuHCl .

There are thus three observations which indicate that DMSO is inducing psi^- mutants:

- 1) the numbers of mutants on the 5% and 6% DMSO plates (Table 6.8),
- 2) the appearance of mutant cells grown in YNB + leu + DMSO,
- 3) the occurrence of sectored colonies after growth in liquid medium containing DMSO.

Growth of MT182/8d in YC + 5% DMSO resulted in a significant frequency of both K^- and petite mutants (Table 6.10). Of the

TABLE 6.10 The number of non-suppressed mutants of MT182/8d found after growth in complete and defined media containing DMSO.

MEDIUM + DMSO (v/v)	NUMBER OF NON-SUPPRESSED COLONIES						PETITE		KILLER	
	COLONIES SCORED	VIABLE CELL TITRE /ml	WHOLE RED	SECTORED		% MUTANT COLONIES	TOTAL COLONIES SCORED	% rho ⁻	TOTAL COLONIES SCORED	NO. k ⁻
				RED	WHITE					
YC	3799	1.80x10 ⁸	0	0	0	0.03	1494	0.3	705	0
YNB + leu	1963	3.08x10 ⁶	0	0	0	0.05	735	0.4	309	1
YC + 5%	2110	1.68x10 ⁷	26	10	0	1.7	684	9.5	343	34
YNB+leu+5%	1807	6.82x10 ⁵	5	2	0	0.4	536	17.7	398	1
YNB+ade+his+leu+5%	1068	6.11x10 ⁵	31	2	0	3.1	404	1.5		NT
YC + 10%	4	13.33	1	0	0	25.0		NT		NT
YNB+leu+10%	555	1.08x10 ⁴	128	11	0	25.0	333	5.4	253	2
YNB+ade+his+leu+10%	0	0	0	0	0	0	0		0	

34 K⁻ mutants obtained all were psi⁺ and 3 were petite. No K⁻ mutants were observed after growth in defined media containing 5% DMSO.

(iv) Effect of DMSO on the psi⁺ phenotype in non-growth conditions

To determine whether DMSO was able to induce psi⁻ mutants, in the absence of growth of both non-suppressed and fully suppressed strains, log phase cells of MT182/8d were suspended in 10ml saline + 5% (V/V) DMSO, and incubated at room temperature for 5 days. Samples were taken at various times and plated onto YC. The results obtained (Table 6.11) conclusively demonstrated that 5% DMSO was unable to induce psi⁻ or petite mutants of MT182/8d under total non-growth conditions.

(v) The isolation of suppressed DMSO-resistant mutants of 507-4/2b

By plating cells of 507-4/2b onto YC + 6% or 7% DMSO, red psi⁻ mutant colonies were obtained (see above and Table 6.8). During these experiments it was noted that some white colonies were also present on the YC + DMSO plates, but in much lower numbers than the non-suppressed mutants. Table 6.12 gives the frequencies with which these suppressed resistant colonies arose spontaneously in several independent experiments and in one experiment where 507-4/2b was irradiated with 20 J / m² (74% survival) prior to plating onto YC + 6% DMSO.

9 spontaneous and 24 UV-induced white DMSO-resistant colonies were picked. Two of the clones obtained after UV treatment were ade⁻ HIS⁺ the remaining 31 were fully suppressed. Two of the UV-induced, and 4 of the spontaneously occurring, DMSO-resistant suppressed strains were crossed to 466/4a, a strain unable to grow

TABLE 6.11 The number of non-suppressed mutants of 507-4/2b after exposure to 5%(v/v) DMSO in saline for up to 6 days at room temperature.

INCUBATION TIME (hr)	COLONIES SCORED	VIABLE CELL TITRE/ml	% SURVIVAL	NO. RED COLONIES	PETITE	
					TOTAL COLONIES SCORED	% rho ₋
0	899	2.52x10 ⁶	100	0	899	0.6
4	788	2.19x10 ⁶	86.9	0	624	0.5
24	1047	2.62x10 ⁶	103.9	0	1047	1.3
72	2463	1.81x10 ⁶	71.8	0	1054	0.3
144	4121	1.80x10 ⁶	71.4	1	532	0.9

TABLE 6.12 Spontaneous and UV-induced frequencies of white DMSO^r mutants of 507-4/2b.

$\frac{\%}{v/v}$ DMSO IN YC	TREATMENT	VIABLE CELLS PLATED	NUMBER OF WHITE COLONIES	FREQUENCY OF WHITE COLONIES /10 ⁴ PLATED
6%	SPONTANEOUS	3.94x10 ⁴	2	0.51
	SPONTANEOUS	5.28x10 ⁴	13	2.46
	SPONTANEOUS	3.1x10 ³	2	6.45
	+UV(20J/m ²)	3.92x10 ⁴	69	17.60
7%	SPONTANEOUS	8.37x10 ⁵	2	0.02

on YC + 6% DMSO, It had the genotype a SUQ5 ade2.1 his5.2 psi⁺. 10 tetrads were dissected from each diploid and, in the 5 crosses where complete tetrads were obtained, DMSO^r : DMSO^s segregated 2:2 in all tetrads. DMSO^r segregated independently of ura3.1 in each set of tetrads. From the sixth diploid only 15% spore viability was obtained. All the diploids were DMSO-sensitive.

The resistance to DMSO in these strains therefore was conferred by a single, recessive, nuclear gene mutation.

(vi) Effect of DMSO on other suppressed strains

Saline suspensions (c. 5×10^6 cells /ml) were made of each of the following strains; 507-4/2b, MT182/8d, 466/4a and MT209/7b (all SUQ5 ade2.1 his5.2 psi⁺), 562/2a (SUP2.1), X2315-14c (SUP3.1), five strains of genotype SUQ5 ade2.1 sal psi⁻, each carrying a different sal mutation, the SUQ5/SUQ5 ade2.1/ade2.1 psi⁺ diploid MT35 and the homozygous mating type mutants of MT35, HM2 and HM3. 10µl aliquots were spotted onto YC, YC + 6% DMSO or YC + 7% DMSO. All strains produced white colonies on YC. The only strains that remained white on DMSO medium were the five sal mutants. The SUP2.1 and SUP3.1 strains were both pink on the DMSO medium, but by replating onto YC, this was shown to be a phenotypic effect. All other strains showed growth of red papillae and, in all cases, the non-suppressed phenotype of these papillae was maintained upon recloning on YC.

6 non-suppressed papillae from the a/a MT35 psi⁺ diploid on YC + 6% DMSO were picked, recloned on YC and transferred to FMA to induce sporulation. Tetrads were dissected from each, and all 6 mutants gave only red non-suppressed spores. The parent strain MT35 gave only suppressed spores. (Table 6.13) Therefore the psi⁺ phenotype of a diploid strain can also be "cured" by growth on 6% DMSO.

TABLE 6.13 Tetrad analysis of MT35 and 6 non-suppressed mutants isolated as papillae on YC + 6% DMSO.

MUTANT NUMBER	TETRAD ANALYSIS			SUPPRESSED: NON SUPPRESSED		TOTAL *1 SPORES S:NS
	NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRAD			
				4:0	0:4	
MT35 PARENT	12	95.8	10	10	0	46:0
N1	8	96.9	7	0	7	0:31
N2	8	100	8	0	8	0:32
N3	8	96.9	7	0	7	0:31
N4	8	93.8	7	0	7	0:30
N5	8	100	8	0	8	0:32
N6	7	92.8	6	0	6	0:26

*₁ includes data from incomplete tetrads.

(vii) Effects of deuterium oxide on the ψ^+ phenotype

Deuterium oxide (D_2O) resembles DMSO in that it forms stronger hydrogen bonds with proton donor groups of biopolymers than does water. 10ml of liquid YC, with either 5%, 20% or 30% (V/V) D_2O , was inoculated with stationary phase cells of 507-4/2b to a cell titre of approximately 2×10^5 cells/ml. The cultures were incubated, with shaking, for 5 days at $28^\circ C$. Samples were then taken and plated onto YC.

The results obtained (Table 6.14) showed, that at the concentrations of D_2O used, no significant loss of ψ^+ phenotype was observed. 12 of the non-suppressed mutants from the D_2O cultures were analysed; 6 were asu, 5 were ψ^- and one was a pnm mutant. The non-suppressed mutant in the control was ψ^- . No petite induction was observed. It was concluded that, at equivalent concentrations, DMSO was far more effective at eliminating the ψ^+ phenotype of 507-4/2b, but that D_2O may have a weak "curing" ability, or possibly selects for non-suppressed revertants.

(viii) Summary

Dimethyl sulphoxide was found to revert strains from ψ^+ to ψ^- at very high frequencies, both in liquid medium (complete or defined), and on solid medium (complete). Evidence suggested that DMSO induced the ψ^- mutation but also selected against the ψ^+ phenotype. To be effective growth was required. Cells from YC + DMSO liquid cultures gave rise to sectored ψ^+/ψ^- colonies as well as wholly ψ^- colonies.

TABLE 6.14 The viable cell titre and frequency of non-suppressed and petite colonies obtained after growth in YC + D20 liquid medium of 507-4/2b.

D20 % ($\frac{v}{v}$)	COLONIES SCORED	VIABLE CELL TITRE/ml	NO. OF NON-SUPPRESSED COLONIES			PETITE	
			WHOLE	SECTORED	MOSAIC	COLONIES SCORED	% - rho
0	4398	1.95×10^8	1	0	0	1308	0.15
5	5583	1.86×10^8	3	1	0	1877	0.11
20	3339	1.00×10^8	0	2	1	1382	0.14
30	3551	0.79×10^8	0	3	4	779	0.13

6.4 Miscellaneous treatments that eliminate the ψ^+ phenotype at a high frequency

This section will deal with preliminary results obtained with 4 other treatments that apparently induce, at a high frequency, the loss of the ψ^+ phenotype.

1. POTASSIUM CHLORIDE

Singh (1977) reported that many nonsense suppressors of S. cerevisiae caused inhibition of growth on hypertonic media, but strains carrying SUQ5, were able to grow on such media. During experiments conducted with YC medium supplemented with potassium chloride (KCl), to determine at which molarity growth of an SUQ5 ade2.1 ψ^+ strain became inhibited (see Chapter 3), it was found that growth in liquid medium with $> 1.5M$ KCl resulted in a large fraction of the cells, in the culture, becoming non-suppressed. This phenomenon was investigated to determine the spectrum of classes of non-suppressed mutants obtained.

(i) Growth in YC + KCl liquid culture

Stationary phase cells of 507-4/2b, suspended in saline, were used to inoculate 10ml. YC cultures, made 1.5M, 2M or 2.5M in KCl, to give an initial viable cell titre of 4.51×10^5 /ml. Samples were taken, after 5 days and 10 days incubation at $28^\circ C$, and sonicated prior to plating onto YC. The results obtained are given in Table 6.15.

1) In YC, made 1.5M in KCl, a molarity at which many nonsense suppressor-bearing strains fail to grow (Singh 1977; but see Chapter 3), 507-4/2b grew as well as in the YC control culture. Growth was severely inhibited in the 2M and 2.5M KCl cultures, with little overall increase in the viable cell titre in either culture being observed, even after 10 days incubation. The cell

TABLE 6.15 The growth of 507-4/2b in YC + KC1 and the number of non-suppressed mutanat and petite colonies after 5 and 10 days indubation.

5 DAYS										10 DAYS																						
(M)	COLONIES SCORED	VIABLE CELL TITRE /ml	NO. OF NON-*					PETITE					NO. OF NON-*					PETITE														
			SUPPRESSED COLONIES					%					COLONIES SCORED					%					SUPPRESSED COLONIES					%				
			MUTANT COLONIES					TOTAL COLONIES SCORED					rho-					MUTANT COLONIES					TOTAL COLONIES SCORED					rho-				
			W	S	M			W	S	M			W	S	M			W	S	M			W	S	M							
0	5909	1.5 x 10 ⁸	1	0	0	0.02	1584	0.06	2415	1.2 x 10 ⁸	0	0	0	0.04	1291	0.15																
1.5	5213	1.3 x 10 ⁸	5	2	0	0.13	1767	0.11	2874	7.8 x 10 ⁷	2	0	0	0.07	1369	0.07																
2	18	1.3 x 10 ⁵	0	0	0	-	NT		3061	1.8 x 10 ⁵	79	8	55	4.64	784	0.26																
2.5	80	2.4 x 10 ⁴	2	2	0	5.0	NT		516	1.0 x 10 ⁵	101	2	6	21.12	516	0.19																

titre at time 0 for these cultures was 2.0×10^4 / ml.

2) The cultures, grown in YC or 2.5M in KCl, contained large numbers of non-suppressed mutants after 10 days incubation with a maximum frequency of 21% of all survivors from the culture grown in YC 2.5M in KCl. In the culture grown in YC 1.5M in KCl there was only a slightly higher frequency of non-suppressed mutants than was observed in the control culture.

3) Growth in YC + KCl medium resulted in no induction of petite mutants. In fact petite mutants appeared to be more susceptible to growth inhibition on YC 1.5M in KCl than the grande parent strains and, therefore, growth in hypertonic media possibly selects for grande strains.

The genotype of some of the non-suppressed mutants was determined by complementation analysis. Seven whole red mutants from the culture grown in YC 1.5M in KCl, 32 from the culture grown in YC 2M in KCl and 34 from the culture grown in YC 2.5M in KCl were recloned and tested. All 73 were ade^- his^- can^r and psi^- . None were petite. The non-suppressed mutant in the control culture was an SUQ5⁺ mutant.

Because the cells were sonicated before plating, the occurrence of a high proportion of mosaic mutant colonies, particularly from the 2M KCl culture, was unexpected. Two of the mosaic mutant colonies were resuspended in saline, sonicated and replated onto YC to give individual colonies (Table 6.16). No wholly red mutant colonies were found with only mosaic, whole suppressed colonies or white/mosaic sector colonies being present. Thus the mutation leading to the red phenotype, rather than the non-mutant phenotype, was unstable. A mutant colony showing a half red sector was also replated onto YC after sonication (Table 6.16).

TABLE 6.16 Replating of two mosaic and one sectoried colony of 507-4/2b after growth in YC + 2M KC1 and sonication.

NATURE OF MUTANT	COLONIES TOTAL	NUMBER OF COLONIES AND % TOTAL WITH THE GIVEN PHENOTYPES				
		WHOLE RED	WHOLE WHITE	MOSAIC	SECTORED RED/WHITE	SECTORED MOSAIC/WHITE
MOSAIC-1	260	0	73 (28.1)	172 (66.2)	0	15 (5.8)
MOSAIC-2	349	0	77 (22.1)	265 (75.9)	0	7 (2.0)
$\frac{1}{2}$ RED/ $\frac{1}{2}$ WHITE SECTORED	484	320 (66.1)	161 (33.3)	0	3 (0.6)	0

Although 3 colonies carrying a red sector were found, all the remaining colonies were either wholly mutant or wholly non-mutant. In this case the mutant phenotype was stable.

One mosaic colony derived from each of the two mosaic colonies isolated, together with a red colony from the red/white sector mutant, were each crossed to an a SUQ5 ade2.1 psi⁺ and an a SUQ5 ade2.1 psi⁻ tester strain (MT193/3b and 468/1a respectively) and individual zygotes isolated by micromanipulation. The red mutant gave red diploids with 468/1a, and white diploids with MT193/3b, showing that it was a psi⁻ mutant. However the two mosaic mutants gave only stable white diploids with both strains. The instability observed was therefore recessive. All diploids failed to sporulate preventing further genetic analysis.

(ii) Experiments to determine whether KCl induces or selects psi⁻ mutations

The results so far presented demonstrate that growth of a psi⁻ strain, in medium 2M or 2.5M in KCl, results in a high frequency of psi⁻ mutants being present in the culture after prolonged incubation. In an attempt to determine whether induction or selection of psi⁻ mutants occur in these media, the ability of KCl, to induce psi⁻ mutants of MT182/8d in medium that will not support growth of non-suppressed mutants was investigated. The experimental protocol was that used in identical experiments described in Sections a) iii and b) iii using GuHCl and DMSO respectively.

The results obtained from the preliminary experiments (Table 6.17) suggested that no induction of psi⁻ mutants occurs.

TABLE 6.17 Growth of MT182/8d in defined media supplemented with KC1 - preliminary results.

GROWTH MEDIUM	COLONIES SCORED	VIALE CELL TITRE /ml	NUMBER OF NON-SUPPRESSED COLONIES
YNB + leu	1040	5.25×10^6	0
YNB + leu + 1.25MKC1	591	6.17×10^6	0
YNB + leu + ade + his + 1.25MKC1	468	2.67×10^6	0
YNB + leu + 1.5MKC1	793	5.06×10^6	0
YNB + leu + ade + his + 1.5MKC1	134	7.20×10^5	0
YNB + leu + 1.75MKC1	1760	5.03×10^6	0
YNB + leu + 2MKC1	1598	2.53×10^5	0
YNB + leu + ade + his + 2MKC1	29	8.29×10^4	0

It should be noted that, even in medium that would support growth of a non-suppressed mutant (YNB + leu + ade + his), no psi⁻ mutants were induced by up to 2M KCl. MT182/8d may be more resistant to KCl-induced loss of the psi⁺ phenotype. Another SUQ5 ade2.1 psi⁺ mutant, MT161-6/2a, gave high numbers of psi⁻ mutants when grown in liquid YC + 1.5M KCl culture for 10 days (7 out of 751 colonies scored) indicating that KCl-induction, or selection, of psi⁻ mutants is not specific to 507-4/2b.

From the data presented it is unclear whether there is induction or selection of psi⁻ mutants by KCl. The occurrence of sectored mutant colonies after plating on YC is evidence in favour of induction. The finding that the psi⁺ phenotype is lost at a high frequency during growth in hypertonic media has recently been independently discovered (F. Sherman personal communication).

2. METHANOL

Haploid psi⁺ strains, grown in liquid culture with 10% (V/V) methanol (MeOH), lose their psi⁺ phenotype at a very high frequency whether or not the strain carries SUQ5 (C. Mundy personal communication). The effect of MeOH, on the suppressed phenotype of two haploid SUQ5 ade2.1 psi⁺ strains MT182/8d and 507-4/2b, was investigated by growing each of the strains in liquid YC culture made 10% (V/V) in MeOH. Stationary phase cells were used to inoculate the cultures, which were grown for up to 3 days at 28°C. An experiment was also performed in which washed, log phase cells of 507-4/2b were suspended in saline + 10% (V/V) MeOH at a viable cell titre of 1.3×10^7 /ml and incubated at room temperature for up to 6 days to see if growth of the psi⁺ strain was required for MeOH to be effective.

The result from these experiments, shown in Table 6.18, demonstrated that:-

(a) growth of a psi⁺ strain in YC + 10% MeOH resulted in a high frequency of non-suppressed mutants being present in the culture,

(b) many sectored mutant colonies of both strains were found following treatment with MeOH. No mosaic colonies were found,

(c) 507-4/2b showed a higher frequency of loss of the suppressed phenotype than MT182/8d, and

(d) growth of the psi⁺ strain was required for MeOH to be effective.

A total of 72 wholly non-suppressed colonies, arising from the 507-4/2b YC + MeOH culture, were analysed; all were ade⁻ his⁻ can^r and psi⁻. One was petite. The red sectors from 12 colonies were recloned and analysed and all were ade⁻ his⁻ can^r and psi⁻. The white sectors from these colonies were also re-streaked onto YC and showed no loss of their suppressed (psi⁺) phenotype. This indicated that such red and white sectored colonies are not the result of a diluting out of psi⁺ "particles" during mitosis. Of the 62 sectored colonies scored, 8 were $\frac{1}{4}$ sectors, 16 were $\frac{1}{2}$ sectors, 8 were $\frac{3}{4}$ sectors and the remaining 30 were hairline sectors (i.e. $< \frac{1}{4}$ sectors).

The 24 non-suppressed mutants of MT182/8d, including 18 isolated as sectors, were analysed; all were ade⁻ his⁻ and psi⁻. All 24 retained the K⁺ phenotype of the psi⁺ parent and in fact showed an increased zone of killing on the killer test plates (KM) compared to the psi⁺ parent. One of these psi⁻ mutants has recently been analysed and shown to contain higher levels of total dsRNA than the psi⁺ parent (C. Waldron personal communication).

TABLE 6.18 The viable cell titre, number of colonies scored and the frequency of non-suppressed and petite mutants after incubation of 507-4/2b in YC + 10% MeOH or saline + 10% MeOH and MT182/3d in YC + 10% MeOH .

STRAIN + MEDIUM	DAYS OF INCUBATION AT 25°C	COLONIES SCORED	VIABLE CELL TITRE /ml	NO. OF MUTANT COLONIES		FREQUENCY /10 ³ SURVIVORS	PETITE	
				WHOLE	SECTORED		TOTAL COLONIES SCORED	% rho -
507-4/2b (YC)	0	-	4 x 10 ^{5*1}	-	-	-	-	-
	1	1378	4.6 x 10 ⁷	0	1	0.72	1378	0.9
	3	2207	7.4 x 10 ⁷	136	62	89.71	NT	NT
507-4/2b (SALINE)	0	1315	1.3 x 10 ⁷	0	0	-	1315	0
	3	2403	7.4 x 10 ⁶	0	0	-	381	0.5
	6	14,059	4.2 x 10 ⁶	1	0	0.07	1210	0.2
MT182/8d (YC)	0	-	7 x 10 ^{5*1}	-	-	-	-	-
	1	694	2.8 x 10 ⁷	2	0	2.88	NT	NT
	3	10,453	4.2 x 10 ⁷	4	18	2.10	NT	NT

NT = not tested.

*1 estimated using a haemocytometer.

The effect of growth, in YC + 10% MeOH, on the suppressed (psi⁺) phenotype of a diploid psi⁺ strain was also investigated. The strain used was MT35 ($\times$ /a SUQ5/SUQ5 ade2.1/ade2.1, his5.2/his5.2 can1.100/can1.100 ura3.1/+ psi⁺). In an initial experiment stationary phase cells of MT35 were used to inoculate 10ml of YC + 10% MeOH liquid medium to an initial cell titre of 5×10^5 /ml. An identical culture, without MeOH, was also prepared. After 3 days incubation cells were plated onto YC and 6 whole and 1 sectored mutant colony were found among 4190 colonies screened arising from the YC + MeOH culture. (Table 6.19). Cells from the white suppressed sector of the one sectored mutant colony were resuspended in saline and used to inoculate two flasks, one with 10ml YC + 10% MeOH and one with 10ml of YC, to an initial cell titre of 4×10^5 /ml. After 3 days cells from both cultures were plated onto YC. The results obtained (Table 6.19) demonstrated that the white sector was stable for the suppressed (psi⁺) phenotype during mitotic growth and that it was no more susceptible, to loss of the suppressed (psi⁺) phenotype during further growth in YC + 10% MeOH, than the parent MT35 strain. Tetrad analysis gave only suppressed spores confirming it to be psi⁺. No significant increase in the frequency of petites following growth of MT35 in YC + 10% MeOH was observed (Table 6.19).

All non-suppressed diploid strains from these experiments were recloned induced to sporulate on FMA, and subjected to a random spore analysis (see Chapter 3 for protocol). All 21 mutants gave only red colonies. 6 of the mutants were also subjected to tetrad analysis. 8 tetrads were dissected from each mutant with spore viability being between 94% and 100%.

TABLE 6.19 The viable cell titre, number of colonies scored and the frequency of non-suppressed and petite mutants after incubation of a diploid psi strain in YC + 10% MeOH.

STRAIN AND MEDIUM (DAYS OF INCUBATION)	COLONIES SCORED	VIABLE CELL TITRE /ml	NO. OF MUTANT COLONIES		FREQUENCY /10 ³ SURVIVORS	PETITE	
			WHOLE	SECTORED		TOTAL COLONIES SCORED	%rho
YC(0)	-	5x10 ^{5*1}	-	-	-	-	-
MT35 YC(3)	2555	1.0x10 ⁸	0	0	-	1115	2.1
YC +MeOH (3)	4190	1.4x10 ⁸	6	1	1.67	1941	2.7
YC(0)	-	4x10 ^{5*1}	-	-	-	-	-
YC(3)	2206	1.1x10 ⁸	0	0	-	NT	NT
WHITE SECTOR YC (MT35) +MeOH(3)	2678	6.6x10 ⁷	9	0	3.4	NT	NT

*₁ estimated using a haemocytometer.

In all cases only non-suppressed spores were obtained. The psi⁻ nature of the spores from one tetrad of one of the mutants was confirmed by complementation analysis.

Both haploid and diploid psi⁺ strains lose their psi⁺ phenotypes at a relatively high frequency following growth in YC + 10% MeOH and, preliminary experiments showed that, both triploid (MT54) and tetraploid (MT57) psi⁺ strains lost their suppressed phenotypes at a high frequency following such treatment (data not shown).

A detailed analysis of the action of methanol and ethanol on the psi⁺ phenotype has been performed by C. Mundy and will be reported in his thesis. His evidence, together with the data obtained here (i.e. the occurrence of sectoring mutant colonies following treatment), conclusively demonstrate that MeOH induces rather than selects for the psi⁻ mutation.

3. ERYTHROMYCIN

Erythromycin (ERY) is an antibacterial antibiotic that blocks protein synthesis on the mitochondrial ribosomes of yeast (Grivell et al., 1973). Resistance to erythromycin (ERY^r) can be coded for either by the nuclear or mitochondrial genome (Thomas and Wilkie 1968b; Linnane et al., 1968).

During experiments on the UV-induction of mutants resistant to low levels of ERY (50ug/ml-400ug/ml), in the psi⁺ strains 507-4/2b, MT195/1b (α SUQ5 ade2.1 his5.2 lys1.1 ura3.1 umr1.1 psi⁺) and MT196/3a (a SUQ5 ade2.1 his5.2 can1.100 rev3.1 psi⁺), the following was observed. When these strains were plated onto YEPGly3 (i.e. YEPGly with 3% rather than 2% glycerol) + ERY medium, discrete resistant colonies were observed, together with a weak background growth after about 7 days incubation. When these plates, plus the colonies, were replica plated onto YC the

majority of the background growth was found to consist of non-suppressed colonies. The resistant colonies were stable for the suppressed phenotype.

In one experiment a total of 1.12×10^8 viable cells of 507-4/2b were plated onto four YEPGly3 + 250 μ g/ml ERY plates. After 7 days 52 resistant colonies were present ($4.6 / 10^7$ cells plated) and, upon transfer, were found to be fully suppressed and stable. A weak background of growth was also observed and, when replica plated onto YC, approximately 50% of the colonies were non-suppressed. In a control plating, onto YC, of the original 507-4/2b suspension, only 1 non-suppressed mutant (an asu mutant) was found among 2748 colonies screened. The cells plated were therefore stable for the suppressed phenotype. By plating at lower cell densities of 507-4/2b, onto YEPGly3 + ERY (250 μ g/ml) (approximately 1000 cells/plate), individual non-suppressed mutants, present as background growth, were obtained. Many of these were sectorial red and white. 26 were recloned and 24 complemented as psi⁻ mutants, the other two being an asu and an SUQ5⁺ mutant. Similar experiments using 507-4/2b, with lower ERY concentrations (50 and 100 μ g/ml), yielded far fewer non-suppressed mutants.

By plating between 10^3 and 10^5 cells/plate of 507-4/2b onto YEPGly3, incubating at 28°C for 7-10 days before replica plating onto YC, it was shown that no non-suppressed mutant colonies were induced by growth on YEPGly3 alone. Survival of 507-4/2b on YEPGly3 was 92.0% of that on YC.

Identical observations were made with both the MT195/1b and MT196/3a strains plated onto YEPGly3 + ERY. In one experiment of 796 "background" colonies scored, by replica plating a YEPGly3 + ERY (50 μ g/ml) + MT195/1b plate onto YC, only 42

(5.3%) were stable suppressed strains. The majority of colonies were mosaics which, upon recloning on YC, gave whole red or whole white colonies only. 29 of the non-suppressed mutants from MT195/1b were tested but all failed to mate with any of the tester strains (α or a). The untreated parent, MT195/1b, was also found to be sterile. Of 25 non-suppressed mutants isolated from MT196/3a on ERY (200 μ g/ml) media, 18 were psi⁻ and 7 failed to mate.

Growth of an SUQ5 ade2.1 psi⁺ in the presence of ERY, therefore, resulted in a high frequency of loss of the psi⁺ phenotype. The effect of ERY on the psi⁺ phenotype of 507-4/2b in liquid medium was investigated by inoculating 10ml YEPGly3 + 50 μ g/ml or 200 μ g/ml ERY, with stationary phase cells of 507-4/2b, to an initial cell density of 3×10^5 cells/ml.

Because the solvent used for the 20mg/ml stock solution of erythromycin was methanol, another chemical agent known to induce high frequency loss of psi⁺ phenotype at concentrations

$\geq 10\%$ (V/V) (C. Mundy personal communication), a control was also grown with 1% (V/V) methanol present. A culture, grown in YC as a control, was also run. Cultures were incubated at 28°C and samples were taken, after 5 and 10 days, and plated onto YC. The results are given in Table 6.20. They show that prolonged incubation (10 days) in YEPGly3 + 200 μ g/ml ERY resulted in a high frequency of non-suppressed mutants being present in the culture. The majority of mutants (95%) gave whole mutant clones when plated onto YC. No mosaic mutant colonies were found. Very few non-suppressed mutants were present in this culture after 5 days incubation. ERY at 50 μ g/ml had no effect on the suppressed phenotype of 507-4/2b and had very little effect on overall growth. ERY at 200 μ g/ml had a significant effect on

TABLE 6.20 Growth of 507-4/2b in the presence of ERY and it's effect on the suppressed phenotype.

MEDIUM	5 DAYS					10 DAYS				
	COLONIES SCORED	VIABLE CELL TITRE /ml	NO. OF NON-SUPPRESSED COLONIES			COLONIES SCORED	VIABLE CELL TITRE /ml	NO. OF NON-SUPPRESSED COLONIES		
			WHOLE	RED/WHITE	%			WHOLE	RED/WHITE	%
YC	1126	1.1×10^8	0	0	0.09	1400	1.4×10^8	0	0	0.07
YPGly3+1% (v/v) MeOH	1462	1.8×10^7	0	0	0.07	1404	2.3×10^7	0	1	0.07
YPGly3+ERY (50µg/ml)	4645	6.5×10^6	1	3	0.09	2434	9.7×10^6	0	0	0.04
YPGly3+ERY (200µg/ml)	3495	4.9×10^6	1	4	0.14	973	2.8×10^5	360	19	38.95

growth, with a decline in cell viability being observed between the 5th and 10th days of incubation. The presence of 1% (V/V) methanol had no effect on the psi⁺ phenotype of 507-4/2b; the one mutant obtained from this culture was an asu mutant.

11 of the mutants from the ERY (200µg/ml) culture (10 days incubation) were recloned and analysed; all 11 were ade⁻ his⁻ can^r psi⁻ mutants.

These results demonstrated that growth of a psi⁺ strain on, or in, medium containing ERY resulted in a high frequency of loss of the psi⁺ phenotype. It was unclear whether induction or selection of psi⁻ mutants was occurring although the presence of sectorised mutant colonies again suggests that there was at least some induction. There is a possibility that non-suppressed mutants are slightly more resistant to ERY since, on the YC + ERY plates, most of the 'background' growth was of non-suppressed colonies.

4. GROWTH IN YNB

The psi⁺ strain 507-4/2b was found to grow very slowly in YNB + ura liquid culture. In one experiment stationary phase cells of 507-4/2b were washed twice in saline and used to inoculate 10ml YNB + ura and 10ml YNB + ura + 10% (V/V) DMSO to an initial viable cell titre of 2.6×10^4 /ml. After 7 days of incubation these cultures showed no visible signs of growth whilst a YC culture, started with the same inoculum used for the YNB + ura cultures, had reached stationary phase by day 3. After 14 days incubation positive signs of growth were seen in the YNB + ura culture. Samples were then taken from all 3 cultures on day 14 and plated onto YC. The results obtained (Table 6.21) demonstrated that non-suppressed mutants were present in both YNB cultures at a frequency $>1\%$ of the total

TABLE 6.21 The growth of 507-4/2b in defined media and the number of non-suppressed mutants obtained after 14 days indubation.

TREATMENT	COLONIES COUNTED	VIABLE CELL TITRE /ml	NO. OF NON-SUPPRESSED COLONIES		
			WHOLE	SECTORED RED/WHITE	MOSAIC
YNB + ura (0 days)	1313	2.6×10^4	0	0	0
YNB + ura (14 days)	1323	1.6×10^7	14	0	0
YNB + ura + 10% v/v DMSO (14 days)	1485	1.4×10^3	48	11	10
YC (14 days)	2748	2.3×10^8	0	0	0

viable cells. 10 mutants from the YNB + ura, and 14 from the YNB + ura + DMSO culture, were recloned and analysed. All 24 were $\text{ade}^- \text{his}^- \text{can}^r$ and psi^- . All were grande.

These results suggest that the psi^+ phenotype is lost at a high frequency by cells that are growing very slowly. There can be no selection for psi^- mutants in YNB + ura because such mutants would require added histidine and adenine for growth.

5. OTHER AGENTS TESTED

The following is a list of chemical agents screened that had no effect on the psi^+ phenotype:-

i. methylene blue (20 $\mu\text{g}/\text{ml}$); this was found however to be a potent inducer of petites.

ii. nystatin (5-10 $\mu\text{g}/\text{ml}$); a polyene antibiotic that causes cell membrane distortions. psi^+ and psi^- strains also showed equal sensitivity to nystatin in well tests.

iii. streptomycin (up to 200 $\mu\text{g}/\text{ml}$); an antibacterial anti-
biotic that is an effective mutagen for cytoplasmic genes in Chlamydomonas (Sager 1972).

iv. neomycin (up to 100 $\mu\text{g}/\text{ml}$); an antibacterial antibiotic.

v. urea a protein-denaturing agent.

vi. protamine sulphate (10-100 $\mu\text{g}/\text{ml}$); a basic protein that, in the absence of K^+ ions, binds to the cell membrane causing distortions and intracellular leakage.

vii. Galloway's Cough Mixture; well it cured my cough!

6.5 Discussion

A characteristic of certain cytoplasmically-inherited phenotypes in yeast is that they can be lost at a high frequency either spontaneously or by treatment with certain chemical or physical agents. The most extensively studied example is the grande

phenotype which is lost spontaneously to give petite mutants, at a frequency between 0.01% and 10%, depending upon the strain. This frequency can be raised to 100% by many agents, both of a specific nature e.g. ethidium bromide, and a non-specific nature e.g. heat (see Table 5.1). The petite mutation is however unique in the specificity and frequency with which it can be induced. Many cytoplasmic genes both in yeast and other organisms mutate spontaneously at frequencies comparable with those observed of nuclear genes.

This chapter describes a number of treatments that effectively cause a high frequency loss of the psi⁺ phenotype. Unlike the nuclear mutagens described in Chapter 3, which induce the psi⁺ to psi⁻ mutation (e.g. UV, NTG), these agents induce almost exclusively psi⁻ mutants. These agents are summarised in Table 6.22. To date GuHCl, DMSO, MeOH and hypertonic media have been conclusively shown to induce, rather than select, psi⁻ mutants (this thesis, C. Mundy personal communication, F. Sherman personal communication).

Cytoplasmic petite mutations are characteristically accompanied by either loss, or gross structural change, of the cytoplasmic genome responsible for the phenotype, namely mitochondrial DNA (see Nagley et al., 1977). They cannot be considered therefore to be in the same class, as nucleotide base changes, as "normal" mutational events. Although relatively stable, the cytoplasmic killer phenotype (K⁺)^{of} yeast can also be eliminated at a high frequency by both specific and non-specific agents (see Table 5.1). Loss of the K⁺ phenotype is accompanied by either loss or physical change of the low molecular weight P2 dsRNA species (Sweeney et al., 1976; Mitchell et al., 1976b) or loss of both /killer/

TABLE 6.22 Summary of agents that cause a high frequency loss of the psi⁺ phenotype.*1

AGENT	SELECTION	INDUCTION*2	EFFECTIVE DOSE RANGE	GROWTH REQUIRED FOR EFFECT	EXCLUSIVELY <u>psi</u> ⁻ MUTANTS INDUCED
GuHCl	against <u>psi</u> ⁻	+ (1,2,3)	1-5mM	+	+
DMSO	against <u>psi</u> ⁺	+ (2,3)	5-10% ($\frac{v}{v}$)	+	> 95%
METHANOL ^a (+ ETHANOL)	NONE	+ (2,3)	7.5-12.5% ($\frac{v}{v}$)	+	+
KCl, HYPER- } GLYCEROL, TONIC } ETHYLENE MEDIA } GLYCOL _b	? against suppression	+ (2)	1.5M-2.5M 10% ($\frac{v}{v}$) 1.75M	?	> 95%
ERYTHROMYCIN	? against suppression	+ (2)	200µg/ml	?	+
SLOW GROWTH (YNB + ura)	?	+ (3)		?	+
HEAT SHOCK (52°C)	against <u>psi</u> ⁻	+ (1)	30-60 min	-	> 80%
cdc8:HOLDING AT 37°C	?	?	24 hours	?	+

*1 includes unpublished data of C. Mundy (a) and F. Sherman (b).

*2 evidence for induction: 1 = selection against mutant,
2 = sector colonies after treatment,
3 = mutants found in media in which they cannot proliferate.

associated ds RNA's P1 and P2 (Mitchell et al., 1976b; Vodkin 1977b). The phenomenon of "curing" i.e. induced high frequency loss of a phenotype, in yeast, can occur with both cytoplasmic DNA or RNA genomes and always results in gross physical alteration or loss of the genome. "Curing" is not a phenomenon associated with nuclear gene-coded phenotypes.

The discovery of agents such as GuHCl, DMSO and MeOH, that will "cure" cells of their psi⁺ phenotype, will greatly assist the search for a cytoplasmic nucleic acid species associated with the psi⁺ phenotype. A major breakdown in our understanding of the petite mutation came with the discovery (Mounolou et al., 1966) that mitochondrial DNA's of different cytoplasmic petite strains differ from the wild type, and one another, in buoyant density. A study of λ 2 μ 7 DNA, in GuHCl or DMSO-induced psi⁻ strains, would help in establishing the relationship, if any, between this extrachromosomal nucleic acid species and the psi⁺ phenotype.

The observation that the psi⁺ phenotype can be lost, whilst the cell remains grande and K⁺, is further evidence for the non-identity of these latter 2 cytoplasmic genetic elements with the psi⁺ phenotype.

One way to determine genetically whether GuHCl DMSO or MeOH cause a physical loss of the λ psi7 genome would be reversion analysis. Psi⁻ mutants can revert, either spontaneously or by UV-induction, to psi⁺ (Young and Cox 1975, Chapter 7 this thesis). The failure of "cured" psi⁻ strains to revert to psi⁺ would imply loss of genetic information rather than a simple nucleotide base change in DNA (or RNA?). Cytoplasmic petite mutants show no reversion to grande (Ephrussi 1953). Reversion

analysis in SUQ5 ade2.1 psi⁻ strains is hampered by the high frequency with which sal mutants arise. These are nuclear gene mutations that allow SUQ5 to suppress efficiently in a psi⁻ background (Young and Cox 1975; Cox 1977b). Such sal revertants could be distinguished from an SUQ5 ade2.1 psi⁺ revertant by replica plating all colonies onto, say, YC + 5mM GuHCl. Only strains of the genotype SUQ5 ade2.1 psi⁺ give red colonies on such medium. Sal mutants and new, ochre suppressor-bearing strains remain white and suppressed. Alternatively one could induce psi⁻ revertants of diploid psi⁺ strains with GuHCl or DMSO. Reversion analysis in diploids would rule out the problems of sal mutants because these are generally recessive (see Chapter 7).

The molecular mechanisms of petite-inducing agents are not generally known although the majority are known to affect DNA metabolism or membrane synthesis (Nagley et al., 1977). There is no apparent property common to the 4 psi⁻ inducing agents GuHCl, DMSO, MeOH and KCl.

Juliani et al., (1973, 1975b) have suggested that GuHCl acts on the proteins of the mitochondrial membrane, rather than directly on the mitochondrial DNA, and that the integrity of the association between mitochondrial DNA and the membrane might be involved in petite induction. No data is available on the effects of GuHCl on membranes in vivo or in vitro. DMSO is a well known membrane-active agent, both in vivo and in vitro (for review see Jacob and Herschler 1975), and evidence has been presented that DMSO increases mitochondrial membrane permeability in vivo (Oliver and Williamson 1977). Friend et al., (1974) have postulated that, although the primary site of action of DMSO in eukaryotes is the cell membrane, it can have secondary effects on transcription possibly by inhibiting binding of repressor

molecules to DNA. DMSO is believed to cure promotor mutations in yeast (G. Fink personal communication to B. Cox). Growth on hypertonic media could also result in changes in the cell membrane as a consequence of the increased osmolarity (see Rose 1976 for a review).

If the $\bar{\psi}$ genome is a nucleic acid species that requires association with the cytoplasmic (or mitochondrial) cell membrane, for successful replication, one could postulate that the ψ^- inducing agents are membrane agents which 'destroy $\bar{\psi}$ -membrane attachment sites' preventing replication of the $\bar{\psi}$ nucleic acid. Bacterial DNA is attached to the membrane of the cell at the origin where replication of the chromosome is initiated and this association may be implicated in initiating new cycles of replication and in ensuring even segregation of replicated chromosomes to daughter cells (Lewin 1975). The ψ^- -inducing agents could, if they act at the level of the cell membrane, prevent successful mitotic segregation of $\bar{\psi}$ to daughter cells. It is interesting to note that the $\bar{\mu}$ DNA of yeast has been reported to be attached, in vivo, to cytoplasmic membranes (Guerineau et al., 1971). An analysis of membrane proteins in ψ^+ and ψ^- strains might prove fruitful.

During growth in hypertonic media yeast synthesises glycerol, to act as an osmoregulator, in response to the water stress (Brown 1978). Wallis and Whittaker (1974) speculate that glycerol causes a breakdown in the control system necessary for maintaining mitochondrial DNA levels. Consequently another possibility is that ψ^- -inducing agents may create water stress with a subsequent increase in the intracellular levels of glycerol. This could then result in the breakdown of the control system necessary for main-

taining the $\bar{\psi}$ genome.

The mode of action of ψ^- -inducing agents is not clear and, until the molecular nature and location of the $\bar{\psi}$ factor is known, it will be difficult to determine. By looking, for example, at whether specific inhibitors of cellular metabolism e.g. cycloheximide, modify the efficiency of these agents, one may be able to get an insight into the primary target site for these agents.

6.6 Summary

A number of agents cause a high frequency/^{loss} of the ψ^+ phenotype of haploid and, in two cases, diploid ψ^+ strains. Four chemical agents, guanidine hydrochloride, dimethyl sulphoxide, potassium chloride and methanol were shown, by a number of criteria, to induce ψ^- mutations rather than select for them. Preliminary studies also demonstrated that growth in YEPGly, in the presence of erythromycin (200ug/ml), and, for one particular strain (507-4/2b), slow growth in defined medium, also resulted in reversion of ψ^+ to ψ^- at a high frequency. The agents are summarised in Table 6.22.

CHAPTER 7

CHROMOSOMAL GENES INVOLVED IN MAINTENANCE OF THE PSI^+ PHENOTYPE

- 7.1 Introduction
 - 7.2 Spontaneous and UV-induced mutation from psi^- to psi^+ in diploids
 - 7.3 Modifiers of the PNM^- phenotype
 - 7.4 Recessive $\text{PNM}(\text{pnm})$ mutations
 - 7.5 Discussion
-

7.1 Introduction

The phenotypic expression of an extrachromosomal element is dependent on the nuclear genotype. For all major classes of extrachromosomal mutants e.g. male sterility, plastid abnormalities respiratory deficiencies, chromosomal gene mutations have been described which have similar effects on the phenotype or which modify, even eliminate, the phenotypic effect of the extra-chromosomal mutation.

Chromosomal genes are known to control the pattern of transmission of cytoplasmic genes from parents to progeny e.g. gene differences controlling mating type in Chlamydomonas (see Sager 1972). Other chromosomal genes are known to lead to heritable changes in the cytoplasm e.g. the iojap gene of Maize induces changes in plastids which are stable in the absence of the iojap gene (Rhoades 1946). In bacteria host genes are essential for the replication of plasmids and DNA and RNA bacteriophages (see Lewin 1977).

In yeast the involvement of chromosomal genes in the expression and replication of extrachromosomal elements has been most extensively studied for the /killer/ plasmid by Wickner and his collaborators (for a recent review see Wickner 1976). At least 25 chromosomal genes, essential for maintenance and replication of the plasmid (mak1 - mak25, pets), have been found (Somers and Bevan 1968; Fink and Styles 1972; Wickner 1974b; Wickner and Leibowitz 1976). Three chromosomal genes (kex1 - kex3) are also essential for expression of plasmid information (Wickner 1974b).

Maintenance of the psi⁺ phenotype is also dependent upon the functional integrity of chromosomal genes. In particular there are

nuclear genes, pnm⁺ (formerly called "R") which, when mutant, cause loss of the psi⁺ phenotype (Young and Cox 1971). PNM⁻ is dominant to pnm⁺ and a heterozygous diploid gradually loses the ability, over successive generations, to produce pnm⁺ psi⁺ spores (Young and Cox 1971; McCready, Cox and McLaughlin 1977). PNM⁻ mutations map at least 2 unlinked nuclear loci designated pnm1⁺ and pnm2⁺ (B. Cox personal communication).

The role of the pnm⁺ loci in maintenance of the psi⁺ phenotype is not understood. In a PNM⁻/pnm⁺ heterozygote it is unclear whether the loss of the psi⁺ phenotype is as a result of a physical loss (by diluting) of the /psi/ genome (i.e. psi⁰) or whether psi⁺ is just "mutated" to a nonfunctional form (i.e. psi⁻). Experiments described in this chapter were carried out in an attempt to gain a better understanding of the phenomenon of PNM⁻ elimination of the psi⁺ phenotype, the role of the pnm⁺ loci and the nature of the psi⁺ phenotype. 3 questions were posed:

(a) can a pnm⁺ psi⁻ spore segregant from a pnm⁺ psi⁺ x PNM⁻ psi⁻ cross revert spontaneously, or be induced, to psi⁺?

(b) can one get modifiers of PNM⁻ phenotype such that elimination of the psi⁺ phenotype is reduced or enhanced?

(c) do other nuclear gene mutations occur which prevent expression or induce loss of the psi⁺ phenotype?

7.2 Spontaneous and UV-induced mutation from PSI⁻ to PSI⁺ in diploids

(1) Induction of PSI⁺ in pnm⁺ strains

(a) CONSTRUCTION OF DIPLOIDS

Haploid strains of the genotype SUQ5 ade2.1 his5.2 psi⁻ revert at a very high frequency to ADE⁺ HIS⁺, but less than 1% of these revertants are psi⁺ (Young and Cox 1975; Cox 1977b).

Because 90% or more of the $ADE^+ HIS^+$ revertants are due to recessive allosuppressor mutations (Cox 1977b), reversion of $\underline{psi}^-$ to $\underline{psi}^+$ was studied in $\underline{psi}^-$ diploid strains homozygous for SUQ5 and ade2.1. In diploids, $ADE^+ HIS^+$ revertants would arise only by a $\underline{psi}^-$ to $\underline{psi}^+$ reversion or by dominant ochre suppressor mutations that do not depend on $\underline{psi}^+$ for their expression.

A number of diploid strains were constructed by crossing haploid strains of the genotype SUQ5 ade2.1 his5.2 pnm⁺ $\underline{psi}^-$ and picking individual zygotes by micromanipulation. The 483 pnm⁺ $\underline{psi}^-$ strains used were isolated by Dr B.S. Cox from a PNM1/pnm⁺ zygote. The 468 pnm⁺ $\underline{psi}^-$ strains, also isolated by Dr B.S. Cox, were ultimately derived from crosses involving a spontaneous $\underline{psi}^-$ mutant (Cox 1965). A $\underline{psi}^-$ diploid, CM237.5, was obtained from C. Mundy. Both parents were spontaneous $\underline{psi}^-$ mutants.

4 different diploids constructed from $\underline{psi}^-$ strains of different origin were used to isolate spontaneous and UV-induced $ADE^+ HIS^+$ revertants. Each of the parent diploids was sporulated on FMA and between 10 and 24 tetrads dissected from each. Spore viabilities varied between 77 and 97% and all diploids segregated only red $ade^- his^-$ spores.

(b) SPONTANEOUS AND UV-INDUCED REVERSION RATES TO $ADE^+ HIS^+$

The 4 diploids were cloned on YC to obtain single colonies of each. For reversion studies a single colony, showing no white papillae or sectors, was suspended in 10ml saline, sonicated where necessary, and plated onto YNB + ura + lys + trp medium to select

for spontaneous ADE^+HIS^+ revertants. A 1ml sample of the cell suspension was also diluted and plated onto YC medium to determine the viable cell titre of the suspension. The remaining original cell suspension was then UV-irradiated with $75 \text{ J} / \text{m}^2$ and cells again plated onto YNB + ura + lys + trp and YC plates.

ADE^+HIS^+ revertants were scored after 10 days incubation at 28°C as colonies greater than or equal to 1mm in diameter (Table 7.1). Of 191 such colonies picked from the different diploids, both before and after UV-irradiation, 147 (76.9%) gave a white colony colour on YC, 33 (17.3%) were pink and 11 (5.8%) were red on YC. Also 239 ADE^+HIS^+ colonies, with diameters less than 1mm, were checked for colony colour on YC and 135 (56.5%) were red, 73 (30.5%) were pink and 31 (13.0%) were white. 10 of the red " ADE^+HIS^+ " revertants checked were found to be ade^-his^- . The pink mutants were fully suppressed. The ADE^+HIS^+ reversion frequencies given in Table 7.1 are therefore underestimates.

Considerable variation in the frequency of ADE^+HIS^+ revertants per 10^6 viable cells plated was seen among the 4 diploids used (Table 7.1). The origin of the psi⁻ phenotype of the parents of the diploids appeared not to influence the reversion rate to ADE^+HIS^+ . UV-irradiation, at $75 \text{ J} / \text{m}^2$, increased the reversion frequencies by factors of X6 to X15 depending on the diploid.

(c) PROTOCOL FOR ANALYSIS OF REVERTANTS

ADE^+HIS^+ revertants from 3 of the diploids, M7, M10, and CM237.5, were examined in detail to see if any revertants were psi⁺. Only those revertants which gave a white or pink colony colour on YC were used including some revertants that had original colony sizes on the YNB + trp + ura + lys plates of $< 1\text{mm}$. These are given the prefix "S" in the analysis, all others being given

TABLE 7.1 Spontaneous and UV-induced reversion to ADE⁺HIS⁺ in psi⁻ diploids homozygous for SUQ5 and ade2.1.

DIPLOID NUMBER	PARENT STRAIN	PSI ^{-*1} DERIVATION	REVERSION TO ADE ⁺ *2				
			SPONTANEOUS		UV (75J/m ²)		
			NO. EXPTS.	TOTAL ADE ⁺ REVERTANTS PLATED	NO. EXPTS.	% SURVIVAL REVERTANTS PLATED	+ ADE ⁺ /10 ⁵
M7	483/7bx483/4d	P/P	4	27	3	24.6	287 917.6
M10	483/7bx168/6d	P/+	3	271	3	12.6	22 307.6
MT3	483/3cx468/6d	P/+	6	951		NT	
CM237.5	?	+/+	2	13	1	39.5	15 10.7 (56J/m ²)
				6.9	1	11.4	5 46.3 (75J/m ²)

*1 P indicates pnm⁺ spore from a PNM⁻/pnm⁺ diploid ; + indicates pnm⁺ spore from a pnm⁺/pnm⁺ diploid.

*2 Total number of ADE⁺ revertants from all experiments given. ADE⁺ frequency is the average of the frequencies from each experiment. % survival is the average value.

the prefix "L".

The protocol for analysing the revertants was:

- (1) a pure clone of the diploid was obtained, its genotype determined and transferred to FMA to induce sporulation,
- (2) after 4-6 days incubation at 28°C sporulation was checked under a light microscope. A loopful of the sporulated culture, from those strains showing at least 5% asci, was resuspended in 100ul of 1% helicase solution and left at room temperature for 2-3 hours to digest the ascus walls,
- (3) following 30 seconds vigorous agitation on a 'Whirlimix', to separate the spores, a loopful of the 'spore suspension' was streaked onto YC and the plate incubated for 4 days at 28°C,
- (4) ADE⁺HIS⁺ diploids that gave < 1% red colonies, following such 'random spore analysis', were subjected to further genetic analysis by dissecting at least 6 tetrads from each,
- (5) diploids giving predominantly tetrads with 4 suppressed: 0 non-suppressed spores could be either psi⁺, homozygous for a dominant, /psi⁻ -independent, ochre suppressor (SUPX) or homozygous for a recessive allosuppressor (salx). To distinguish between these, suppressed spores were taken and crossed to both an SUQ5 ade2.1 psi⁻ and an SUQ5 ade2.1 psi⁻ PNM⁻ tester and occasionally to an SUQ5 ade2.1 sal3.5 psi⁻ PNM⁻ tester. At least two single cell diploids were isolated from each cross by micromanipulation and the phenotype of the resulting diploid clone ascertained. Table 7.2 gives the predicted complementation patterns.

The results of the 'random spore analysis', on the ADE⁺HIS⁺ revertants of the 3 diploids used, are given in Table 7.3.

(d) ANALYSIS OF REVERTANTS FROM M7

The two psi⁻ pnm⁺ haploid parents of M7 were obtained from a PNM⁻/pnm⁺ heterozygote and were psi⁻ as a consequence of

TABLE 7.2 Complementation test for distinguishing ψ^+ , SUPX and sal x spores.

SPORE GENOTYPE	CROSSED TO *1	
	x SUQ5 ade2.1 ψ^-	x SUQ5 ade2.1 PNM $^-$ ψ^- x SUQ5 ade2.1 sal3.5 PNM $^-$ ψ^-
ψ^+	S (4:0)	NS (0:4)
SUPX	S (2:2)	S (2:2)
sal x	NS (2:2)	NS (2:2)
		NS (2:2)
		S (3:1)
		NS*2 (3:1)

*1 S = suppressed NS = non-suppressed. Figures in brackets are the predicted S:NS segregation in tetrads .

*2 If sal3, a S diploid would be found giving 4S:ONS asci .

TABLE 7.3 Classification of ADE⁺HIS⁺ revertants on the basis of 'random spore analysis'.

DIPLOID	ORIGIN	NUMBER OF REVERTANTS	NUMBER OF PETITES	'RANDOM SPORE ANALYSIS'		
				TOTAL TESTED	ONLY WHITE SEGREGANTS	RED AND WHITE SEGREGANTS
M7	SPONTANEOUS	25	0	24	2	22
	UV	41	2	33	9	24
M10	SPONTANEOUS	55	0	35	12	23
	UV	8	2	3	2	1
CM237.5	SPONTANEOUS	3	0	3	0	3
	UV	12	1	9	3	6

'PNM⁻ - elimination'.

From the random spore analysis of 66 ADE⁺HIS⁺ revertants of M7, 11 (16.7%) gave predominantly (> 99%) suppressed colonies (Table 7.3). Tetrads from each of these 11 diploids were dissected and the results of this analysis are given in Table 7.4. Many of the UV-induced revertants gave very poor spore viability. All 11 diploids examined gave an excess of suppressed spores. The parent M7 was heterozygous for ura3.1/URA⁺ and ura3.1 and URA⁺ segregated 2:2 in all sets of tetrads.

The mutation giving rise to the suppressed phenotype of 2 spontaneous (7S18, 7L5) and 4 UV-induced revertants (7L23, 7L25, 7L45, 7L46) was determined by crossing spores from a complete tetrad, where possible, or at least 3 spores, to the various tester strains listed in Table 7.2. From the phenotypes of the diploids obtained it was concluded that 7S18, 7L5 and 7L25 were psi⁺ whilst 7L23, 7L45 and 7L46 were homozygous for a recessive allosuppressor mutation. By complementation analysis, with sal4 and sal5 tester strains (obtained from Dr B.S. Cox), 7L46 was found to be homozygous for sal4. 7L23 and 7L45 were homozygous for either sal1 or sal2.

To confirm that 7S18, 7L5 and 7L25 were psi⁺ the diploids, from crosses to the SUQ5 ade2.1 psi⁻ and SUQ5 ade2.1 psi⁻ PNM⁻ testers, and the suppressed spores from one complete tetrad from each of these revertants, were sporulated and at least 10 tetrads dissected from each. The results obtained (Table 7.5) confirmed that they were indeed psi⁺ i.e. all spores from crosses to the SUQ5 ade2.1 psi⁻ testers were suppressed and spores from crosses to the SUQ5 ade2.1 psi⁻ PNM⁻ tester strain were predominantly non-suppressed.

TABLE 7.5 Genetic analysis of one tetrad from each of 3 psi^+ revertants of M7.

x SUQ5 ade2.1 psi ⁻										x SUQ5 ade2.1 psi ⁻ PNM ⁻				
STRAIN	NO. DISSECTED	SPORE VIABILITY %	NO. COMPLETE TETRADS	SUPPRESSED:NON-SUPPRESSED			NO. DISSECTED	SPORE VIABILITY %	NO. COMPLETE TETRADS	SUPPRESSED-NON-SUPPRESSED				
				4:0	3:1	2:2				0:4	1:3	2:2		
7L5	1a	11	86.4	7	7	0	0	10	90.0	8	8	0	0	
	1b	32	93.0	26	26	0	0	10	100.0	10	10	0	0	
	1c	16	95.3	14	14	0	0	7	100.0	7	8	0	0	
	1d	34	86.0	26	26	0	0	10	100.0	10	8	2	0	
7S18	1a	12	87.5	9	8	1	0	10	100.0	10	10	0	0	
	1b	10	100.0	10	10	0	0	10	100.0	10	6	4	0	
	1c	10	100.0	10	10	0	0	10	100.0	10	7	2	1	
	1d	10	100.0	10	10	0	0	10	100.0	10	9	1	0	
7L25	1a	11	97.7	10	10	0	0	10	92.5	8	8	0	0	
	1b	10	100.0	10	10	0	0	11	93.2	9	7	2	0	
	1c	11	97.7	10	7	1	2	11	97.7	10	10	0	0	
	1d							12	93.8	10	8	2	0	

*1 Diploid failed to sporulate.

A similar analysis was done with the spores from 1 tetrad of 7L46, the presumptive sal4/sal4 heterozygote, together with a preliminary analysis of 2 suppressed spores from 7L45. The results of the 7L45 analysis (Table 7.6) confirmed the presence of a sal mutation in the spores tested. For 7L46 the results were obscured by poor viability in crosses to 1b, 1c and 1d (Table 7.6), but 1a clearly carried a sal mutation with all complete tetrads, bar one, giving 2 suppressed:2 non-suppressed spores. The poor viability for the 1b and 1d crosses could be explained if the 7L46 revertant was also heterozygous for a recessive, suppressible lethal gene mutation (designated osl). Crosses to 7L46/1b and 7L46/1d gave only 2 suppressed : 2 non-suppressed spores, or 2 suppressed : 1 non-suppressed: 1 dead spore, or 2 suppressed : 2 dead spores i.e.

osl	sal	osl	sal	osl	+		
osl	sal	osl	+	osl	+		
+	+	or	+	sal	or	+	sal
+	+		+	+		+	sal
2S:2NS		2S:1NS:1 dead		2S:2 dead			
(PARENTAL DITYPE)		(TETRATYPE)		(NON-PARENTAL DITYPE)			

For crosses to 7L46/1c the spore germination was slightly different although viable suppressed spores were still the predominant class (29 out of 40 spores).

If the osl mutation was present it was not linked to sal4 (PD=3, TT=19, NPD=5).

The two spontaneous psi⁺ revertants, 7L45 and 7S18, both gave rise to a few non-suppressed spores (Table 7.4). The genotypes of the 4 from 7L5 and the 3 from 7S18 were found to be SUQ5 ade2.1 psi⁻ by standard complementation analysis.

TABLE 7.6 Genetic analysis of 2 sal/sal revertants of M7.

MUTANT NUMBER	CROSSED TO	SPORE GERMINATION					SUPPRESSED:NON-SUPPRESSED								TOTAL S : NS	
		DISSECTED NO VIABLE SPORES					%	3:1	2:2	2:1	1:2	2:0				
		NUMBER	4	3	2	1							0	VIAB.		
7L46/1a	SUQ5ade2.1psi ⁻	6	5	1	0	0	0	95.8	0	5	0	1	0	11	12	
	SUQ5ade2.1PNM ⁻ psi ⁻	10	10	0	0	0	0	100	1	9	0	0	0	21	19	
7L46/1b	SUQ5ade2.1psi ⁻	7	1	6	0	0	0	78.6	0	1	6	0	0	14	8	
	SUQ5ade2.1PNM ⁻ psi ⁻	10	1	5	4	0	0	67.5	0	1	5	0	4	20	7	
7L46/1c	SUQ5ade2.1psi ⁻	10	0	7	3	0	0	67.5	0	0	5	1	4	19	7	
	SUQ5ade2.1PNM ⁻ psi ⁻	10	0	0	5	4	0	35.0	0	0	0	0	4	10	4	
7L46/1d	SUQ5ade2.1psi ⁻															
	SUQ5ade2.1PNM ⁻ psi ⁻	10	1	8	1	0	0	75.0	0	1	8	0	1	20	10	
7L45-1	SUQ5ade2.1psi ⁻	10	6	4	0	0	0	90.0	0	6	2	2	0	18	18	
7L45-2	SUQ5ade2.1psi ⁻	12	9	0	1	0	2	79.2	0	9	0	0	1	20	18	

ND: not done, diploid failed to sporulate.

These experiments with M7 demonstrated that when the PNM⁻ mutation eliminates the psi⁺ phenotype in a heterozygous PNM⁻/pnm⁺ diploid, the pnm⁺ psi⁻ spores can still revert to psi⁺. Whether the new psi⁺ phenotype was "normal" was investigated further in the 7L25 psi⁺ revertant.

(e) ANALYSIS OF THE PSI⁺ PHENOTYPE OF 7L25

Using 7L25, experiments were performed to determine whether:

i/ the new psi⁺ phenotype could be lost at a high frequency when grown in YC supplemented with 10% (V/V) methanol as is observed with 'standard' psi⁺ strains (C. Mundy personal communication),

and ii/ if the kinetics of elimination of the psi⁺ phenotype, in the presence of a PNM⁻ mutation, followed that of standard psi⁺ strains.

Both sets of experiments were conducted on the 4 spores 7L25/1a-1d and the 'standard' psi⁺ strains 507-4/2b and 465/1b.

(i) Methanol Elimination

Each of four 10ml cultures of YC + 10% (V/V) methanol (MeOH) were inoculated with stationary phase cells of one of the four 7L25 psi⁺ spore clones, as were four 10ml YC control cultures. The starting cell density was c. 1×10^5 cells/ml. The cultures were incubated with vigorous agitation, at 28°C for 3 days, and then a loopful of each culture streaked onto YC to obtain single colonies. From MeOH cultures of all four 7L25 psi⁺ strains, non-suppressed colonies were obtained at a frequency of 1% - 5% of the total colonies on the YC plates. 10 non-suppressed colonies from each strain were analysed by complementation. All, except two from 7L25/1a (which were SUQ5⁺ ade2.1 psi⁺), complemented as SUQ5 ade2.1 psi⁻ strains. No non-suppressed colonies were obtained from any of the YC control cultures.

Both 507-4/2b and 465/1b were treated similarly and both strains gave approximately 5-10% non-suppressed colonies after growth in 10% (V/V) meOH. 47 and 19 non-suppressed clones respectively were analysed and all were SUQ5 ade2.1 psi⁻.

The psi⁺ phenotype of spores obtained from 7L25 was therefore lost at a high frequency during growth in YC + 10% (V/V) MeOH.

(ii) PNM⁻ elimination of the psi⁺ phenotype

For each of the four 7L25 psi⁺ spores (7L25/1a-1d), and for the 'standard' psi⁺ strains 465/1b and 507-4/2b, the following was carried out. Mass-matings between log phase cells of the psi⁺ strain and either 483/1a (α) or 483/1b (a) strains (both of the general genotype SUQ5 ade2.1 psi⁻ PNM1) were set up on YC plates. Approximately 10 zygotes were picked by micromanipulation, from the mass-mating after 4 hours incubation at 28°C, and allowed to proliferate. After 6 hours an inoculum from the mass-mating was transferred to an FMA plate to induce sporulation in the zygotes. The isolated individual zygotes gave rise to visible clones after 30 hours incubation, at which time 3 were transferred to FMA.

Similarly, after a further 1,2 and, in some cases, 3 days of incubation, 3 more zygotes were transferred to FMA to induce sporulation.

Approximately 12 tetrads were dissected from each of the 3 zygote clones transferred, for each psi⁺ strain, at each incubation period. For all 6 psi⁺ strains analysed spore viability was greater than 90%. The number of suppressed and non-suppressed spores (i.e. psi⁺ and psi⁻) was scored in all complete tetrads obtained (Table 7.7 and 7.8). Several spore clones derived from

TABLE 7.7 Sporulation of diploid clones of different ages from
crosses between 7L25/1a-d and PNM⁻ strain (483/1a or 483/1b).

	HOURS OF GROWTH BEFORE SPORULATION	NUMBER OF COMPLETE TETRAIDS	TETRAD ANALYSIS (S:NS)			SPORE TOTALS		% RED SPORES
			0:4	1:3	2:2	S	: NS	
1a x 483/1a	6	24	24	0	0	0	96	100
	32	36	36	0	0	0	144	100
	56	34	31	2	1	4	132	97.1
	80	33	33	0	0	0	132	100
1b x 483/1b	6	29	22	5	2	9	107	92.2
	28	38	20	15	3	21	131	86.2
	52	32	26	5	1	7	121	94.5
	76	20	14	6	0	6	74	92.5
1c x 483/1a	6	25	25	0	0	0	100	100
	76	29	29	0	0	0	116	100
1d x 483/1b	6	27	10	13	4	21	87	80.6
	52	13	5	5	3	11	41	78.8
	76	14	8	4	2	8	48	85.7

TABLE 7.8 Sporulation of diploid clones of different ages from
crosses between two standard psi⁺ strains and a
PNM⁻ strain (483/1b).

	HOURS GROWTH BEFORE SPORULATION	NUMBER OF TETRADS	TETRAD ANALYSIS (S:NS)			SPORE TOTALS N:NS	% RED SPORES
			0:4	1:3	2:2		
483/1b x 507-4/2b	8	32	13	14	5	24 104	81.2
	24	35	9	15	11	37 103	73.6
	45	32	14	11	7	25 103	80.5
	69	34	10	18	6	30 106	77.9
	93	29	8	10	11	32 84	72.4
483/1b x 465/1b	6	25	9	11	5	21 79	79.0
	32	27	15	11	1	13 95	88.0
	56	27	27	0	0	0 108	100
	80	32	18	12	2	16 112	87.5
	96	28	26	2	0	2 110	98.2

crosses to each of the 6 strains were found to have a discrete white (psi⁺?) and red (psi⁻?) sector. Such spores were classified as psi⁻ for the purposes of the analysis. The results were plotted as % red spores against the number of hours of growth on YC of the zygote prior to transfer to FMA (Figure 7.1). Data from Table 7.5 was not included because the zygotes were grown for an undefined length of time prior to sporulation.

Two striking observations were made from this analysis:

(A) In the case of 507-4/2b, no progressive elimination of the psi⁺ phenotype was observed, but rather a slight decline in the frequency of psi⁻ spores was observed (Figure 7.1). The data of Young and Cox (1971) is also plotted on Figure 7.1 for comparison. 465/1b did show a progressive elimination of the psi⁺ phenotype, but this elimination was much more efficient than reported by Young and Cox (1971).

(B) Complete elimination of the psi⁺ phenotype occurred within 6 hours of mixing the cells in the two psi⁺ strains, 7L25/1a and 7L25/1c, whilst with the other two members of the tetrad, 7L25/1b and 7L25/1d, gave 92.2 and 80.6% psi⁻ spores respectively after mixing with the PNM⁻ strain (i.e immediately after zygote formation) with no real increase in the frequency of psi⁻ spores with further incubation of the PNM1/pnm⁺ zygote.

The latter result could reflect the number of psi⁺ "particles" present in the 4 psi⁺ 7L25 spore clones; in 1a and 1c there are very few (although enough to maintain the psi⁺ phenotype) with significantly more psi⁺ "particles" in 1b and 1d. Both 1a and 1c, although ADE⁺HIS⁺, gave pale pink colonies and 1b and 1d gave white ADE⁺HIS⁺ colonies. This segregation pattern for colour was 2:2 in all tetrads dissected from 7L25. Also 5 non-suppressed spores were obtained from the 7L25/1c x SUQ5 ade2.1 psi⁻ diploid (Table 7.5)

FIGURE 7.1 The elimination of the psi⁺ phenotype from
diploids heterozygous for PNM1.

(●) 507-4/2b x 483/1a

(▲) 465/1b x 483/1a

(○) 7L25/1a x 483/1a

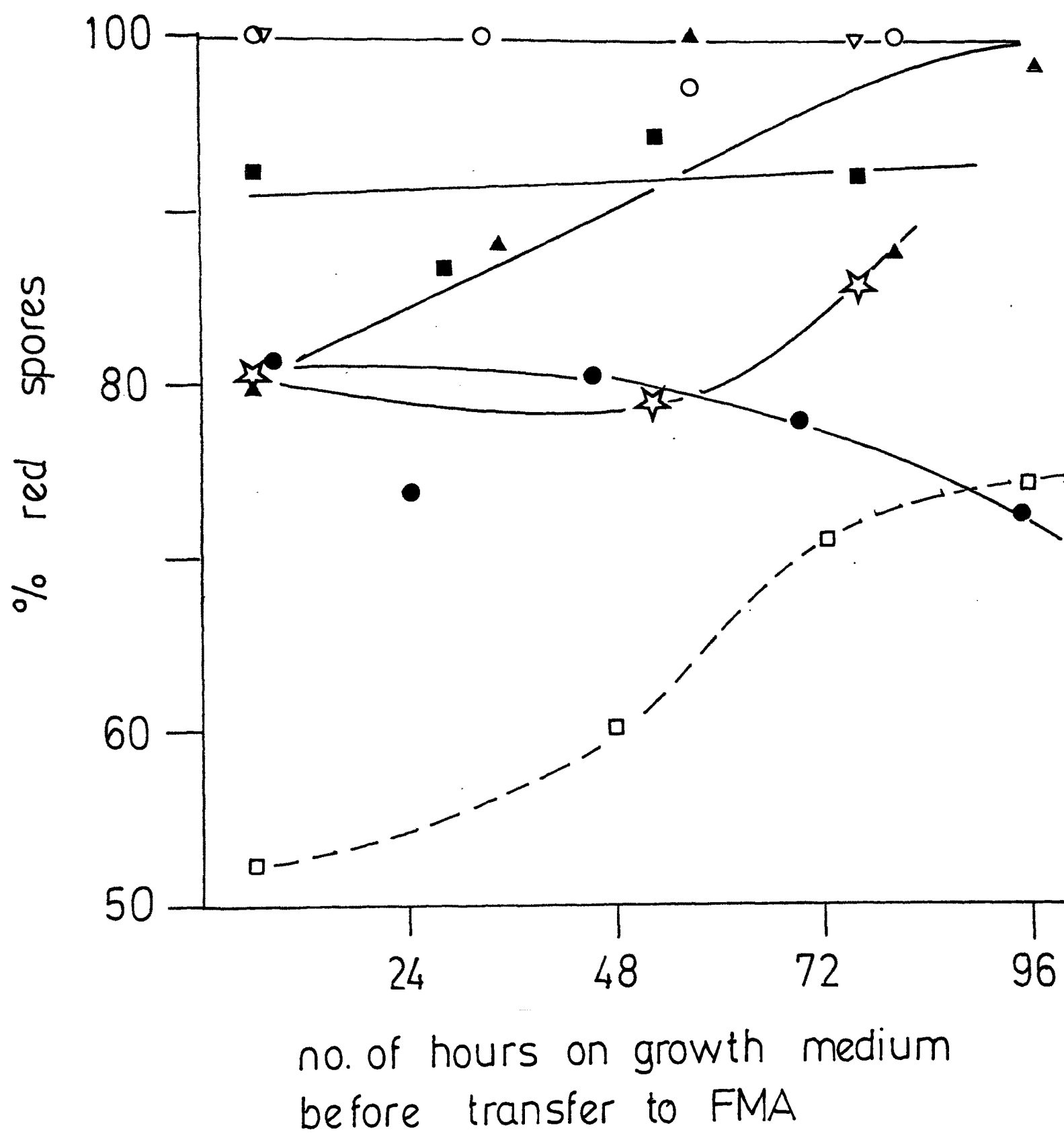
(■) 7L25/1b x 483/1b

(▼) 7L25/1c x 483/1a

(☆) 7L25/1d x 483/1b

— — □ — —

represents the data of Young and Cox
(1971).



All 5 were psi⁻, although no psi⁻ segregants were obtained from the similar cross to 1a or 1b (Table 7.5). 7L25/1c was also crossed to the SUQ5 ade2.1 psi⁺ strain, 507-4/2b. From the suppressed diploid 21 complete tetrads were obtained, 19 containing 4 suppressed spores each, and two tetrads with 3 psi⁺ and 1 psi⁻ spores. Both 7L25 and the 7L25/1a-d tetrad remained stable with respect to their psi⁺ phenotype during growth on YC.

Another possible explanation for the results with the 7L25 tetrad is that, of the 6 psi⁺ strains analysed in this study, only two (7L25/1a and 7L25/1c) were of mating type a and therefore crossed with 483/1a. Although carrying the same PNM⁻ mutation there is a possibility that the nuclear genetic background of the PNM⁻ strain has an effect on its efficiency of elimination of the psi⁺ phenotype in the PNM⁻/pnm⁺ diploid.

The difference between the efficiency of elimination observed here and that obtained by Young and Cox (1971) can be explained in terms of the sporulation media used. FMA, used in this study, allows the cells to undergo several doublings prior to sporulation. Acetate medium, used by Young and Cox, prevents any cell growth prior to sporulation.

(f) ANALYSIS OF REVERTANTS OF M10

Only one parent of M10 (483/7b) was a pnm⁺ psi⁻ segregant from a PNM⁻/pnm⁺ diploid, the other parent (468/6d) being derived from a spontaneous psi⁻ mutant isolated by Dr B.S. Cox. Reversion to ADE⁺HIS⁺ was studied in M10, as a control for M7, and to see if any significant recombination could occur between two independently derived psi⁻ strains.

The M10 diploid was stable for the non-suppressed phenotype and had a spontaneous reversion rate to $ADE^+ HIS^+$ of $56.3/10^5$ cells plated. This is comparable with the reversion rate of M7 ($65.1/10^5$ cells plated) implying that significant spontaneous allelic recombination was not occurring in M10. The UV-induced reversion frequency of M10 was also significantly lower than the UV-induced reversion frequency of M7 (Table 7.1) implying that significant UV-induced allelic recombination was not occurring in M10.

14 (36.8%) of the $ADE^+ HIS^+$ revertants gave predominantly white colonies after random spore analysis (Table 7.3) and all 14, together with a spontaneous $ADE^+ HIS^+$ revertant (10g) picked up on a YC plate, were subjected to tetrad analysis (Table 7.9). All, bar 10g, gave almost exclusively suppressed spores. 10g was heterozygous for a dominant $\bar{psi}7$ -independent, ochre suppressor. 4 other M10 revertants (10S4, 10S11, 10S14 and 10S20) gave some non-suppressed spores. In all cases the non-suppressed spores complemented as SUQ5 ade2.1 psi⁻ mutants.

Five of the revertants (10S1, 10S10, 10S11, 10S13 and 10S14) were analysed further by taking a tetrad from each and crossing each strain to the SUQ5 ade2.1 psi⁻ and SUQ5 ade2.1 psi⁻ PNM1 tester strains as described for M7 revertants (see Table 7.2). All strains from the 5 revertants gave the complementation patterns of psi⁺ strains i.e. red diploids with the PNM⁻ tester and white diploids with the pnm⁺ tester. All spore cultures also gave red diploids with the SUQ5 ade2.1 sal3.5 psi⁻ PNM2 tester.

It was concluded that all 5 $ADE^+ HIS^+$ revertants tested were psi⁺.

TABLE 7.9 Genetic analysis of ADE⁺HIS⁺ revertants of M10.

REVERTANT NUMBER	ORIGIN	TETRAD ANALYSIS			SUPPRESSED:NON-SUPPRESSED				*1 SPORE TOTALS S : NS
		NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS					
					4:0	3:1	2:2	0:4	
M10	PARENT	21	96.4	18	0	0	0	18	0 : 81
109		12	70.8	5	0	0	5	0	16 : 18
10L26	UV	12	60.4	7	7	0	0	0	29 : 0
10L29		9	75.0	2	2	0	0	0	27 : 0
10L30		12	70.8	6	6	0	0	0	34 : 0
10S1		6	75.0	4	4	0	0	0	18 : 0
10S2		6	95.8	5	5	0	0	0	23 : 0
10S4		6	87.5	4	3	1	0	0	20 : 1
10S10	SPONTANEOUS	6	100.0	6	6	0	0	0	24 : 0
10S11		6	87.5	5	4	1	0	0	20 : 1
10S13		6	91.7	5	5	0	0	0	22 : 0
10S14		6	95.8	5	3	1	1	0	20 : 3
10S15		6	66.7	3	3	0	0	0	16 : 0
10S16		6	100.0	6	6	0	0	0	24 : 0
10S20		6	66.7	3	0	3	0	0	12 : 4

*1 indicates data from incomplete tetrads.

(g) ANALYSIS OF REVERTANTS OF CM237.5

The psi⁻ diploid CM237.5 (constructed by C. Mundy) was derived by crossing two, independently isolated, spontaneous psi⁻ mutants (C. Mundy personal communication). Spontaneous and UV-induced reversion to ADE⁺HIS⁺ was measured in this diploid to compare with M7 and M10 reversion rates and to look for any evidence of allelic recombination between the two psi⁻ "genomes".

CM237.5 had as much lower spontaneous and UV-induced reversion frequency to the ADE⁺HIS⁺ phenotype than M7 or M10 (Table 7.1). Therefore no significant spontaneous or UV-induced allelic recombination was apparent.

12 of the 15 revertants of CM237.5 isolated sporulated well, 3 of which (TF10, TF13 and TF14) gave predominantly white colonies on random spore analysis (Table 7.3). These three, together with the 9 revertants that gave red and white colonies in the 'random spore analysis', were subjected to tetrad analysis (Table 7.10).

The 3 revertants that gave only white colonies on 'random spore analysis' segregated only suppressed clones from tetrads. One complete tetrad from TF13 and 2 spore clones from TF10 and TF14 were crossed to SUQ5 ade2.1 psi⁻ and SUQ5 ade2.1 psi⁻ PNM⁻ testers. From the diploid phenotypes it was concluded that TF10 and TF14 were homozygous for a recessive sal mutation whereas TF13 was homozygous for a dominant, /psi⁻-independent, ochre suppressor. The TF13/1a x SUQ5 ade2.1 psi⁻ diploid was sporulated and all 5 tetrads dissected segregated 2 suppressed: 2 non-suppressed spores. This confirms TF13 as being psi⁻ because if

TABLE 7.10 Genetic analysis of ADE⁺HIS⁺ revertants of CM237.5.

REVERTANT NUMBER	ORIGIN	NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS	SUPPRESSED : NON-SUPPRESSED			SPORE TOTALS S : NS
					4:0	3:1	2:2	0:4
CM237.5	PARENT	12	97.7	11	0	0	0	11
TF1	SPONTANEOUS	5	85.0	3	0	0	3	0
TF2		2	87.5	1	0	0	1	0
TF3		6	91.7	4	0	0	4	0
TF4	UV	6	16.7	0	0	0	0	0
TF5		6	95.8	5	0	0	5	0
TF6		5	100.0	5	0	0	5	0
TF7		6	29.2	0	0	0	0	0
TF8		6	83.3	2	0	0	2	0
TF10		7	25.0	0	0	0	0	0
TF13		6	100.0	6	6	0	0	0
TF14		7	42.8	0	0	0	0	0
TF15		7	28.6	0	0	0	0	0

it was psi⁺ one would have seen two suppressors segregating in these tetrads.

All the remaining 9 revertants appeared to be heterozygous for a dominant /psi⁻ -independent, ochre suppressor (SUPX). 2 red spores, derived from each revertant, were found to be psi⁻ by complementation analysis. To confirm the presence of SUPX one suppressed spore clone from each of 6 such revertants was crossed to SUQ5⁺ ade2.1 psi⁺ tester strains (Table 7.11). All gave suppressed diploids.

When the diploids, from crosses to the SUQ5⁺ ade2.1 psi⁺ testers, were analysed by tetrad analysis, 4 revertants (TF1/1a, TF2/1a, TF3/1c and T5/1a) segregated 2 suppressed: 2 non-suppressed spores, whereas TF4/1b and TF6/1c gave an overall segregation of 3 suppressed: 1 non-suppressed spores. The latter segregation pattern (3:1) can be explained by the presence of SUPX; one gets two, independently segregating ochre suppressors from a psi⁺ diploid; SUQ5 and SUPX. For the remaining 4 revertants which gave a 2:2 segregation either;

- (a) a second-site mutation in the SUQ5 gene had occurred to make the suppressor /psi⁻ -independent,
- or (b) a new dominant, /psi⁻ -independent ochre suppressor, very closely linked, to SUQ5, had arisen,
- or (c) a dominant allosuppressor (SALX) had arisen which was not itself a suppressor in a psi⁺ background.

An attempt to identify which of the above was responsible for the suppressed phenotype of the 4 revertants was made by crossing TF1/1a, TF2/1a, TF3/1c and TF5/1a to SUQ5⁺ ade2.1 psi⁻ strains (468/1b or 468/2d). Viable suppressed diploids were obtained in each case, but all failed to sporulate.

TABLE 7.11 Genetical analysis of presumptive SUQ5 SUPX ade2.1 psi⁻ spores in crosses to SUQ5⁺ ade2.1 psi⁺ strain.

SPORE NUMBER	MATING TYPE	TETRAD ANALYSIS		NO. COMPLETE TETRADS	SUPPRESSED:NON-SUPPRESSED			SPORE TOTALS	*1
		NO. TETRADS DISSECTED	% SPORE VIABILITY		4:0	3:1	2:2		
								S	:
TF1/1b	a	6	100.0	6	0	0	6	12	: 12
TF2/1a	α	6	95.8	5	0	0	5	12	: 11
TF3/1c	a	7	96.4	6	0	1	5	14	: 13
TF4/1b	a	8	90.6	6	1	4	1	21	: 8
TF5/1a	α	6	91.7	4	0	0	4	12	: 10
TF6/1c	α	6	100.0	6	1	4	1	18	: 6

*1 includes incomplete tetrad data.

All suppressed clones from tetrads of crosses with TF1, TF2, TF3 and TF5 were sensitive to 6% (V/V) dimethyl sulphoxide (DMSO) in YC as were the original diploid revertants. On the other hand all spores from TF4 and TF6 and the original diploids were resistant to DMSO. The mutation(s) giving the suppressed phenotype in TF1, TF2, TF3 and TF5 therefore also rendered the cells sensitive to DMSO. Both phenotypes were dominant.

(2) Induction of ψ^+ in PNM strains

Reversion experiments, similar to those reported in the previous section (7.2.1) were repeated using diploids homozygous for PNM1, or heterozygous at one or both of the known PNM⁻ loci, in an attempt to isolate mutants with either an altered ψ^+ phenotype (mutant ψ^- factor?), which was not eliminated in the presence of PNM⁻, or mutations (both extracistronic and intracistronic) which eliminate or suppress the PNM⁻ mutant phenotype. Obtaining such mutants would assist analysis both of the PNM⁻ phenotype and of the ψ^- factor itself.

Three kinds of experiment were carried out. In all three reversion from non-suppressed to suppressed phenotype was selected in diploid strains to reduce the number of recessive sal mutants among the revertants. All revertants were subjected to analysis as described in the previous section; namely random spore analysis and tetrad analysis.

In the first experiment a diploid (MT90) was treated which was homozygous for the PNM1 mutation and therefore started as ψ^- . In the second a PNM1 haploid strain was mated to a ψ^+ strain which had been UV-irradiated or grown in a YC + 10% MeOH liquid culture for 24 hours. The diploid zygotes, heterozygous for PNM1, were subjected to selection. In this experiment

counter selection was applied against each haploid parent, and against dominant nonsense suppressors, by plating the mating mixture, after 24 hours incubation, on hypertonic medium (Singh 1977). In the third experiment a similar diploid was constructed and treated. It differed from the diploids used in the second experiment in being heterozygous at two PNM⁻ loci.

The results are summarised in Table 7.11a.

The four psi⁺ revertants, obtained from the 153 ADE⁺ HIS⁺ revertants analysed, were all found to be pnm⁺/pnm⁺ by crossing the four spores from one tetrad of each to standard SUQ5 ade2.1 psi⁺ strains. In all cases only suppressed diploids were observed which segregated only suppressed spores. It was concluded that the diploids had regained their psi⁺ phenotype following a mitotic cross-over; namely PNM1/pnm⁺ → pnm⁺/pnm⁺. The psi⁻ parent diploid segregated 11 psi⁺ and 76 psi⁻ spores demonstrating that the psi⁺ phenotype had been completely eliminated.

These results demonstrate that a PNM⁻/pnm⁺ psi⁻ diploid does not lose the genetic information required to revert back to psi⁺ but such an event can only happen following loss of the PNM⁻ mutant allele by mitotic cross-over.

(3) Summary

These experiments have confirmed that the psi⁺ phenotype can be regained in a psi⁻ strain either spontaneously or be induced by UV-light. The elimination of the psi⁺ phenotype by the PNM⁻ mutation does not result in the loss of information required for this "reversion" to psi⁺. Psi⁻ to psi⁺ revertants are normal with regard to being inherited cytoplasmically, and elimination of the phenotype by 10% MeOH and by the PNM1 mutation. The

TABLE 7.11a The number of revertants obtained and their genotypes established by subsequent

analysis in three experiments. The protocol of the experiments is described in the text.

STRAIN *1	SELECTION MEDIUM	VIABLE CELLS PLATED	TREATMENT	NO. ADE ⁺ HIS ⁺ REVERTANTS ANALYSED *2	GENOTYPES OF REVERTANTS		
					SUPX/+	SUPX/SUPX	salx/salx psi ⁺
MT90	YNB + lys	1.4 x 10 ⁷	0	48	48	0	0
(<u>PNM 1</u>)	+ trp + leu	6.7 x 10 ⁵	UV (100J/m ²)	21 (7)	10	0	4
(<u>PNM 1</u>)							0
MT182/8a		c. 1 x 10 ⁶	0			0	0
(SUQ5 psi ⁺)							2
x							
483/7b	YNB + 1.5M	c. 1 x 10 ⁶	UV (75J/m ²)	56 (1)	45	0	2
(SUQ5 PNM1)	KCl	c. 1 x 10 ⁶	MeOH (10%v/v)			0	4
MT11							1
(<u>PNM1</u> <u>PNM2</u>)	YNB	c. 1 x 10 ⁶	0	28 (10)	18	0	0
+ +							

*1 all diploids were homozygous for SUQ5 ade2.1 his5.2.

*2 figure in brackets is the number of revertants that failed to sporulate.

kinetics of PNM⁻ elimination were found to differ from those previously published (Young and Cox 1971; McCready, Cox and McLaughlin 1977) in 5 of the 6 psi⁺ strains looked at i.e. no progressive loss of the phenotype was observed.

Reversions to the ADE⁺HIS⁺ phenotype by an SUQ5/SUQ5 ade2.1/ade2.1 his5.2/his5.2 psi⁻ diploid were found to be due to one of four events: reversion to psi⁺; a dominant, /psi⁻-independent ochre suppressor; homozygosity for a sal mutation. From the genetic analysis conducted, no conclusion about a fourth class of revertant (TF1, TF2, TF3 and TF5 of CM37.5) could be made other than they were /psi⁻-independent and nuclear mutations.

No evidence for spontaneous or UV-induced allelic recombination between different psi⁻ strains was found.

7.3 Modifiers of the PNM⁻ phenotype

(a) A HAPLOID PNM⁺ MUTANT THAT SHOWS ENHANCED STABILITY OF THE PSI⁺ PHENOTYPE

During studies with kar1 strains (see Appendix 3) : a red zygotic clone from the cross MT152/1d $\times$ ade2.1 his5.2 kar1 leu1 psi⁺ x 483/1b a SUQ5 ade2.1 his5.2 can1.100 PNM1 psi⁻ was plated onto YC. Of 604 colonies that arose one had a suppressed phenotype but, upon recloning on YC, this phenotype was found to be very unstable with colonies giving rise to large numbers of non-suppressed sectors. Both the suppressed and non-suppressed forms of this mutant (designated WPC-1) were haploid, of mating type a and LEU⁺ suggesting that it was a mutant of the 483/1b parent. Zygotes isolated by micromanipulation from kar1 x KAR⁺ crosses often given rise exclusively to one or other of the haploid parent strains (see Appendix 3).

Because the 483/1b strain carried the mutation PNM1 the WPC-1 mutant was analysed in some detail to see if it was psi⁺ and whether the PNM1 mutation was still "active" i.e. does the strain carry a defective psi⁺ phenotype partially resistant to elimination by PNM1 or is the efficiency of the PNM1 gene modified?

(i) INSTABILITY OF THE SUPPRESSED PHENOTYPE

The original mutant colony was suspended in saline and plated onto YC to give individual colonies. No cell clumping in saline was observed. After 4 days an apparently stable white colony was picked (although several "hairline" pink sectors were present), suspended in saline and replated onto YC to give individual colonies. This was repeated once more. The phenotypes of the colonies that arose are given in Table 7.12. From the first plating a pink and a red colony were also taken, suspended in saline and plated onto YC to give individual colonies.

Loss of the suppressed phenotype by WPC-1 appeared to be in 2 clearly distinguishable stages; white to pink, then pink to red. In no case was a white colony with a red sector seen (Table 7.12). All "stable" white colonies scored had several "hairline" pink sectors when examined under a stereo microscope, but only those with distinct pink sectors were scored as white/pink/sector colonies. No truly stable white colony was isolated.

Thus there appeared to exist a replicating instability in WPC-1 because cells, individually plated from the original mosaic WPC-1 colony, themselves produced mosaic colonies with a frequency at least a factor of 10^5 higher than predicted of normal spontaneous nuclear mutation. The white to pink change occurred at a

TABLE 7.12 Phenotypes of colonies arising from white, pink or red isolates

of WPC-1 upon plating onto YC.

PHENOTYPE OF COLONY PLATED	TOTAL	COLOUR OF COLONIES ON YC ^{*1}					
		"WHITE" ^{*2}		SECTORED PINK & WHITE	PINK	SECTORED PINK & RED	RED
WHITE 1	130 (100)	77 (59.2)	33 (25.4)	17 (13.1)	2 (1.5)	1 (0.8)	0
WHITE 2	463 (100)	327 (70.6)	65 (14.0)	68 (14.7)	2 (0.4)	1 (0.2)	0
WHITE 3	347 (100)	302 (87.0)	36 (10.4)	5 (1.4)	0	2 (0.6)	1 (0.3)
PINK 1	1176 (100)	1 (0.1)	0	1163 (98.9)	12 (1.0)	0	0
RED 1	669 (100)	0	0	0	0	669 (100)	0

*1 number of colonies (% of total colonies counted).

*2 those colonies with hairline pink sectors.

much higher frequency than the pink to red change.

The suppression patterns of 12 white, 18 pink and 4 red colonies were determined. The whites were a $ADE^+HIS^+CAN^S$, the pinks a $ade^{(+)}HIS^+can^{(s)}$ with the reds being a $ade^-his^{(+)}can^r$. i.e. a progressive loss of suppression of the 3 markers was observed with the change in colour of the mutant to red. No class was unstable for /rho and all colonies tested were K⁺.

(ii) GENETIC ANALYSIS OF WPC-1

A white, a pink and a red mutant of WPC-1 (WPC-1W, WPC-1P, WPC-1R respectively) were each crossed to (1) an SUQ5 ade2.1 psi⁺ strain (507-4/2b) to check for the presence of a PNM⁻ mutation (2) to an SUQ5 ade2.1 psi⁻ strain (468/1d) to check their /psi phenotype and (3) to another SUQ5 ade2.1 psi⁻ PNM1 (483/1a) to look for the presence of a PNM⁻ modifying gene mutation. All diploids were grown for at least 4 days prior to inducing sporulation. At least 10 tetrads were dissected from each cross and the results are given in Table 7.13.

Three distinct spore phenotypes were seen; white suppressed and pink or red non-suppressed. The crosses with the SUQ5 ade2.1 psi⁻ tester demonstrated that WPC-1W and WPC-1P were psi⁺ whilst WPC-1R was psi⁻. However the crosses to WPC-1W and WPC-1P gave an excess of non-suppressed spores (> 65%) suggesting that these mutants still carried an active PNM⁻ mutation. This was confirmed in the crosses to 507-4/2b where again an excess (> 60%) of non-suppressed spores were obtained, the WPC-1W mutant being the least efficient, and WPC-1R the most efficient at eliminating the psi⁺ phenotype. The efficiency of the latter mutant was identical to that scored for the parent strain 483/1a crossed to 507-4/2b (see Figure 7.1).

TABLE 7.13 Genetic analysis WPC-1 mutant phenotypes.

PHENOTYPE OF WPC1	CROSSED TO *3	DIPLOIDS		% SPORE VIABILITY	SEGREGATION OF SPORE COLOUR							SPORE TOTALS		
		NUMBER	PHENOTYPE *2									*1		
					2W:2P	2W:2R	2P:2R	2W: 1P:1R	1W: 1P:2R	1W: 1P:2R	4R:OW	S	NS	%NS
WHITE	x SUQ5 psi ⁺	10	6xS 4xS/NS	84.7	1	1	0	1	3	2	0	21	37	63.8
	x SUQ5 psi ⁻	6	2xS 4xNS	88.5	1	2	2	0	1	1	0	15	31	67.4
	x SUQ5PNM1 psi ⁻	3	3xNS	93.8	0	0	17	0	0	0	0	0	75	100
PINK	x SUQ5 psi ⁺	6	6xS/NS	61.8	0	0	1	2	0	1	0	14	33	70.2
	x SUQ5 psi ⁻	6	6xNS	95.0	0	0	2	0	6	0	0	7	31	81.6
	x SUQ5PNM1 psi ⁻	3	3xNS	97.5	0	0	9	0	0	0	0	0	39	100
RED	x SUQ5 psi ⁺	7	7xNS	97.5	0	2	2	0	1	4	0	10	29	74.4
	x SUQ5 psi ⁻	8	8xNS	100.0	0	0	10	0	0	0	0	0	40	100
	x SUQ5PNM1 psi ⁻	3	3xNS	100.0	0	0	0	0	0	0	10	0	40	100

*1 data from incomplete tetrads included; S= white NS = red or pink .

*2 where 2 different phenotypic classes of diploid were obtained the suppressed diploid was analysed by tetrad analysis.

*3 strains were 507-4/2b, 468/1a and 483/1a respectively.

Crosses to the SUQ5 ade2.1 psi⁻ PNM1 strain yielded only non-suppressed spores for all 3 mutants although, in the case of WPC-1W and WPC-1P, a clear 2:2 segregation for pink:red colony colour was observed.

(iii) MODEL FOR WPC-1

To explain the observed phenotypes of WPC-1, and the results of the genetic analysis in Table 7.13, the following model is proposed. WPC-1 carries a recessive mutation (mod) which stabilises psi⁺ in a PNM⁻ background. This modifier has two states mod and mod¹ with the mod $\longrightarrow$ mod¹ reversion occurring spontaneously at a frequency of 10^{-1} . The tetrad analysis of WPC-1W and WPC-1P (Table 7.13) demonstrates that both are psi⁺ because, when crosses to the SUQ5 ade2.1 psi⁻ strain 468/1d, some fully suppressed white (psi⁺) spores are obtained. WPC-1R is psi⁻ because no suppressed spores are obtained in such a cross. A mod¹ PNM1 psi⁺ strain loses its psi⁺ phenotype at a frequency of 10^{-2} to give a mod¹ PNM1 psi⁻ strain i.e.

WPC-1W	:	<u>mod</u>	<u>PNM1</u>	<u>psi⁺</u>
WPC-1P	:	<u>mod¹</u>	<u>PNM1</u>	<u>psi⁺</u>
WPC-1R	:	<u>mod¹</u>	<u>PNM1</u>	<u>psi⁻</u>

The observed spore segregations in Table 7.13 can be accounted for if one assumes that mod or mod¹ have weak nonsense suppressor or allosuppressor phenotypes. In crosses between either WPC-1W or WPC-1P and either SUQ5 ade2.1 psi⁺ or SUQ5 ade2.1 psi⁻ strains one would predict basically a 2 pink (mod or mod¹) : 2 red (MOD⁺) segregation with those spores remaining psi⁺ (and pnm⁺) being white:

	<u>GENOTYPE</u>			<u>TETRAD PHENOTYPE</u>
Parental ditype	mod	PNM	ψ^-	IW : 2P : 1R
	mod	PNM	ψ^-	or
	+	+	ψ^+ or ψ^-	2P : 2R or
	+	+	ψ^+ or ψ^-	2W : 2P
Tetratype	mod	PNM	ψ^-	2W : 1P : 1R
	mod	+	ψ^+ or ψ^-	or
	+	PNM	ψ^-	1W : 2P : 1R or
	+	+	ψ^+ or ψ^-	2P : 2R
Non-parental ditype	mod	+	ψ^+ or ψ^-	2W : 2R
	mod	+	ψ^+ or ψ^-	or
	+	PNM	ψ^-	1W : 1P : 2R or
	+	PNM	ψ^-	2P : 2R

- all these classes of tetrad phenotypes were observed (Table 7.13). WPC-1R x SUQ5 ade2.1 ψ^+ would give the same results as above whilst the WPC-1R ψ^- would segregate no white spores, all tetrads being 2 red : 2 pink. This again was observed (Table 7.13). In crosses between either WPC-1W and WPC-1P and the SUQ5 ade2.1 PNM1 ψ^- strain the weak 'suppressor' phenotype of the mod or mod¹ mutation would give 2 pink : 2 red tetrads with no ψ^+ segregants arising from the PNM1/PNM1 diploid. To account for the 0 pink : 4 red segregation in tetrads, from the WPC-1R x SUQ5 ade2.1 PNM1 ψ^- cross, one could postulate that, in the absence of ψ^+ , mod¹ is converted to MOD⁺ by PNM1 at meiosis

A similar modifier, which appears to stabilise the ψ^+ phenotype in a "weak" PNM background and which acts as a weak (alb) suppressor, has recently been identified (C. Mundry personal communication). Its effects in ^aPNM1 background have not been investigated.

(b) A HAPLOID PSI^+ STRAIN THAT SHOWS A HIGH FREQUENCY OF REVERSION TO PSI^-

Several strains of the genotype SUQ5 ade2.1 his5.2 cdc21 psi^+ were found to show a high spontaneous reversion to psi^- (see Chapter 5 for details), Cdc21 mutants are unstable for the grande phenotype at the permissive temperature (Newlon et al., 1976) and are rapidly converted to petite at the non-permissive temperature (see Chapter 5). To determine whether the psi^+ instability was a function of the cdc21 mutation, or some other mutation present in these strains, a genetic analysis of MT155/3c (a SUQ5 ade2.1 his5.2 cdc21 psi^+) was undertaken.

Nineteen spontaneous red mutants of MT155/3c were isolated by repeated replating of suppressed colonies on YC plates incubated at 23°C. The frequency was approximately $5/10^3$ cells plates. Five were grande and 14 were petite with all being ts at 37°C. All 19 mutants were ade⁻his⁻ and complemented an α ade1 strain but failed to complement the SUQ5 ade2.1 psi^- strain 468/1d. They all showed weak, but detectable, complementation with the SUQ5⁺ ade2.1 psi^+ strain 465/1d.

Four of these putative psi^- mutants were picked for analysis; R1 and R2 were grande and R3 and R4 were petite. Three fully suppressed clones of MT155/3c were also picked for analysis; W1 was grande and W2 and W3 petite. Each of the 7 mutants was crossed to 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi^+) and upto 5 individual zygotes isolated for each. The zygote clones were then induced to sporulate and tetrads dissected from one zygote clone from each cross. The results are given in Table 7.14.

TABLE 7.14 Genetic analysis of suppressed and non-suppressed mutants of MT155/3c;

crosses to 507-4/2b (SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺)

MUTANT	MUTANT PHENOTYPE	DIPLOID (x 507-4/2b) PHENOTYPE	% SPORE VIABILITY	NO. COMPLETE TETRAIDS	SEGREGATION IN COMPLETE TETRAIDS		
					S : NS	CDC ⁺ :cdc21	URA ⁺ :ura3.1
R1	ade ⁺ his ⁺ rho ⁺ K	RED ade ⁻ his ⁻	100	9	9x0 : 4	9x2 : 2	9x2 : 2
R2	ade ⁺ his ⁺ rho ⁺ K	RED ade ⁻ his ⁻	97.5	9	9x0 : 4	9x2 : 2	9x2 : 2
R3	ade ⁺ his ⁺ rho ⁺ K	RED ade ⁻ his ⁻	95.0	8	8x0 : 4	8x2 : 2	8x2 : 2
R4	ade ⁺ his ⁺ rho ⁺ K	RED ade ⁻ his ⁻	92.5	8	8x0 : 4	8x2 : 2	8x2 : 2
W1	ADE ⁺ HIS ⁺ rho ⁺ K	WHITE ⁺ ADE ⁺ HIS ⁺	91.7	8	8x4 : 0	7x2 : 2 1x1 : 3	8x2 : 2
W2	ADE ⁺ HIS ⁺ rho ⁺ K	WHITE ⁺ ADE ⁺ HIS ⁺	92.5	8	8x4 : 0	8x2 : 2	8x2 : 2
W3	ADE ⁺ HIS ⁺ rho ⁺ K	WHITE ⁺ ADE ⁺ HIS ⁺	37.5	4	3x4 : 0 1x2 : 2	4x2 : 2	4x2 : 2

This analysis demonstrated that the putative psi⁻ mutants in fact carried a dominant mutation in a psi⁺ maintenance gene. All segregants from the R mutant crosses to 507-4/2b were non-suppressed. It was concluded that R1 - R4 carried a PNM⁻ mutation (Young and Cox 1971). Cdc21 was recessive for both its petite -inducing and ts phenotypes. The 3 suppressed mutants W1-W3 however gave only suppressed spore clones in crosses to 507-4/2b demonstrating these strains to be PNM⁺ psi⁺.

MT155/3c therefore appeared to carry an inactive PNM⁻ mutation which became "switched on" during mitotic growth at a high frequency. This "switching on" was not necessarily accompanied by loss of the grande phenotype.

4 non-suppressed spore cultures, taken from one tetrad dissected from the R4 x 507-4/2b cross, were each crossed to a number of standard tester strains and individual zygotes isolated (Table 7.15). Two sterile and 2 α spores were found. One of the α spores, 3d, complemented as an SUQ5 ade2.1 psi⁻ mutant and was cdc21. The second α spore, 3a, gave mosaic diploid clones when crossed to both the SUQ5 ade2.1 psi⁺ strain (466/4a) and the SUQ5⁺ ade2.1 psi⁺ strain (465/2a). 3a therefore appeared to carry an inefficient, dominant PNM⁻ mutation; 3a was CDC21⁺.

A single mosaic zygotic clone from 3a x 466/4a and 3a x 465/2a were each suspended in saline sonicated and replated onto YC. In each case > 95% of the colonies were mosaic, the rest being red, stable, non-suppressed diploids. The maintenance of the original mosaic phenotype, upon replating, demonstrated that these diploids carried a replicating instability.

A stable red diploid from the 3a x 466/4a cross was sporulated and tetrads dissected. Red:white spores segregated in the ratios

TABLE 7.15 A complementation analysis of a single tetrad from R4 (MT155/3c) x 507-4/2b.

SPORE NUMBER	COLOUR OF SPORE CULTURE	GROWTH AT 37°C	PHENOTYPE OF ZYGOTE CLONES				MATING TYPE	
			x ade1	x SUQ5 psi ⁻	x SUQ5 ⁺ psi ⁺	x SUQ5 ⁺ psi ⁺ kar x SUQ5psi ⁺		
3a	RED	+	WHITE	RED	MOSAIC	RED	MOSAIC	α
3b	RED	+	_____	_____	STERILE	_____	_____	-
3c	RED	-	_____	_____	STERILE	_____	_____	-
3d	RED	-	WHITE	RED	WHITE	RED, RED & WHITE WHITE	WHITE	α

TABLE 7.15 A complementation analysis of a single tetrad from R4 (MT155/3c) x 507-4/2b.

SPORE NUMBER	COLOUR OF SPORE CULTURE	GROWTH AT 37°C	PHENOTYPE OF ZYGOTE CLONES				MATING TYPE	
			x ade1	x SUQ5 psi ⁻	x SUQ5 ⁺ psi ⁺	x SUQ5 ⁺ psi ⁺ kar x SUQ5psi ⁺		
3a	RED	+	WHITE	RED	MOSAIC	RED	MOSAIC	α
3b	RED	+	_____	_____	STERILE	_____	_____	-
3c	RED	-	_____	_____	STERIE	_____	_____	-
3d	RED	-	WHITE	RED	WHITE	RED, RED & WHITE WHITE	WHITE	α

of 4 x 4 0, 3x3:1 and 1 x 2:2 with his5.2 being poorly suppressed in all spore cultures. This confirms that 3a carried a PNM⁻ mutation. A stable red diploid from the 3a x 465/2a cross was also subjected to tetrad analysis. Red:white spores segregated in the ratios of 3 x 4:0 and 5 x 3:1 with the his5.2 marker being poorly suppressed in the SUQ5 - carrying spores. The PNM⁻ mutation, carried by 3a, was therefore active in both SUQ5/SUQ5 and SUQ5/SUQ5⁺ diploids.

Two^{of} the non-suppressed revertants of MT155/3c (R2 and R3) were crossed to 465/4d (SUQ5⁺ ade2.1 psi⁺) and in each case mosaic diploid clones were obtained. A stable red colony was obtained from each cross, by recloning on YC, and sporulated. R2 x 465/2a segregated red and white spores in the ratios 2 x 4:0, 4 x 3:1 and 5 x 2:2 whilst R3 x 465/2a segregated red and white spores in the ratios 2 x 4:0, 8 x 3:1 and 1 x 2:2. The PNM⁻ mutation present in R2 and R3 therefore was effective in SUQ5/SUQ5⁺ diploids but, as observed with 3a, it gave rise to a replicating instability in such cross, from suppressed to non-suppressed. Stable, fully non-suppressed, zygote clones were obtained in crosses to the SUQ5 ade2.1 psi⁺ 507-4/2b (see Table 7.14).

To determine whether MT155/3c carried a modifier of PNM⁻ mutations the suppressed derivative W1 was crossed to the SUQ5 ade2.1 PNM1⁻ psi⁻ strain 483/1a. The isolated zygotes all gave rise to mosaic clones which, upon replating, after sonication, gave either red, pale pink or mosaic colonies. A red and a pale pink diploid were sporulated. The red (MT174-1) segregated red and white spores in the ratios 6 x 4:0 and 1 x 3:1, whilst the pink (MT174-2) segregated red and white spores in the ratios 2 x 4:0 6 x 3:1 and 2 x 2:2. The psi⁺ phenotype was apparently less efficiently eliminated in MT174-2 than in MT174-1.

In summary MT155/3c appeared to carry both a PNM⁻ mutation and a modifier of PNM⁻ (suppressor?) which prevented expression of the PNM⁻ phenotype. This modifier was "lost" or reverted at a high frequency to an inactive state during vegetative growth at an approximate frequency of 5×10^{-3} . This resulted in a reversion of psi⁺ to psi⁻ in the cell, the reversion being stable. The modifier, in crosses to the previously characterised PNM1 mutation, appeared to have an effect on the efficiency of PNM1 in eliminating the psi⁺ phenotype. The modifier was therefore at least partially dominant. The crosses between the non-suppressed revertants of MT155/3c (R2 and R3) and the SUQ5⁺ ade2.1 psi⁺ strain 465/4d demonstrated that the PNM⁻ mutation was inefficient in an SUQ5/SUQ5⁺ diploid giving rise to a replicating instability.

These results demonstrate that cdc21 is not the PNM⁻ mutation (see Table 7.15). Since MT155/3c was one of the parent strains of the MT161-6/2a strain used in thymidylate starvation experiments (see Chapter 5) it was concluded that MT161-6/2a also carried the PNM⁻ mutation and the modifier. The results, presented in Chapter 5, Table 5, demonstrate that holding MT161-6/2a at the non-permissive temperature did not result in an increase in the frequency of non-suppressed revertants. The psi⁺ instability of MT161-6/2a was not a function of the cdc21 mutation.

(c) SUMMARY

The two mutants analysed (MT155/3c and WPC-1) both appeared to carry modifiers of the PNM⁻ phenotype. In WPC-1 the modifier appeared to stabilise the psi⁺ phenotype in a PNM⁻ background whilst MT155/3c carried a modifier which prevented expression of

the PNM⁻ mutation. These modifiers were highly unstable being "lost" or reverted to an inactive state at very high frequencies during normal vegetative growth.

7.4 Recessive PNM (pnm) mutations

During an analysis of non-suppressed mutants obtained by several different mutagenic treatments of 507-4/2b (see Chapter 3), a class of nuclear mutation was obtained which failed to complement SUQ5 ade2.1 psi⁻ strains, but did complement SUQ5⁺ ade2.1 psi⁺ strains, i.e. they complemented as psi⁻ mutants. Tetrad analysis of the mutant x SUQ5⁺ ade2.1 psi⁺ crosses suggested that these were nuclear, rather than cytoplasmic, psi⁻ mutants (Table 3). Consequently a preliminary genetic analysis was carried out on some of these "nuclear psi⁻" (pnm) mutants and a complementation test devised to distinguish pnm from the "cytoplasmic psi⁻" mutants.

1) Genetic analysis

A preliminary genetic analysis of 17 of these mutants was carried out by crossing each to 465/2a (a SUQ5⁺ ade2.1 psi⁺). All 17 gave suppressed diploids confirming that loss of the suppressed phenotype in these mutants was due to a recessive mutation. A set of tetrads was dissected from each cross and in all cases an excess of non-suppressed spores was obtained (Table 7.16). If the mutants were true cytoplasmic psi⁻ mutants one would predict a 2 suppressed (SUQ5) : 2 non-suppressed (SUQ5⁺) spore segregation in tetrads from a cross to 465/2a. This was found to be true of a MeOH-induced psi⁻ strain, 507 Me3psi⁻ (Table 7.16). If the mutants carried a single nuclear gene mutation, that was recessive with respect to its elimination of the psi⁺ phenotype, one would predict (assuming the mutant gene was not linked to SUQ5) an overall 1 suppressed: 3

TABLE 7.16 Genetic analysis of pnm x 465/2a (ade2.1 psi⁺ SUQ5⁺) crosses.

MUTANT NUMBER	*1	TETRAD ANALYSIS		SEGREGATION IN COMPLETE TETRADS			SPORE		GOODNESS OF FIT	*3
		NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS	(SUPPRESSED:NON-SUPPRESSED)		TOTALS			
					0:4	1:3		2:2		
									χ^2	p
1		12	85.4	6	2	2	9	32	0.07	0.7-0.8
2		12	97.9	11	2	1	10	37	0.18	0.5-0.7
3		12	81.2	7	2	1	7	32	0.69	0.3-0.5
4		12	79.2	6	0	1	12	26	0.56	0.3-0.5
5		12	10.4	0	0	0	3	2	-	-
6		10	95.0	8	3	1	9	29	0.0	0.95
7		11	100.0	11	2	0	9	35	0.27	0.5-0.7
8		10	97.5	9	5	1	5	34	2.47	0.1-0.2
9		7	100.0	7	3	0	4	24	1.19	0.2-0.3
10		12	95.8	10	7	0	3	43	7.42	0.001-0.01
11		12	97.9	11	1	3	13	34	0.06	0.7-0.8
12		12	81.2	8	5	1	7	32	0.69	0.3-0.5
13		10	70.8	6	5	0	1	33	7.69	0.001-0.01
14		12	95.8	10	3	3	11	35	0.0	0.95
15		20	62.5	8	1	4	15	35	0.40	0.5-0.7
16		9	94.4	7	3	1	7	27	0.16	0.5-0.7
17		10	92.5	7	1	1	11	25	0.04	0.8-0.9
507Me3psi ⁻		22	87.5	15	0	15	37	40	-	-

*1 Origin of the mutants used 1-4, UV (100J m²); 5-14, UV (80J/m²); 15, UV (200J/m²); 16, NTG (25µg/ml); 17, UV (75J/m²) - MT182/8d.
 *2 includes data from incomplete tetrads.
 *3 the null hypothesis is that pnm and SUQ5 segregate independantly with an expected tetrad segregation of 1S:3NS. Degrees of freedom=1 and therefore 'Yates' correction' was used.

non-suppressed spore segregation from a cross to 465/2a.

Statistical analysis of the tetrad data (Table 7.16) showed that only three mutants (6, 11 and 14), in crosses to 465/2a, gave spore segregation data suggestive of a recessive pnm mutation unlinked to SUQ5. All other mutants in crosses to 465/2a gave a significant ($p < 0.8$) excess of non-suppressed segregants suggesting either;

- (a) the pnm mutation in these mutants was partially dominant in a heterozygous pnm⁻/pnm⁺ diploid,
- or (b) the pnm mutation was linked to SUQ5.

To determine which alternative was correct two tetrads, containing only non-suppressed spores, were taken from the 8 x 465/2a cross and two from the 10 x 465/2a cross and subjected to the standard complementation test (see Chapter 3). The results are given in Table 7.17. Several striking results came from this analysis:

- i/ haploid spores that were psi⁻ did not necessarily carry a pnm mutation,
- ii/ in both the mutants SUQ5 and pnm did not appear to be linked, and
- iii/ aberrant segregation of the mating type loci occurred. In 3 of the 4 tetrads tested only 2 fertile α spores were found but, because only one spore had the pnm mutation, it was concluded that this was an aberrant tetrad.

A similar analysis was carried out with 3 other mutants 2, 7 and 11 (Table 7.18). In two of these crosses (involving mutants 2 and 7) defects in mating ability were seen, but in neither case was a pnm carrying spore found, even in the one tetrad with 4 fertile spores. The tetrad from 11 x 465/2a did have pnm segregating and normal segregation of the mating type loci.

TABLE 7.17 Complementation analysis of tetrads containing 4 non-suppressed spores from two putative SUQ5-linked pnm mutants x 465/2a.

CROSS	SPORE NUMBER	MATING TYPE	CROSSED TO *1				GENOTYPE OF SPORE
			ade1	SQ5 ⁻ psi ⁻	SQ5 ⁺ psi ⁺	SQ5 ⁺ psi ⁺ kar1	
8 x 465/2a	3a	S	R				
	3b	S	R				
	3c	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	3d	α	W	R	W	W	SUQ5 psi ⁻
	8a	α	W	R	W	W	SUQ5 psi ⁻
	8b	a	W	R	W	R	SUQ5 psi ⁻ pnm
	8c	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	8d	α	W	W	R	R	SUQ5 ⁺ psi ⁺
10 x 4465/2a	9a	S	R				
	9b	α	W	R	R	R	SUQ5 ⁺ psi ⁻ (pnm)?
	9c	α	W	R	R	R	SUQ5 ⁺ psi ⁻ (pnm)?
	9d	S	R				
	10a	α	W	R	R	R	SUQ5 ⁺ psi ⁻ (pnm)?
	10b	S	R				
	10c	S	R				
	10d	α	W	R	R	R	SUQ5 ⁺ psi ⁻ (pnm)?

*1 standard tester strains used (see Table 2.5) R = no complementation, W = complementation.
*2 S = sterile ie. showed no mating with α or a ade1.

TABLE 7.18 Complementation analysis of tetrads containing 3 non-suppressed spores from 3 further putative pnm mutants x 465/2a.

CROSS	SPORE NUMBER	MATING TYPE	CROSSED TO				GENOTYPE OF SPORE
			ade1	SUQ5 psi ⁻	SUQ5 ⁺ psi ⁺	SUQ5 ⁺ psi ⁺ kar	
2 x 465/2a	3a	S	R				
	3b	S	R				
	3c	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	3d	α	W	R	W	W	SUQ5 psi ⁻
	7a	a	W	W	R	R	SUQ5 ⁺ psi ⁺
	7b	S	R				
	7c	a	W	W	R	R	SUQ5 ⁺ psi ⁺
	7d	S	R				
7 x 465/2a	2a	S	R				
	2b	S	R				
	2c	α	W	R	W	W	SUQ5 psi ⁻
	2d	α	W	R	W	W	SUQ5 psi ⁻
	3a	α	W	R	W	W	SUQ5 psi ⁻
	3b	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	3c	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	3d	a	W	R	W	W	SUQ5 psi ⁻
11 x 465/2a	9a	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	9b	a	W	R	W	R	SUQ5 psi ⁻ pnm
	9c	α	W	W	R	R	SUQ5 ⁺ psi ⁺
	9d	a	W	R	W	R	SUQ5 psi ⁻ pnm

Two of the mutants, in crosses to 465/2a, gave some red/white spore clones; in the cross of mutant 10 x 465/2a, 7 of the 43 non-suppressed spores carried small white sectors (1-5 per clone); in the case of mutant 14 x 465/2a, 3 of the 30 non-suppressed spores gave rise to clones carrying a single, small, white sector. No sectoring was observed in the segregation from crosses involving the other pnm mutants.

All 17 mutants, when crossed to an SUQ5 ade2.1 psi⁻ strain (468/1a), gave only red diploids indicating that it was loss of the psi⁺ phenotype rather than a restriction of expression of the psi⁺ phenotype that was occurring in the pnm mutants. Seven tetrads dissected from one such cross (17 x 468/1a) gave only non-suppressed spores confirming this suggestion.

11 of the putative pnm mutants were crossed to MT193/3b (SUQ5 ade2.1 psi⁺). In all cases fully suppressed diploids were obtained. Tetrads were dissected from each of these 11 crosses and the segregation of suppressed : non-suppressed spores scored. A strain carrying a recessive nuclear mutation in a gene required for maintenance of the psi⁺ phenotype would be expected to show a 2:2 segregation in these crosses. The results obtained (Table 7.19) suggest this to be true although several crosses gave tetrads with 3 or, in the case of mutant 11, 4 suppressed spores. 4 crosses (involving mutants 3, 8, 10 and 11) each gave one tetrad containing 3 non-suppressed spores. Only one cross (6 x MT193/3b) gave a regular 2:2 segregation in all tetrads dissected. In the cross between the methanol-induced psi⁻ strain 507Me3psi⁻ and MT193/3b only suppressed spores were obtained (Table 7.19).

TABLE 7.19 Genetic analysis of pnm x MT193/3b (SUQ5 ade2.1 psi⁺).

MUTANT NUMBER	*1	TETRAD ANALYSIS			SUPPRESSED : NON-SUPPRESSED				*2 SPORE TOTALS S : NS	SEGREGATION OF rho ⁺ :rho ⁻
		NO. TETRADS DISSECTED	% SPORE VIABILITY	NO. COMPLETE TETRADS	SUPPRESSED : NON-SUPPRESSED					
					4:0	3:1	2:2	1:3		
1		22	71.6	11	0	2	9	0	24 : 20	11x4 : 0
2		21	94.0	16	0	2	14	0	34 : 30	16x4 : 0
3		22	85.2	12	0	2	9	1	25 : 23	12x4 : 0
6		10	100	10	0	0	10	0	20 : 20	6x4 : 0 4x3 : 1
8		10	95.0	9	0	0	8	1	17 : 19	8x4 : 0 1x3 : 1
9		10	85.0	7	0	3	4	0	17 : 11	7x4 : 0
10		10	87.5	8	0	0	7	1	15 : 17	8x4 : 0
11		10	92.5	7	3	0	3	1	19 : 9	7x4 : 0
13		10	87.5	6	0	1	5	0	13 : 11	3x4 : 0 3x3 : 1
14		10	90.0	7	0	2	5	0	16 : 12	7x4 : 0
16		5	100.0	5	0	0	5	0	10 : 10	NT
507 Me3 psi ⁻		10	100.0	10	10	0	0	0	40 : 0	10x4 : 0

NT = not tested.
*1 = origin given in Table 7.14.
*2 = only includes data from complete tetrads.

A preliminary analysis of 26 non-suppressed segregants from 7 different pnm x MT193/3b crosses showed that 17 were α 1 was a and 8 were sterile.

In summary the pnm mutations result in loss of the psi⁺ phenotype rather than prevent expression of the psi⁺ phenotype. They are recessive in SUQ5/SUQ5 diploids whilst being apparently partially dominant in an SUQ5/SUQ5⁺ diploid. The data in Table 7.17 and Table 7.18, together with the tetrad analysis of the pnm mutant x MT193/3b (SUQ5 ade2.1 psi⁺) cross (Table 7.19) suggest that the pnm mutation is unstable being "lost" following meiosis. The data presented also suggests that the pnm mutation may inactivate the a-mating type during meiosis, but it has recently been demonstrated that crosses to the parent 507-4/2b often segregate a-sterile spores (C. Mundy personal communication, B. Cox personal communication). This would explain why only α pnm segregants were obtained and does not therefore represent linkage of pnm to the mating type locus on chromosome III.

(2) Maintenance of other extrachromosomal phenotypes in pnm mutants

All 17 putative pnm mutants were grande and no effect on segregation of the grande phenotype in crosses to MT193/3b was seen (Table 7.19). The segregation of the K⁺ phenotype was also normal in three of the crosses between these pnm K⁻ mutants (507-4/2b, the parent strain, was K⁻) and MT193/3b (a K⁺ strain). All haploid progeny were K⁺. The pnm mutation does not, therefore, affect the maintenance or segregation of the /rho or /killer factors.

(3) Demonstration that pnm are nuclear and not cytoplasmic mutations

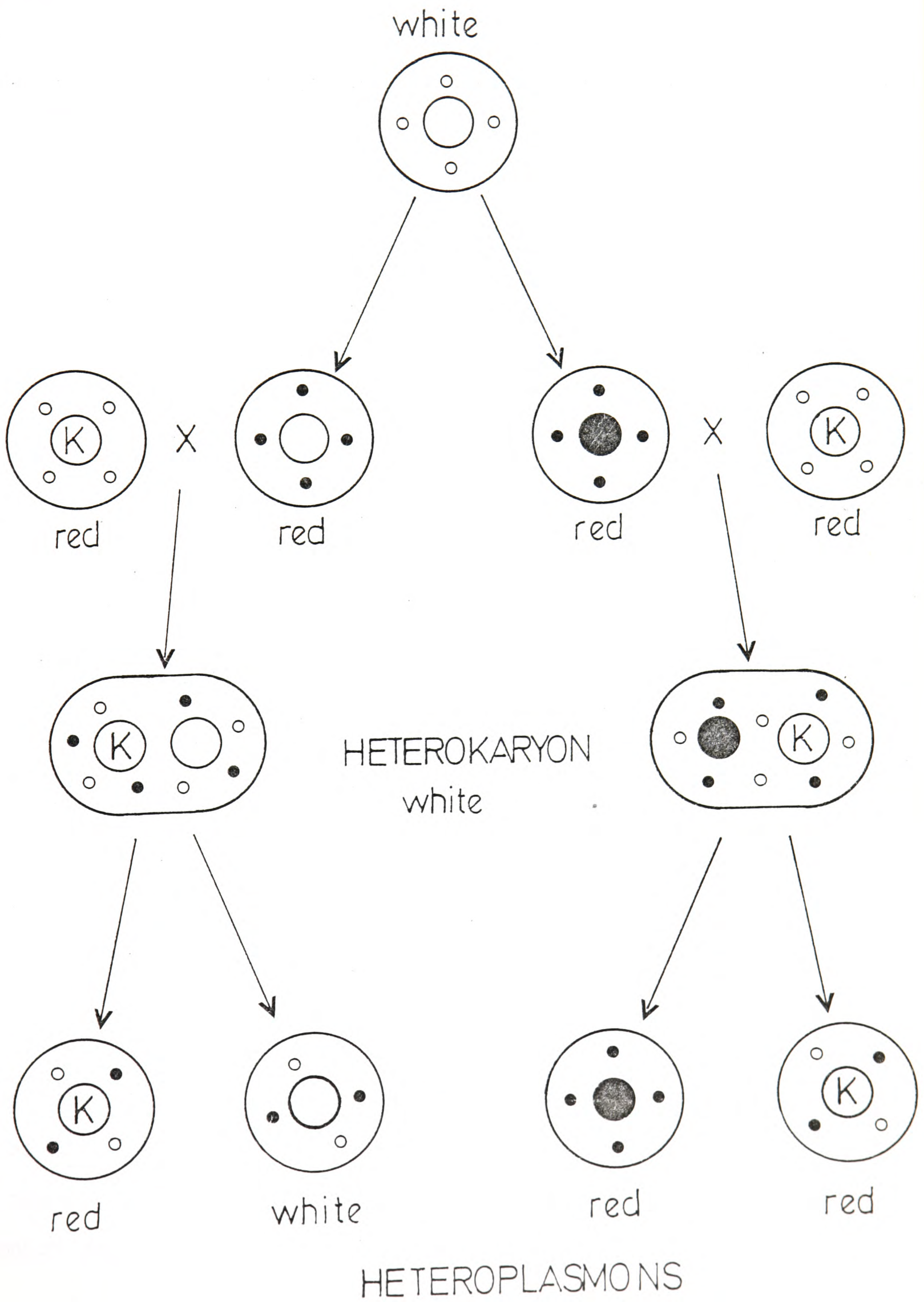
Yeast mutants defective in karyogamy (i.e. fusion of haploid nuclei during conjugation), when crossed to wild type strains, give rise to a high proportion of zygotes which remain as heterokaryons and these segregate haploid daughter cells (heteroplasmons) spontaneously (Conde and Fink 1976, and Appendix 3). Such mutants were used to demonstrate that the pnm mutants isolated were the result of a nuclear rather than a cytoplasmic mutation.

I. Theoretical basis of the 'kar-test'

Figure 7.2 represents schematically the theoretical basis of the test to distinguish "nuclear" psi⁻ mutants from "cytoplasmic" psi⁻ mutants. The kar1 mutation results in a defect in karyogamy which is dominant in kar1 x KAR⁺ crosses (Conde and Fink 1976). When heterokaryons, formed between a cytoplasmic psi⁻ mutant (SUQ5 ade2.1 psi⁻) and a kar1 psi⁺ mutant (SUQ5 ade2.1 kar1 psi⁺), breakdown into their haploid components (heteroplasmons) after failure of karyogamy (which they do under non-selective conditions, Conde and Fink 1976), the SUQ5 ade2.1 nucleus will now be in a cell that has received cytoplasm from both parents and is, therefore, psi⁺ (see Appendix 3 for details). If, however, the psi⁻ mutant also has a nuclear mutation that prevents the cell from maintaining the psi⁺ phenotype (as would be predicted for a pnm mutation) then no psi⁺ heteroplasmons should be found following breakdown of the heterokaryon. Providing non-suppressible, complementing auxotrophic markers are present in the psi⁻ mutant and in the kar1 psi⁺ mutant one should be easily able to distinguish a suppressed heteroplasmon from the rare diploid that is formed in kar1 x KAR⁺

FIGURE 7.2 A schematic representation of the 'kar test' used to distinguish psi⁻ mutants from pnm mutants.

- (○) psi⁺ cytoplasmic background
- (●) psi⁻ cytoplasmic background
- nucleus with the genotype SUQ5 ade2.1
- nucleus with the genotype SUQ5 ade2.1 pnm
- (K) nucleus with the genotype SUQ5⁺ ade2.1 kar1



crosses (Conde and Fink 1976).

Table 7.20 summarises the predicted phenotypes and genotypes of cells obtained from a SUQ5⁺ ade2.1 leu1 kar1 psi⁺ strain crossed to either a SUQ5 ade2.1 pnm ura3.1 psi⁻ or a SUQ5 ade2.1 pnm⁺ ura3.1 psi⁻ strain.

II. Strain Construction

The strain MT152/3c (a ade2.1 his5.2 can1.100 leu1 kar1 psi⁺) was constructed by crossing MT191/8c (× ade2.1 his5.2 psi⁺) x JC8 (a leu1 kar1). The JC8 strain was obtained from Dr G. Fink (Cornell, U.S.A.). Only one, out of 5 zygotes picked, gave rise to any significant numbers of diploids as judged by sporulation. 9 tetrads (100% spore viability) were picked from this cross (MT152/-). All gave 2:2 segregations for LEU⁺:leu⁻, ADE⁺:ade⁻ and HIS⁺:his⁻. All red spores were crossed for an ade1 tester strain by cross-streaking on a YC plate. Weak complementation (i.e. low frequency of diploid formation) was taken to indicate the presence of the kar1 mutation. 8 out of 18 ade⁻ spores tested showed such weak complementation and one of these spores, MT152/3c, was used for the 'kar test'.

III. Experimental Confirmation

3 known "cytoplasmic" psi⁻ mutants (507Me1psi⁻, 507Me3psi⁻ and 468/1d), and 2 of the putative pnm mutants (507pnm1, 507pnm2) identified by genetic analysis, (see Table 7.16) were crossed to MT152/3c and a number of individual zygotes picked by micro-manipulation and allowed to proliferate on YC. Approximately 75% of the zygotes isolated formed viable zygotic colonies and the phenotypes of these are given in Table 7.21. The 3 "cytoplasmic" psi⁻ mutants each gave rise to 3 different zygotic colony

TABLE 7.20 Predicted phenotypes and genotypes of cells derived from a cross between a nuclear or cytoplasmic ura⁻ psi⁻ mutant and an SUQ5 ade2.1 leu1 kar1 psi⁺ mutant.

PSI ⁻ MUTANT (ura ⁻)	CELL DERIVED FROM A CROSS TO AN SUQ5 ⁺ ade2.1 leu1 kar1 psi ⁺ MUTANT		
	COLONY PHENOTYPE	NATURE	GENOTYPE
SUQ5 ade2.1 psi ⁻	RED	} HETEROPLASMONS	ade ⁻ leu ⁻ URA ⁺
	WHITE		ADE ⁺ LEU ⁺ ura ⁻
	WHITE	DIPLOID	ADE ⁺ LEU ⁺ URA ⁺
	RED & WHITE	HETEROKARYON	mixture
SUQ5 ade2.1 pnm psi ⁻	RED	HETEROPLASMONS	ade ⁻ leu ⁻ URA ⁺
	WHITE	DIPLOID	ade ⁻ LEU ⁺ ura ⁻
	RED	HETEROKARYON	mixture

TABLE 7.21 Phenotypes of zygotic colonies arising from crosses between α KAR⁺ ura3.1 psi⁻ mutants
 and MT152/3c α ade2.1 his5.2 can1.100 leu1 kar1 psi⁺.

PSI ⁻ MUTANT ^{*1}	NUMBER OF VIABLE ZYGOTIC COLONIES	PHENOTYPE OF ZYGOTIC COLONIES			
		RED	WHITE	RED + PINK + WHITE	RED + PINK
507 Me1 psi ⁻	9	2	3	4	0
507 Me3 psi ⁻	8	2	2	4	0
468/1d	19	4	6	9	0
TOTAL	36	8	11	17	0
507 prn1	4	2	0	0	2
507 prn2	4	2	0	0	2
TOTAL	8	4	0	0	4

^{*1} all mutants, except 468/1d, were isolated from 507-4/2b (α SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺
 468/1d is α SUQ5 ade2.1 his5.2 ura3.1 psi⁻.

phenotypes of which only the red+pink+white class contained heterokaryons (for discussion see Appendix 3). In the pnm crosses only the red+pink colonies were found to be URA^+LEU^+ and were taken to contain heterokaryons. Diploids from this latter cross are normally white. Of the 4 pure red clones 1 was ura^-LEU^+ and 3 were URA^+leu^- .

1 heterokaryon clone from each of the 5 crosses was suspended in saline and plated onto YC to give approximately 100 colonies/plate. From 4 of the crosses cells were also plated onto OM2-leu, to select for LEU^+ cells, to determine whether any suppressed heteroplasmons (white, ura^-LEU^+) were present in the zygote clones, at a low frequency. The results are given in Table 7.22.

White colonies arising on YC (Table 7.22) were X5 to X50 less frequent than heterokaryon clones derived from the pnm mutants. 80 out of 80 white colonies tested from the "cytoplasmic" psi⁻ crosses were ura^-LEU^+ (i.e. suppressed SUQ5 psi⁺ heteroplasmons) whilst all 20 tested from the pnm mutant crosses were URA^+LEU^+ and sporulated on FMA. They were therefore diploid colonies.

Of the colonies isolated on OM2-leu, by replica-plating onto YC, the majority (>95%) from the pnm mutant crosses were found to be URA^+LEU^+ heterokaryon colonies. All of the white LEU^+ colonies, arising from the two pnm mutants, were found to be diploids i.e. URA^+LEU^+ and showed good sporulation on FMA medium. Of the LEU^+ colonies, arising from the "cytoplasmic" psi⁻ crosses, the majority (50-60%) were white and all 49 such colonies that were analysed, were found to be ura^- i.e. were SUQ5 psi⁺ heteroplasmons. No diploids were found.

TABLE 7.22 Phenotypes of colonies arising from heterokaryon-zygotic colonies from 3 "cytoplasmic" psi⁻ mutants (Me1, Me3 and 468/1d) and 2 pnm psi⁻ mutants crossed to MT152/3c (a ade2.1 his5.2 can1.100 leu1 kar1 psi⁺).

		PSI ⁻ MUTANT	TOTAL COLONIES SCORED	PHENOTYPE OF COLONIES			% WHITE COLONIES
				RED	WHITE	RED/PINK/ WHITE	
YC		507Me1psi ⁻	352	243	63	46	17.9
		507Me3psi ⁻	255	215	37	3	14.5
		468/1d	534	366	109	59	20.4
		TOTAL	1141	824	209	108	18.3
		507pnm1	770	9	28	733	3.6
		507pnm2	691	3	3	685	0.4
		TOTAL	1461	12	31	1418	2.1
OM2		507Me1psi ⁻	266	0	161	105	60.5
		507Me3psi ⁻	88	0	49	39	55.7
		TOTAL	354	0	210	144	59.3
		507pnm1	571	10	25	536	4.4
		507pnm2	808	1	1	806	0.1
		TOTAL	1379	11	26	1342	1.9

The data presented demonstrates that no SUQ5 ade2.1 pnm psi⁺ heteroplasmons were obtained from the two SUQ5 ade2.1 pnm psi⁻ x SUQ5⁺ ade2.1 PNM⁺ psi⁺ kar1 crosses made. pnm mutants therefore carry a nuclear gene defect which prevents the psi⁺ phenotype from being maintained. For the 3 previously genetically characterised "cytoplasmic" psi⁻ mutants psi⁺ heteroplasmons were obtained, at a high frequency (10-20%), from a kar1 x KAR⁺ zygotic colony demonstrating that:

- a) they carried all the nuclear genes required for maintenance of the psi⁺ phenotype, and
- b) the psi⁺ phenotype is inherited independently of the nucleus (this will be discussed further in Appendix 3).

IV. 'kar' complementation test

It was found that, by cross-streaking log phase cells of the psi⁻ mutant across a down-streak of log phase cells of MT152/2c on a YC plate, a rapid test for distinguishing true "cytoplasmic" psi⁻ mutants, from those psi⁻ mutants with a nuclear gene mutation, was possible. The 17 "cytoplasmic" psi⁻ mutants tested gave very good complementation, but the 4 pnm mutants tested, gave very weak complementation. This difference in complementation could be enhanced by replica-plating the cross streaks, after 3-4 days incubation at 28°C, onto a fresh YC plate followed by a further incubation period. Alternatively, providing the psi⁻ mutant was also ura⁻, the initial cross streaks could be replica-plated onto YNB + ura - leu. Only suppressed LEU⁺ colonies grew on such medium (heterokaryons were found to grow very poorly on this medium) and the "cytoplasmic" psi⁻ mutants tested, normally produced a confluent growth on this medium, whilst the pnm psi⁻ mutants only produced a few individual ADE⁺ HIS⁺ URA⁺ LEU⁺ colonies which may have been diploids.

V. Conclusions

The pnm mutants are defective in a nuclear gene(s) required for the maintenance of the psi⁺ phenotype. pnm mutants can be distinguished from cytoplasmic psi⁻ mutants by a simple complementation test using a kar1 mutant.

7.5 Discussion

Maintenance of the psi⁺ phenotype is dependent on the functional integrity of the nuclear genome. Dominant mutations which result in elimination of the psi⁺ phenotype (PNM) occur in at least two loci (Young and Cox 1971; B. Cox personal communication). Recessive PNM (pnm) mutations were also found (Section 7.4). Evidence, presented in Chapter 5, also indicated that the CDC8 gene may be required for maintenance of the psi⁺ phenotype. The psi⁺ phenotype is therefore similar to other extrachromosomal mutants of yeast, and other organisms, in having chromosomal genes which modify the phenotypic expression of the extrachromosomal element.

When a strain is either PNM⁻ or pnm⁻ the psi⁺ phenotype is lost. This is not a block in expression of the phenotype because the psi⁻ phenotype is maintained in all spore progeny when PNM⁻ psi⁻ or pnm⁻ psi⁻ strains are crossed to pnm⁺ psi⁻ strains. Few pnm⁺ psi⁺ spores are obtained.

In PNM⁻ or pnm⁻ mutants the K⁺ and grande phenotypes are stable suggesting that these nuclear genes are not involved in the maintenance of extrachromosomal genomes per se. The /URE3/ plasmid (Aigle and Lacroute 1975) is also maintained in PNM⁻ mutants (M. Aigle personal communication to C. Waldron).

Chromosomal genes are required for the maintenance of the K^+ phenotype, at least 26 being known to date (Somers and Bevan 1968; Fink and Styles 1972; Wickner 1974b, 1978; Wickner and Leibowitz 1976). In the mak⁻ mutants, so far reported, the P2 /killer/ dsRNA is not perpetuated in the presence of the mak1-mak9 (Wickner and Leibowitz 1976) or mak10 (Mitchell et al., 1976a) nuclear gene. Strains that carry the nuclear petS mutation are unable to maintain either mitochondrial DNA or the /killer/ - associated, P2 dsRNA, but the PETS⁺ gene is not required for maintenance of psi⁺ or the /URE3/ plasmid (Leibowitz and Wickner 1977). The psi⁺ phenotype is stable in mak1 - mak9 mutants (B.S. Cox personal communication) and in mak10 mutants (see Appendix 1). These results are further evidence for the non-identity of /psi/ with other extrachromosomal elements of yeast.

Both the petS and mak mutants result in a physical loss of the genome required for the K^+ or grande phenotypes (Wickner and Leibowitz 1976; Mitchell et al., 1976a). It has been suggested that the /psi/ genome stops replicating in a PNM⁻ mutant and is, therefore, "diluted out" during cell division (Young and Cox 1971; McCready et al., 1977) i.e. there is loss of the /psi/ genome. Experiments, presented in this chapter, demonstrated that pnm⁺ psi⁻ haploid spores, derived from a pnm⁺ psi⁺ x PNM⁻ psi⁻ diploid, were able to revert to psi⁺ either spontaneously or be induced by UV-irradiation. Also that PNM⁻/pnm⁺ diploids contain the genetic information required to regain the psi⁺ phenotype in the event of the PNM⁻ mutant allele being lost by a mitotic cross-over event. Loss of a genetic determinant does not appear to occur in a PNM⁻/pnm⁺ diploid. If it did no reversion to psi⁺ would be expected. It was concluded that the pnm⁺ psi⁻ segregants were not psi⁰, but, until the nucleic acid species

associated with the psi⁺ phenotype is identified, this cannot be conclusively demonstrated. PNM⁻ strains still carry the /2u7 DNA (C. Mundy personal communication) confirming that PNM⁻psi⁻ segregants do not show a physical loss of this extra-chromosomal element and the PNM⁺ genes are not required for its maintenance and replication.

The progressive elimination of the psi⁺ phenotype seen in PNM⁻/pnm⁺ diploids previously (Young and Cox 1971; McCready *et al.*, 1977) was not confirmed in this study. Some mutants e.g. 7L25/1a 7L25/1c, when crossed to a PNM1 mutant, gave no psi⁺ spore progeny even when zygotes were immediately sporulated. Other mutants e.g. 507-4/2b, 7L25/1b gave an approximately constant proportion of psi⁺ progeny (10-20%) when crossed to a PNM1 mutant, even after the zygote had been allowed to proliferate on YC for up to 100 hours prior to transferring to sporulation medium. It is unclear whether these differences reflect the "levels" of psi⁺ particles present in these strains i.e. the lower the numbers, the quicker the psi⁺ phenotype is eliminated. Another possibility is that nuclear genetic background has an effect on the efficiency with which a PNM⁻ mutation eliminates the psi⁺ phenotype.

The two mutants examined, which showed modification of the PNM⁻ phenotype (MT155/3c and WPC-1; Section 7.3), suggests that the PNM⁻ mutation is deleterious to the cell and, as a consequence, the cell tends to accumulate genetic modifiers which "suppress" the PNM⁻ phenotype or stabilise the psi⁺ phenotype in a PNM⁻ background. Genetic modifiers, which arise at high frequencies in strains carrying deleterious amber suppressors and reduce the efficiency of amber suppression, have recently been reported (Liebman *et al.*, 1976). Some standard laboratory PNM⁻

stock strains became "less efficient" with prolonged storage (B. Cox personal communication). These PNM⁻-modifiers are analogous to the KRB mutants, recently described, which are chromosomal mutations which allow the K⁺ cytoplasmic phenotype of yeast to be maintained in mak7 and petS mutants (Wickner and Leibowitz 1977).

Expression and/or maintenance of the psi⁺ phenotype is, at least in part, under the control of nuclear genes. It is unclear whether these genes are involved in the structure and function of the /psi/ factor, in transmission of the psi⁺ phenotype during mitosis or meiosis, in replication of the /psi/ genome, or mutability of the /psi/ genome. Possible models for the pnm⁺ genes will be discussed in Chapter 9.

CHAPTER 8

A GENETIC APPROACH TO DETERMINING THE $\overline{\text{PSI}}$ GENE PRODUCT

- 8.1 Introduction
- 8.2 Inhibitors of 80S ribosomes : their effect on the efficiency of SUQ5 in suppressing nonsense codons
- 8.3 Mutants with altered 80S ribosomes : their effect on the efficiency of SUQ5 in suppressing nonsense codons
- 8.4 Discussion
- 8.5 Summary

8.1 Introduction

So far this thesis has been concerned with the molecular nature of the $\overline{[\psi]}$ factor. The following Chapter will describe some experiments performed in an attempt to determine the gene product of the ψ factor and in particular whether $\overline{[\psi]}$ affects 80S ribosomal components.

Mutations which interfere with the process of nonsense suppression must affect the activity or synthesis of one or other of the components of the translation machinery.

Through genetic studies in the prokaryote Escherichia coli it is now well established that the ribosome plays a role in controlling the accuracy of translation. Two types of mutation (known to result in structural alterations of the 30S ribosomal subunit proteins) have been shown to affect the efficiency of nonsense suppression in E.coli:

- (i) the restrictive mutations strA (affecting ribosomal protein S12) and neaA (affecting ribosomal protein S17) decrease the efficiency of tRNA nonsense and missense suppressors (Gorini 1974, Bollen et al 1975), and
- (ii) the ram mutations ramA (affecting ribosomal protein S4) and ramC (affecting ribosomal protein S5) increase the efficiency of amber suppression (Gorini 1974, Piepersberg et al 1975).

From the situation in E.coli, it is possible to infer that $\overline{[\psi]}$ codes for a component of the cytoplasmic 80S ribosome.

The ribosome is an integrated structure and alterations in its structure can result not only from the addition of external reagents, but also from mutations in genes coding for the ribosomal proteins. Such reagents and mutations are

available in yeast. Even a slight modification of a ribosomal protein may change the structure and function of the ribosome, as Gorini and his colleagues^u have shown (Gorini 1974).

Two approaches were used to determine whether ψ codes for a component of the cytoplasmic 80S ribosomes:

- a) by looking at the effects of antibiotics, known to act at the level of the 80S ribosome in yeast, on mutants with different efficiencies of nonsense suppression, and
- b) by looking at the effect of mutations in the 80S ribosome on the efficiency of nonsense suppression.

If a ribosomal mutation or a ribosomal antibiotic was found to restore the suppressor activity of SUQ5 in a ψ^- background this would be indirect evidence for a difference, in a ribosomal component, between ψ^+ and ψ^- strains.

8.2 Inhibitors of 80S ribosomes: their effect on the efficiency of SUQ5 in suppressing nonsense codons

The effects of 4 different antibiotics, known to act in yeast at the level of the 80S ribosome, on the efficiency of SUQ5 was investigated.

(1) Cycloheximide

This is one of the glutarimide antibiotics used to inhibit protein synthesis in yeast. It affects a step in elongation of peptide chains, possibly the translocation of nascent peptidyl-tRNA from the A site to the P site by interfering with the action of elongation factor II (EF2) (Baliga and Munro 1971). However at low concentrations (10 μ g/ml) it has also been shown that cycloheximide (CHX) can selectively inhibit the initiation of protein synthesis (Cooper and Bossinger 1976).

(i) Sensitivity of ψ^+ and ψ^- strains

NON-ISOGENIC STRAINS

49 haploid strains, derived from 9 genetically different diploids, were screened for sensitivity to CHX. 26 psi⁺ and 23 psi⁻ strains were tested, with 16 and 12 respectively also carrying SUQ5. 10 μ l drops of a fresh saline suspension (5×10^6 cells/ml) were spotted onto a control YC plate and YC plates containing 0.01, 0.03, 0.075, 0.1, 0.2, 0.3 and 0.5 μ g/ml CHX. After 6 days incubation at 28°C growth or non-growth was scored (Table 8.1).

There appeared to be a significant difference between psi⁺ and psi⁻ strains in their sensitivity to CHX. The mean and standard deviation of the 2 populations was calculated and found to be:

$$\text{TOTAL } \text{psi}^+ = 0.067 \pm 0.05 \mu\text{g/ml}$$

$$\text{TOTAL } \text{psi}^- = 0.104 \pm 0.06 \mu\text{g/ml}$$

The difference between the means was $0.032 \pm 0.002 \mu\text{g/ml}$ and, at $p = 0.05$, this difference was found to be significant using the "t-test".

ISOGENIC STRAINS

To eliminate any possible nuclear genetic background effects on the levels of resistance to CHX, sensitivity was checked of a psi⁺ (507-4/2b) and an isogenic psi⁻ strain derived directly from 507-4/2b by growth in YC + 10% methanol liquid medium (see Chapter 6). The psi⁻ mutant (507 Me psi⁻) was shown to be such by constructing a diploid with a standard SUQ5 ade2.1 psi⁻ strain (468/1a). The non-suppressed diploid gave rise to 5 tetrads all of which showed the expected segregation of 4 red: 0 white spores. 507 Me psi⁻ also complemented the SUQ5⁺ ade2.1 psi⁺ (465/2a) and SUQ5 ade2.1 kar1 psi⁺ (MT152/3c) confirming

TABLE 8.1 The sensitivity of a number of different psi⁺ and psi⁻ strains to cycloheximide.

GENOTYPE	NO. TESTED	LEVEL OF RESISTANCE ($\mu\text{g/ml}$) [*]						
		0.01	0.03	0.075	0.1	0.2	0.3	0.5
SUQ5 <u>psi</u> ⁺	16	5	4	2	4	1	0	0
+ <u>psi</u> ⁺	10	1	2	2	4	1	0	0
TOTAL <u>psi</u> ⁺	26	6	6	4	8	2	0	0
SUQ5 <u>psi</u> ⁻	12	1	1	2	7	1	0	0
+ <u>psi</u> ⁻	11	0	1	1	7	1	1	0
TOTAL <u>psi</u> ⁻	23	1	2	3	14	2	1	0

* ie. the concentration of cycloheximide up to which the given number of strains grew confluenty.

it as a cytoplasmic psi⁻ mutant.

Sensitivity to CHX was determined using a more accurate "well-test" (see Materials and Methods) over a concentration range of 0-100 µg/ml CHX. No difference between the psi⁺ and psi⁻ strain was found (Figure 8.1a). Three other, non-isogenic, strains were run in the well test; 21-/4c (SUQ5⁺ ade2.1 psi⁻), 465/2a (SUQ5⁺ ade2.1 psi⁺) and MT247/1b (SUQ5 ade2.1 psi⁺ cyh2). The two CYH⁺ strains were slightly more resistant than 507-4/2b (psi⁺ and psi⁻) at low CHX concentrations, whilst being slightly more sensitive at high CHX concentrations (Figure 8.1a). MT147/1b was substantially more resistant than any of the 4 psi⁺ and psi⁻ strains tested.

(ii) Sensitivity of other mutants which show altered SUQ5 efficiencies

A preliminary screen by McCready (1972) of her antisuppressor mutants asu1-asu8 indicated that none conferred resistance to CHX. These observations were confirmed using well plates with 10 and 50 µg/ml CHX. The zones of inhibition, scored after 7 days incubation at 28°C, are given in Table 8.2. The asu5 strain appeared to be very sensitive to CHX. To see if this was a direct result of the asu5 mutation the strain was crossed to 466/4a (ASU⁺) and 8 tetrads dissected. The diploid, being homozygous for SUQ5, psi⁺, and heterozygous for asu5, gave 2 suppressed (ASU⁺) and 2 non-suppressed (asu5) spores. One tetrad and the two parent strains were checked for sensitivity to 10 µg/ml CHX using a well plate (Table 8.3). All 4 spores showed similar sensitivity to the original asu5 parent and were therefore significantly different to the ASU⁺ parent. This implies that the CHX-sensitive phenotype was not due to the asu5 mutation, but that it was a cytoplasmically inherited phenotype.

A possible explanation for this cytoplasmic inheritance could

FIGURE 8.2 A comparison of the sensitivities of isogenic ψ^+ and ψ^- strains (507-4/2b , 507Me3) to four different ribosomal drugs using the well test.

a) cycloheximide.

(●)	507-4/2b	SUQ5 ψ^+
(○)	507Me3	SUQ5 ψ^-
(■)	2101-4/4c	SUQ5 ⁺ ψ^-
(▲)	465/2a	SUQ5 ⁺ ψ^+
(△)	MT147/1b	SUQ5 ψ^+ cyh2

b) trichodermin.

(●)	507-4/2b	SUQ5 ψ^+
(○)	507Me3	SUQ5 ψ^-
(△)	MT172/4b	SUQ5 ψ^+ tcm1

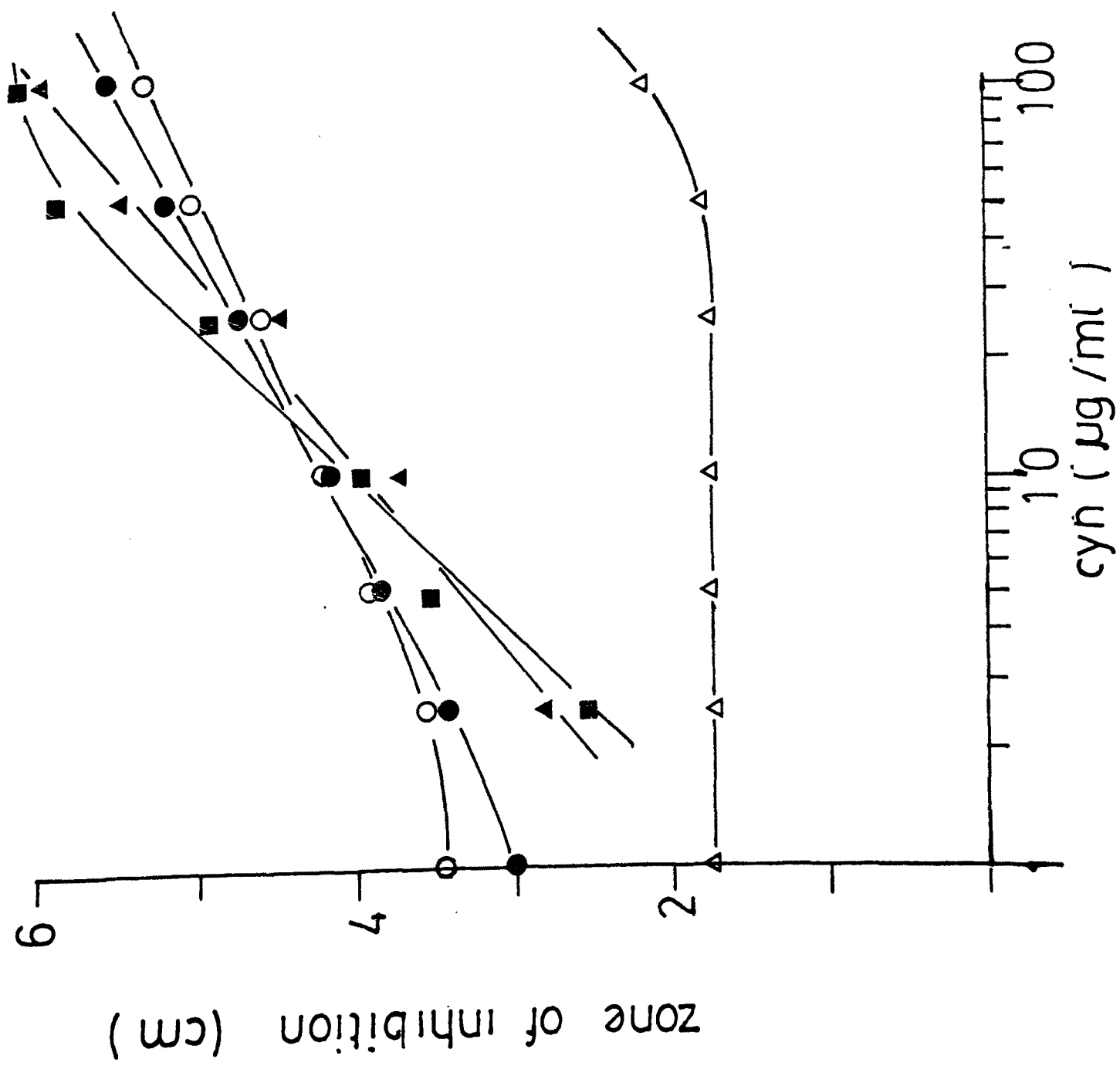
c) cryptopleurine.

(●)	507-4/2b	SUQ5 ψ^+
(○)	507Me3	SUQ5 ψ^-

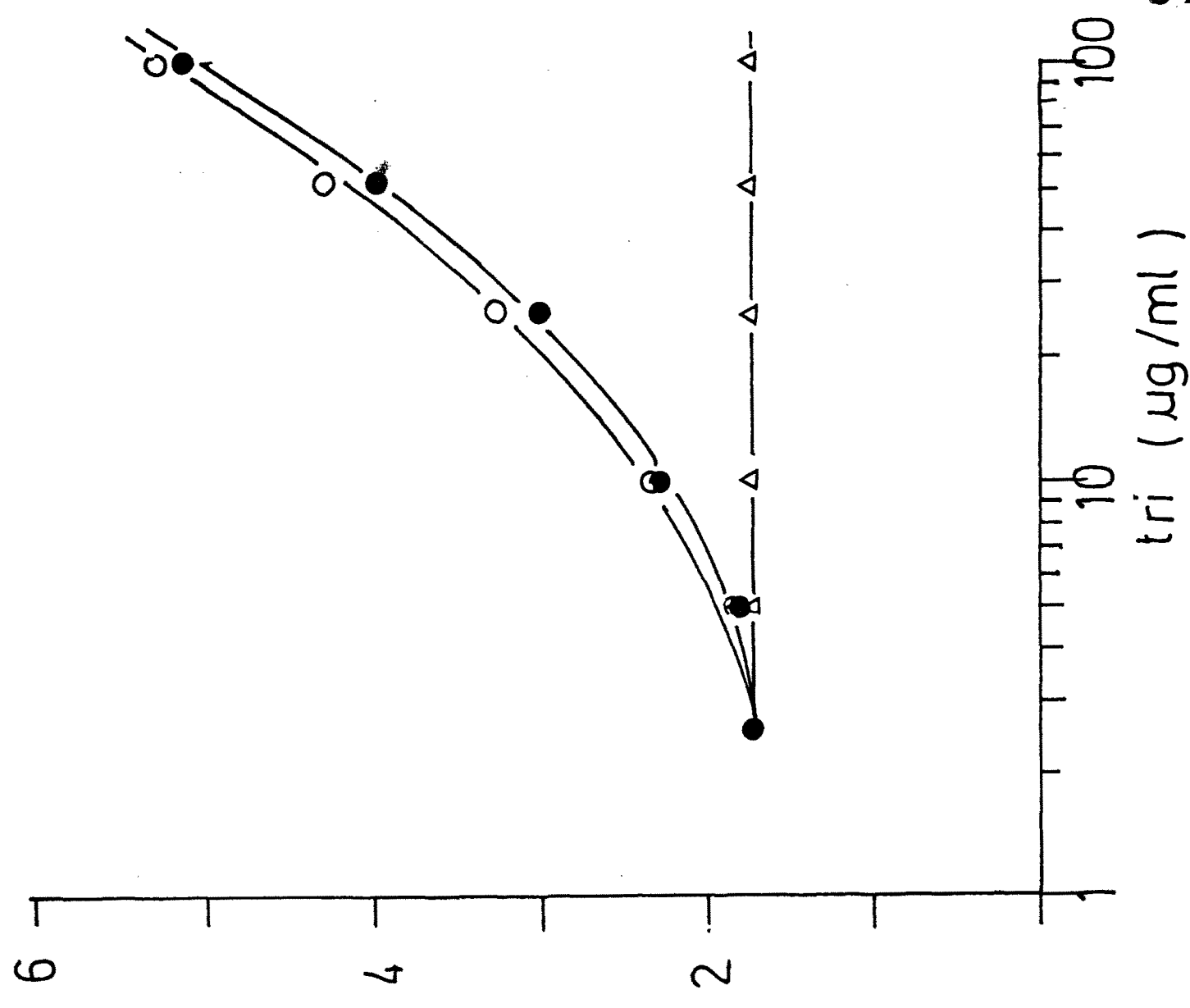
d) anisomycin.

(●)	507-4/2b	SUQ5 ψ^+
(○)	507Me3	SUQ5 ψ^-

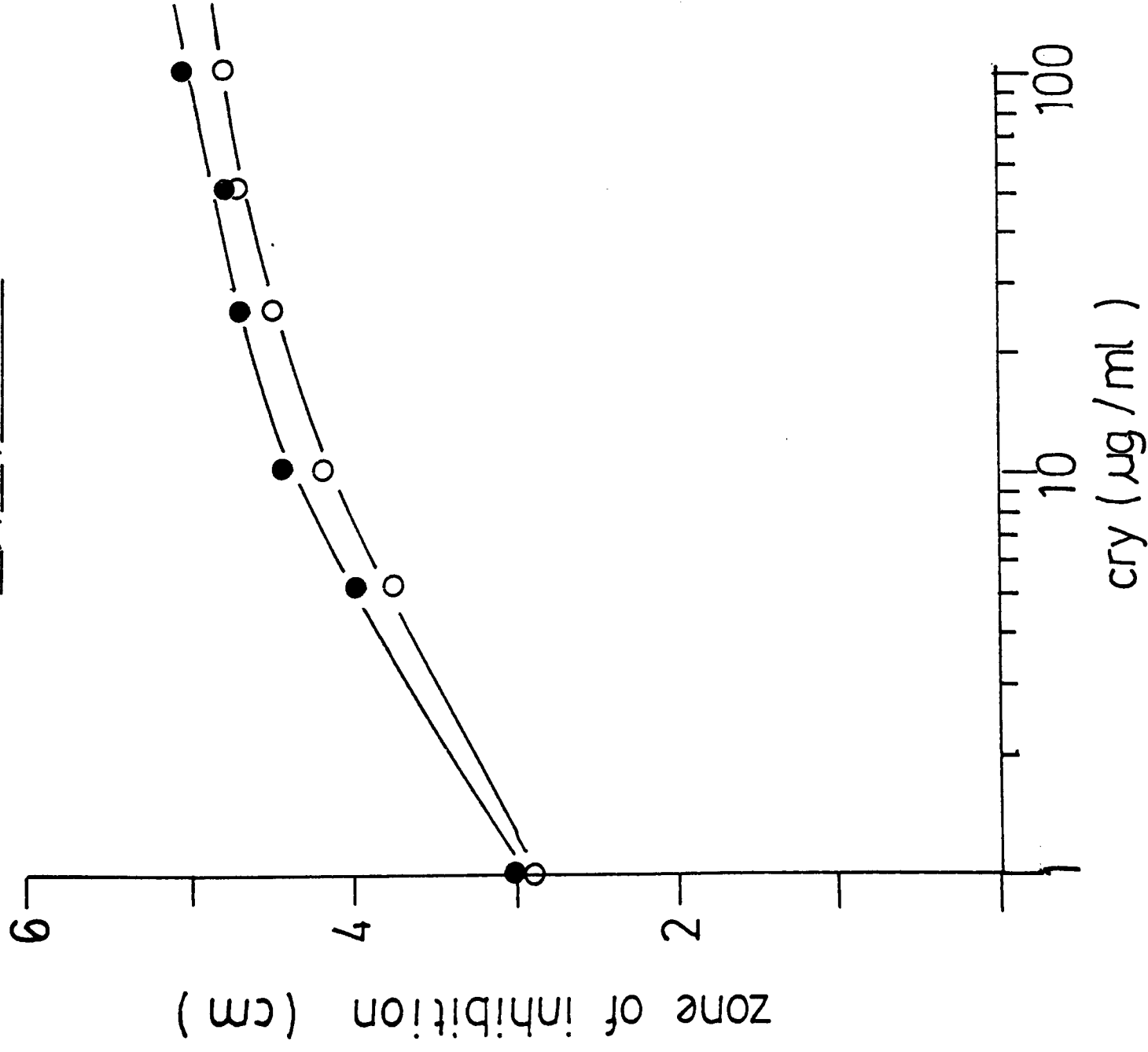
a) cycloheximide



b) trichodermin



c) cryptopleurine



d) anisomycin

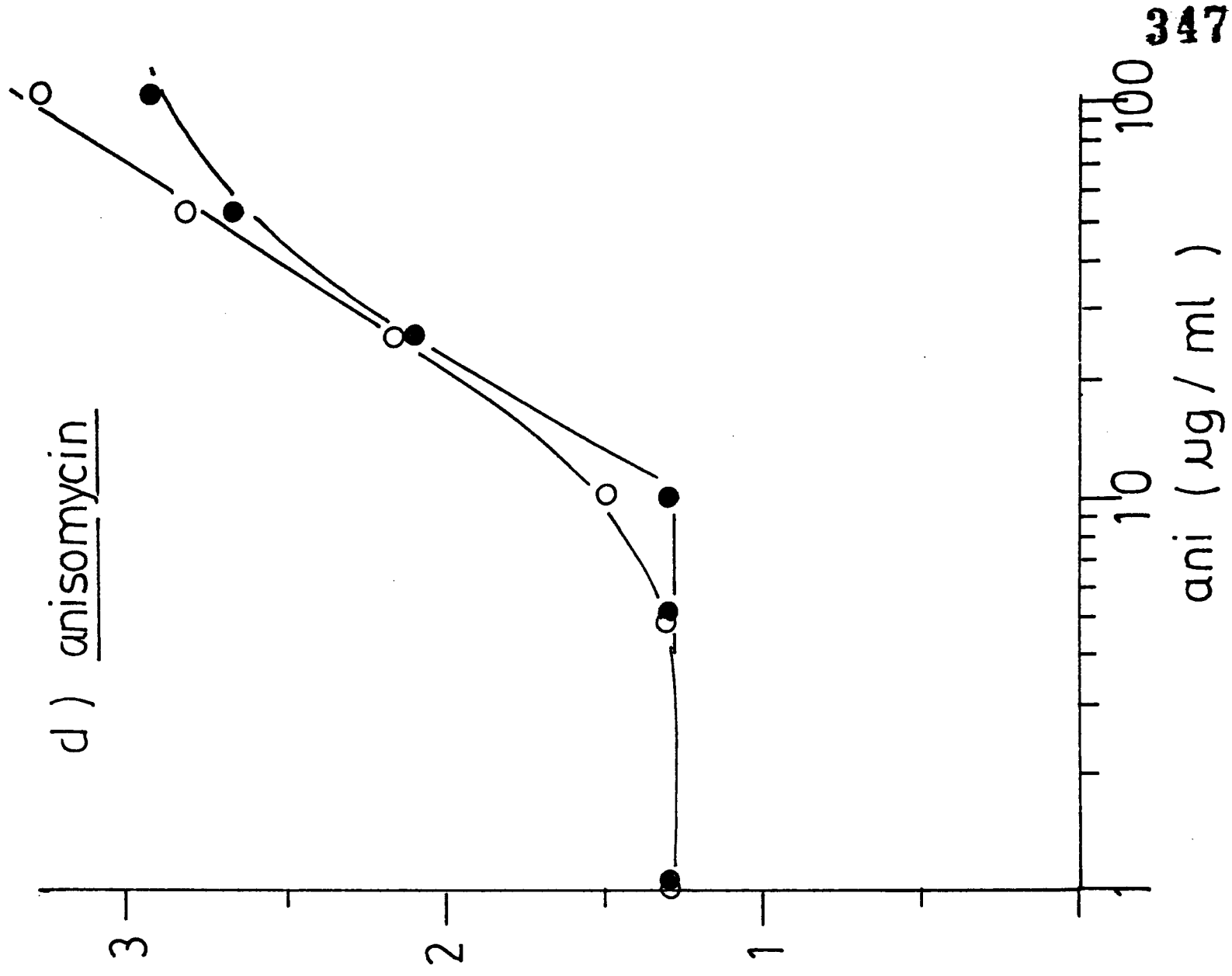


TABLE 8.2 The sensitivity of strains carrying antisuppressor mutations to cycloheximide and trichodermin.

MUTATION	STRAIN	ZONES OF INHIBITION (cm)*1			
		CYCLOHEXIMIDE		TRICHODERMIN	
		10	50	10	50 µg/ml
asu1	1.4	4.1	5.6	2.7	3.8
asu2	2.2a	4.6	6.0	2.4	4.2
asu3	3.3	4.1	5.2	2.6	4.3
asu4	4.1a	4.0	5.4	2.6	3.9
asu5	5.1	5.0	6.2	2.9	5.4
asu6	6.3	3.6	5.4	2.4	3.8
asu7	7.1a	3.8	5.2	2.5	4.2
asu8	8.1a	3.6	5.2	2.5	3.4
ASU ⁺	*2	3.86 [±] 0.39	5.04 [±] 0.19	2.48 [±] 0.38	3.88 [±] 0.36
cyh2ASU ⁺	MT147/1b	1.75	1.75	-	-
tcm1ASU ⁺	MT172/4b	-	-	-	-

*1 well size = 1.75cm .

*2 5 different ASU⁺ strains were tested (2 psi⁺ and 3 psi⁻) and the average figure is given with the standard deviation .

TABLE 8.3 The sensitivity of asu5 mutants to 10 µg/ml cycloheximide.

		STRAIN	GENE	ZONE OF INHIBITION(cm)
PARENTS	{	466/4a	ASU ⁺	3.9
	{	5.1	asu5	5.0
466/4a x asu 5.1	{	1a	asu5	5.2
	{	1b	ASU ⁺	5.1
	{	1c	ASU ⁺	5.1
	{	1d	asu5	4.8

be that the asu5 strain had an altered cell membrane component that increased the cell permeability towards CHX and that this component was apparently inherited cytoplasmically. Heritable membrane alterations have been shown in both Paramecium aurelis. (Beisson and Sonneborn 1965) and epithelial cells (Pietras 1978).

The sensitivity of strains carrying one of the 5 allosuppressor mutations, sal1 - sal5 (Cox 1977 b), to CHX was also determined using a well test with 10 and 50 µg/ml CHX (Table 8.4). The sal4.1 mutant, which appeared to be very sensitive to CHX, was examined further; sal4.1 was crossed to 468/1d (α SUQ5 ade2.1 psi⁻ SAL⁺) and the 6 tetrads isolated from the non-suppressed diploid each had 2 suppressed (sal4.1) and 2 non-suppressed (SAL⁺) spores. Sensitivity to CHX did not segregate with the sal4.1 gene or as a single gene mutation (Table 8.5).

The variations in response to CHX of both the asu and sal mutant strains were probably attributable to genetic background effects.

It was concluded from this study that conversion of a psi⁺ strain to psi⁻ was not accompanied by any change in sensitivity to CHX and that the nuclear mutations affecting the efficiency of suppression by SUQ5 (ie. adu or sal) conferred no significant resistance (or sensitivity) to CHX.

(2) Trichodermin

Trichodermin (TRD) is a 12, 13-epoxytrichothecene fungal toxin and a potent inhibitor of yeast protein synthesis. This drug blocks the peptidyl transferase reaction in vitro (Carrasco et al 1973) but in vivo TRD appears to preferentially inhibit termination of protein synthesis (Stafford and McLaughlin 1973). TRD acts at the level of the 80S ribosome.

(i) Sensitivity of psi⁺ and psi⁻ strains

TABLE 8.4 The sensitivity of strains, carrying allosuppressor mutations (sal), to cycloheximide and trichodermin

sal MUTATION	STRAIN	ZONES OF INHIBITION (cm)			
		CYCLOHEXIMIDE		TRICHODERMIN	
		10	50 (µg/ml)	10	50
1.1	502/2a	2.9	4.4	1.75	2.3
1.2	502/2a	-	5.2	-	3.6
1.3	502/2a	-	4.4	-	2.4
2.1	?	-	-	2.4	3.8
2.4	?	-	-	2.3	3.8
3.5	584/1d	4.7	6.0	2.5	4.0
4.1	502/2a	4.8	5.7	2.4	4.2
4.2	503/4b	-	4.4	-	2.7
4.3	503/4b	3.3	4.6	1.75	2.2
4.4	503/4b	2.4	3.5	1.75	2.3
4.5	503/4b	2.9	4.4	1.75	2.3
5.1	502/2a	4.6	5.6	2.4	3.8
5.2	503/4b	-	4.4	-	2.7
5.3	503/4b	3.4	4.8	1.75	2.4
SAL ⁺	-	3.86 ± 0.39	5.04 ± 0.19	2.48±0.38	3.88±0.36

N.B. The diameter of the well was 1.75cm. A dash (-) indicates "not tested". The full genotypes of the strains are:

502/2a a SUQ5 ade2.1 his5.2 lys1.1 can1.100 psi⁻
503/4b α SUQ5 ade2.1 lys1.1 psi⁻
584/1d α SUQ5 ade2.1 his5.2 lys1.1 can1.100 PNM2 psi⁻

No viable stocks of the 502/2a or 503/4b strains were available for testing (B.S. Cox, pers. comm.)

TABLE 8.5 The sensitivity of sal4.1 mutants to 10 µg/ml cycloheximide.

STRAIN		GENE	ZONE OF INHIBITION(cm)
PARENTS	{ 468/1d	SAL ⁺	4.1
	{ 502/2a sal4.1	sal4.1	4.8
468/1d x 502/2a sal 4.1	{ 4a	sal4.1	5.0
	{ 4b	SAL ⁺	4.5
	{ 4c	sal4.1	3.7
	{ 4d	SAL ⁺	4.6

NON-ISOGENIC STRAINS

A preliminary screen of 25 different haploid strains, derived from 10 genetically different diploids, for sensitivity to TRD was performed. 13 were psi⁺ (including 7 carrying SUQ5) and 12 psi⁻ (including 6 carrying SUQ5). 10ul drops of a fresh saline suspension were spotted onto YC control and YC + TRD (0.025-5.0 µg/ml). TRD was initially dissolved in 2% DMSO with the final concentration of DMSO being < 0.1%. Growth or non-growth was scored after 7 days incubation of the plates at 28°C (Table 8.6). The psi⁺ strains again appeared to be more sensitive to the lethal effects of TRD.

The calculated mean and standard deviation for each of the 2 populations was calculated:

$$\underline{\text{psi}}^+ = 0.731 \pm 0.250 \mu\text{g/ml}$$

$$\underline{\text{psi}}^- = 0.875 \pm 0.204 \mu\text{g/ml}$$

The difference between the two means ($0.144 \pm 0.026 \mu\text{g/ml}$) was found to be significant at $p=0.05$ by the "t-test".

ISOGENIC STRAINS

507 Me psi⁻ showed no significant difference in sensitivity to TRD, compared to the parent psi⁺ strain 507-4/2b, in a series of well plates over a drug concentration range of 0-100 µg/ml (Figure 8.2.b). A strain carrying the tcm1 mutation (MT172/4b), conferring resistance to TRD, was also run as a control. Both 507-4/2b and 507 Me psi⁻ were significantly more sensitive to TRD than the tcm1 strain.

(ii) Sensitivity of other mutants which show altered SUQ5 efficiency

None of the asu mutants (Table 8.2) or the sal mutants (Table 8.4) were found to be significantly different in their sensitivities to TRD compared to the controls. It was noted that mutants slightly more sensitive to CHX eg. asu5, or

TABLE 8.6 The sensitivity of a number of different psi⁺ and psi⁻ strains to trichodermin.

GENOTYPE		NO. TESTED	LEVEL OF RESISTANCE (μg/ml)*							
			0.01	0.05	0.1	0.25	0.5	0.75	1.0	5.0
SUQ5	<u>psi</u> ⁺	7	0	0	0	1	4	2	0	0
	+ <u>psi</u> ⁺	6	0	0	0	0	0	1	5	0
TOTAL	<u>psi</u> ⁺	13	0	0	0	1	4	3	5	0
SUQ5	<u>psi</u> ⁻	6	0	0	0	0	0	3	3	0
	+ <u>psi</u> ⁻	6	0	0	0	0	1	1	4	0
TOTAL	<u>psi</u> ⁻	12	0	0	0	0	1	4	7	0

* ie. the concentration of trichodermin up to which the given number of strains grew confluentlly,

slightly more resistant to CHX eg. sal4.4, showed a similar sensitivity to TRD.

(3) Cryptopleurine

Cryptopleurine (CRY) is a phenanthrene alkaloid and an inhibitor of an elongation step in protein synthesis in yeast primarily acting as an inhibitor of translocation (Bucher and Skogerson 1976). Resistance to this drug results from an alteration in an 80S ribosome component (Grant et al 1974).

Using the isogenic strains 507-4/2b and 507 Me psi⁻ a "well test" was conducted with a range of concentrations of CRY up to 100µg/ml. CRY was dissolved in 100% DMSO, the DMSO control giving a zone of inhibition of 2.8cm. No significant difference between the two strains was found with respect to sensitivity (Figure 8.2c)

In a preliminary "well test" screen, for sensitivity to CRY (50µg/ml), the asu5 mutant 5.1 was not found to be significantly more sensitive than any of the other 7 asu mutants (data not shown).

(4) Anisomycin

Anisomycin (ANI) is an antibiotic, isolated from various Streptomycetes, which inhibits yeast protein synthesis. It has been shown that the transpeptidation step is reversibly inhibited in vitro by the binding of ANI to the peptidyl transferase centre of the 60S ribosomal subunit (Barbacid and Vazquez 1975).

In a "well test" no difference to sensitivity to ANI was observed between 507-4/2b and 507 Me psi⁻ (Figure 8.2d) The solvent used, methanol, had only a very slight effect on viability with the zone of inhibition being 1.4cm.

(5) Summary & Conclusions

An initial screen of non-isogenic strains suggested that psi⁻ strains were significantly more resistant to CHX or TRD than psi⁺

strains. The average level of 'resistance' observed for psi⁻ strains was substantially less than observed for a strain known to carry a single recessive nuclear resistance mutation eg. D6/1d (a ade2.40 his4 cyh2 psi⁻ : obtained from Dr. B.S. Cox) was able to grow on YC containing 5µg/ml CHX; the mean level of resistance for the psi⁻ strains was 0.1µg/ml CHX. These observations however did not take into account any variation in genetic background. Therefore an isogenic psi⁺ and psi⁻ strain were compared using a more accurate "well test" to determine their degree of sensitivity to CHX and TRD. No significant difference was observed. There was also no difference in sensitivity to two other antibiotics ANI and CRY. It was concluded that the [psi] phenotype had no effect on the levels of resistance to a number of drugs known to act at the level of the 80S cytoplasmic ribosome.

Strains carrying either an asu or sal mutation were not significantly different in their sensitivity to CHX and TRD. Differences observed were assumed to be due to variation in the genetic background of the strains tested.

8.3 Mutants with altered 80S ribosomes: their effect on the efficiency of SUQ5 in suppressing nonsense codons.

To determine whether ribosomal mutants have any effect on the efficiency of suppression, experiments were carried out to answer the following questions:

1. Do known ribosomal mutations "suppress" nonsense mutations in vivo in either an SUQ5 psi⁻, SUQ5⁺ psi⁺ or SUQ5⁺ psi⁻ background?, and
2. Do spontaneously arising or UV-induced mutants, resistant to CHX, have any effect on the efficiency of suppression by SUQ5?

The number of mutations known to affect the ribosome directly

in yeast is relatively small (McLaughlin 1974). The majority of those so far isolated came from selection of mutants resistant to antibiotics known to act at the level of the ribosome:

Mutants resistant to Cycloheximide

All yeast mutants resistant to high levels of CHX ($\geq 5\mu\text{g/ml}$) are altered in the same gene product coded for by the CYH2⁺ gene (C.S. McLaughlin, personal communication). Resistance conferred by a mutation in this gene has been shown, using an in vitro, polyU-dependant, polypeptide synthesising system, to be due to an alteration in the 60S ribosomal subunit (Jiménez et al 1972).

Mutants resistant to Trichodermin

A mutant resistant to TRD, designated tcm1, has been isolated conferring resistance via an altered 60S ribosomal sub unit (Schindler et al 1974). Another mutant, tod2, has been isolated which confers resistance to TRD and other 12, 13-epoxytrichothecenes, but has been shown by Stafford (1972) to be altered in cellular permeability.

Mutants resistant to Anisomycin

Mutants resistant to TRD have been isolated that display cross resistance, both in vitro and in vivo, to ANI (Schindler 1974, Jiménez et al 1975). The mutation conferring resistance is expressed in the 60S ribosomal subunit.

Mutants resistant to Cryptopleurine

Mutants resistant to 5mM CRY are known to fall into at least 3 different loci (Meade et al 1977) the majority mapping at the cry1 locus. In vitro resistance of polyphenylalanine synthesis to CRY is conferred by the 40S ribosomal subunit (Grant et al 1974, Bucher and Skogerson 1976). cry1 mutants show no cross resistance to CHX or TRD. The original hypothesis of Grant et al (1974), that the cry1 locus coded for a 40S ribosomal component

(ie. ribosomal protein or ribosomal RNA), has recently been challenged by the observations of Meade et al (1977) who showed that resistance to CRY is not expressed in diploids that are monosomic for chromosome III and that the level of resistance is directly related to the ratio of resistant alleles to the number of chromosome sets. They suggest that the cry1 locus could code for a ribosome-modifying enzyme.

I. The effects of ribosomal drug resistant mutants on suppressor efficiency

Table 8.7 lists the strains used in this study which carry ribosomal drug resistance markers which result in altered ribosomal components (McLaughlin 1974).

The effect on suppression of these drug resistance mutations was investigated by crossing each strain with an SUQ5 ade2.1 his5.2 psi⁺ strain (α = 507-4/2b or 465/1b; a = 466/4a or 518/1c) to obtain, if possible, a strain with the drug resistance mutation in an SUQ5 ade2.1 his5.2 psi⁺ background. For all mutants tested (with the exception of D587-4B-cry1 which, upon crossing to 518/1c, gave a diploid that failed to sporulate) these crosses produced viable, fully suppressed spores carrying one or other of the ribosomal drug resistance markers as well as non-suppressed (SUQ5⁺ psi⁺) spores carrying these mutations (Table 8.8).

A control cross was carried out between 507-4/2b and the A364A parent strain carrying no ribosomal mutations (obtained from Dr. S. Oliver, University of Kent). However, of 24 tetrads dissected from the resulting diploid, no complete viable tetrad was obtained and the overall spore viability was 15.6%. The following is therefore a summary of crosses between 507-4/2b and another A364A derived strain carrying either cdc8 or cdc21 which have no effect on nonsense suppression (see Chapter 5).

TABLE 8.7 The origin and genotypes of strains carrying drug resistance markers used in this study.

DRUG	STRAIN	DRUG RESISTANCE*1 MARKER	RESISTANCE LEVEL (µg/ml)	OTHER GENETIC MARKERS	SOURCE *2
TRD	79.20.3 TP1	tcm 1?	50	ade1 ade2 ura1 leu2 gal1 psi ⁻	1
	A364A TP2	tcm 1?	50	lys1 tyr1 his7 gal1 ade1 ade2 ura1 psi ⁻	1
	79.20.3 TP3	tcm 1?	50	ade1 ade2 ura1 leu2 gal1 psi ⁻	1
CRY	D587-4B	cry 1	5	his 1	2
	A364A cry1	cry 1	5	lys1 tyr1 his7 gal1 ade1 ade2 ura1 psi ⁻	1
	A364A cry3	cry 1	10	lys1 tyr1 his7 gal1 ade1 ade2 ura1 psi ⁻	1
CHX	D6/1d	cyh 2	5	ade2.40 his4 psi ⁻	3
	D6/	cyh 2	5	ade2.40 psi ⁻	3
	A364A C3	cyh 2	2	lys1 tyr1 his7 gal1 ade1 ade2 ura1 psi ⁻	1
	A364A C40	cyh 2	2	lys1 tyr1 his7 gal1 ade1 ade2 ura1 psi ⁻	1

*1 TP1 - TP3 not formally proven to be alleles of tcm1 (C.S. McLaughlin pers. comm.).

*2 1 = C.S. McLaughlin (Univ. of California), 2 = L. Skogerson (Columbia), 3 = B. Cox.

TABLE 8.8 Genetic analysis of original drug resistant mutants x SUQ5 ade2.1 his5.2 psi⁺ strain.

(DIPLOID NO.)	DIPLOID *1	SPORE VIABILITY %	NUMBER OF WHOLE TETRAADS*2 ANALYSED	EXPECTED RATIO ade ⁻ :ADE ⁺	OBSERVED TOTALS ade ⁻ :ade ⁺	NUMBER OF*3 SUPPRESSED rid ^r SPORES	EXPECTED RATIO his ⁻ :HIS ⁺	OBSERVED TOTAL his ⁻ :HIS ⁺	SEGREGATION IN TETRAADS ura:URA ⁺ rid ^r :RID ^s
D6/1d x 507-4/2b(MT21)	NS	92.10	31(2)	3:1	91:25	13	3:5	46:70	29x2:2 26x2:2 2x3:1 1x1:3
D6/3c x 465/1b(MT147)	NS	84.85	23(0)	3:1	67:25	11	1:3	27:65	23x2:2
A364A C3 x 507-4/2b(MT173)	S	95.00	8(0)	3:1	24:8	3	5:3	21:11	1x4:0 5x3:1 2x2:2 8x2:2
A364A C40 x 507-4/2b(MT180)	S	25.00	1(0)	3:1	3:1	1	5:3	2:2	1x3:1 1x2:2
D587-4B x 518/1c(MT58)	S ^{*4}	-	-	-	-	-	-	-	-
A364 cry 1 x 507-4/2b(MT170)	S	97.50	9(1)	3:1	23:9	4	5:3	20:12	1x4:4 5x3:1 2x2:2 8x2:2
A364A cry 3 x 507-4/2b(MT171)	S	80.00	6(0)	3:1	20:4	4	5:3	16:8	4x3:1 2x2:2 6x2:2
79.20.2TP1 x 466/4a(MT181)	WS	85.00	10(0)	3:1	31:9	4	1:3	11:29	- 10x2:2

(DIPLOID NO.)	DIPLOID *1	SPORE VIABILITY %	NUMBER OF WHOLE TETRADS*2 ANALYSED	EXPECTED RATIO ade ⁻ :ADE ⁺	OBSERVED TOTALS ade ⁻ :ADE ⁺	NUMBER OF*3 SUPPRESSED rid ^r SPORES	EXPECTED RATIO his ⁻ :HIS ⁺	OBSERVED TOTAL his ⁻ :HIS ⁺	SEGREGATION IN TETRADS ura:URA rid ^r :RID ^s
A364A TP2 x 507-4/2b(MT172)	S	92.50	7(0)	3:1	21:7	5	5:3	17:11	1x4:0 5x3:1 1x2:2 7x2:2
79.20.3TP3 x 466/4a(MT179)	WS	97.92	11(2)	3:1	28:8	4	1:3	10:26	1x4:0 3x3:1 5x2:2 9x2:2
A364A A15 x 507-4/2b(MT175)	S	97.50	9(0)	3:1	25:11	4	5:3	22:14	4x4:0 4x3:1 1x2:2 9x2:2

*1 Phenotype of diploid: S = fully suppressed; WS = weakly suppressed, NS = non-suppressed.

*2 Figure in bracket is the number of aberrant tetrads in the total number of tetrads analysed.
These were discarded in the analysis.

*3 This is the number of suppressed spores which were resistant to the ribosomal drug under test (ie. rid^r:RID^s).

*4 These diploids failed to sporulate.

A total of 15 complete tetrads were scored, all showing a 2:2 segregation for temperature sensitivity;

	<u>Segregation in tetrads</u>					
	<u>ade⁻ : ADE⁺</u>		<u>his⁻ : HIS⁺</u>		<u>ura⁻ : URA⁺</u>	
OBSERVED	46	: 14	39	: 21	45	: 15
PREDICTED	45	: 15	37.5	: 22.5	45	: 15

A similar control cross between D6/6d (a ade2.40 his4 CYH^S psi⁻) and 507-4/2b yield red, non-suppressed diploids (see Appendix 3). In 11 complete tetrads obtained suppressed:non-suppressed spores segregated 2 x 2:2 7 x 1:3 and 2 x 0:4 (total 11 suppressed: 33 non-suppressed spores).

Thus none of the ribosomal mutations prevent SUQ5 from suppressing in a psi⁺ background nor do they suppress ade2.1 or his5.2 in the absence of SUQ5 in a psi⁺ background. This was confirmed by crossing strains of the genotype SUQ5 ade2.1 his5.2 psi⁺, carrying a drug resistance mutation (rib^r), with an SUQ5⁺ ade2.1 his5.2 psi⁺ strain (Table 8.9). No deviations from the predicted 2 suppressed: 2 non-suppressed spore segregation was seen.

To determine the effect of ribosomal mutations on nonsense suppression in a psi⁻ background, strains of the genotype SUQ5 ade2.1 his5.2 psi⁺ rib^r, were crossed to an SUQ5 ade2.1 his5.2 PNM1 psi⁻ strain (either 483/1a or 483/1b). The resulting genetic analysis showed that none of the mutations suppress ade2.1 in a psi⁻ background, nor do they enhance the efficiency of SUQ5 in a psi⁻ background (Table 8.9). Only the suppression of ade2.1 was checked in these crosses.

It is evident from these crosses that mutations giving rise to an alteration in either the 40S or 60S ribosomal subunit; a) do not increase the efficiency of SUQ5 in a psi⁻ background,

TABLE 8.9 Genetic analysis of suppressed⁺ (SUQ5 ade2.1 psi⁺) strains carrying ribosomal mutations; crosses to an SUQ5 ade2.1 psi⁻ (465/2a or 465/4d) and SUQ5 ade2.1 PNM 1 psi⁻ (483/1a or 483/1b) strain.

STRAIN	RIBOSOMAL MUTATION	CROSSED TO	DIPLOID	SPORE VIABILITY %	SEGREGATION OF R:W IN TETRADS	
					OBSERVED	EXPECTED* ₁
MT147/1b	cyh 2	x 465/2a x 483/1b	S NS	100.00 85.71	10:10 28:0	10:10 28:0
MT173/3a	cyh 2 (C)	x 465/2a x 483/1b	S NS	89.28 100.00	11:9 28:0	10:10 28:0
MT180/1d	cyh 2 (C40)	x 465/4d x 483/1d	S NS	45.65 55.43	17:10* ₂ 11:1	13.5:13.5 12:0
MT181/8d	tcm 1 (TP1)	x 465/2a x 483/1b	S NS	79.16 91.66	8:8 19:1	8:8 20:0
MT172/4b	tcm 1 (TP 2)	x 465/4d x 483/1a	S NS	100.00 96.43	14:14 24:0	14:14 24:0
MT179/2c	tcm 1 (TP 3)	x 465/4d x 483/1a	S NS	67.86 100.00	24:24 26:2	24:24 28:0
MT170/4d	cry 1	x 465/2a x 483/1b	S NS	91.25 95.00	29:31 60:4	30:30 64:0
MT171/6b	cry 3	x 465/2a x 483/1b	S NS	95.83 93.75	22:22 40:0	22:22 40:0
MT175/3b	ani ^r	x 465/2a	S	* ₃	-	-

*₁ Although all spores resulting from a PNM2/+ diploid are expected to be psi⁻ this is not always found in standard crosses (see Chapter 7).

*₂ No complete viable tetrads obtained from 23 tetrads dissected. Figures given are for surviving incomplete tetrads of the 27 spores. 13 were cyh^r and 14 CYHs. *₃ all 5 diploids failed to sporulate.

- b) do not act as antisuppressors, and
 c) cannot suppress nonsense mutations in the absence of SUQ5
 in a psi⁺ or psi⁻ background.

II. The effects of spontaneous and UV-induced CYH^r mutants on suppressor efficiency

A large number of spontaneous and UV-induced mutants resistant to both high and low levels of CHX were isolated in 3 different strains. The effect of these mutations on the efficiency of nonsense suppression was examined.

210-1/4c

The parent strains (obtained from Dr. R.K. Mortimer, University of California) had the genotype:

a ade2.1 his5.2 can1.73 lys1.1 gal1.1 trp5.2 leu2.1 try6.1 ileu1.1
psi⁻

Mutants resistant to CHX were obtained by either plating unirradiated cells directly onto YC + CHX or by UV-irradiating a log phase culture, resuspended in saline, with 100J/m² (approximately 8% survival) and plating onto YC + CHX. Mutants resistant to 0.5 and 1 µg/ml were obtained following UV together with spontaneous mutants resistant to 2µg/ml CHX (Table 8.10). A total of 141 mutants were isolated and all were ade⁻ his⁻ lys⁻ and red (ie. non-suppressed).

Two of the UV-induced mutants were subjected to further analysis. The growth of one of the mutants (MTC2) in liquid YC and YC + CHX (1µg/ml) was compared to that of the parent strain and D6/1d, a psi⁻ strain known to carry the cyh2 mutation (Figure 8.3). MTC2 and D6/1d were found to grow well in YC + CHX (1 µg/ml). The 210-1/4c parent strain failed to grow.

MTC2 and MTC39 were crossed to 507-4/2b as was the 210-1/4c parent strain. All 3 crosses gave fully suppressed ADE⁺ HIS⁺ diploids. The MTC2 x 507-4/2b diploid was resistant to $\leq 0.3\mu\text{g/ml}$ CHX and the MTC39 x 507-4/2b was resistant to $\leq 0.1\mu\text{g/ml}$ CHX.

TABLE 8.10 Spontaneous and UV-induced cycloheximide-resistant mutants of 210-1/4c.

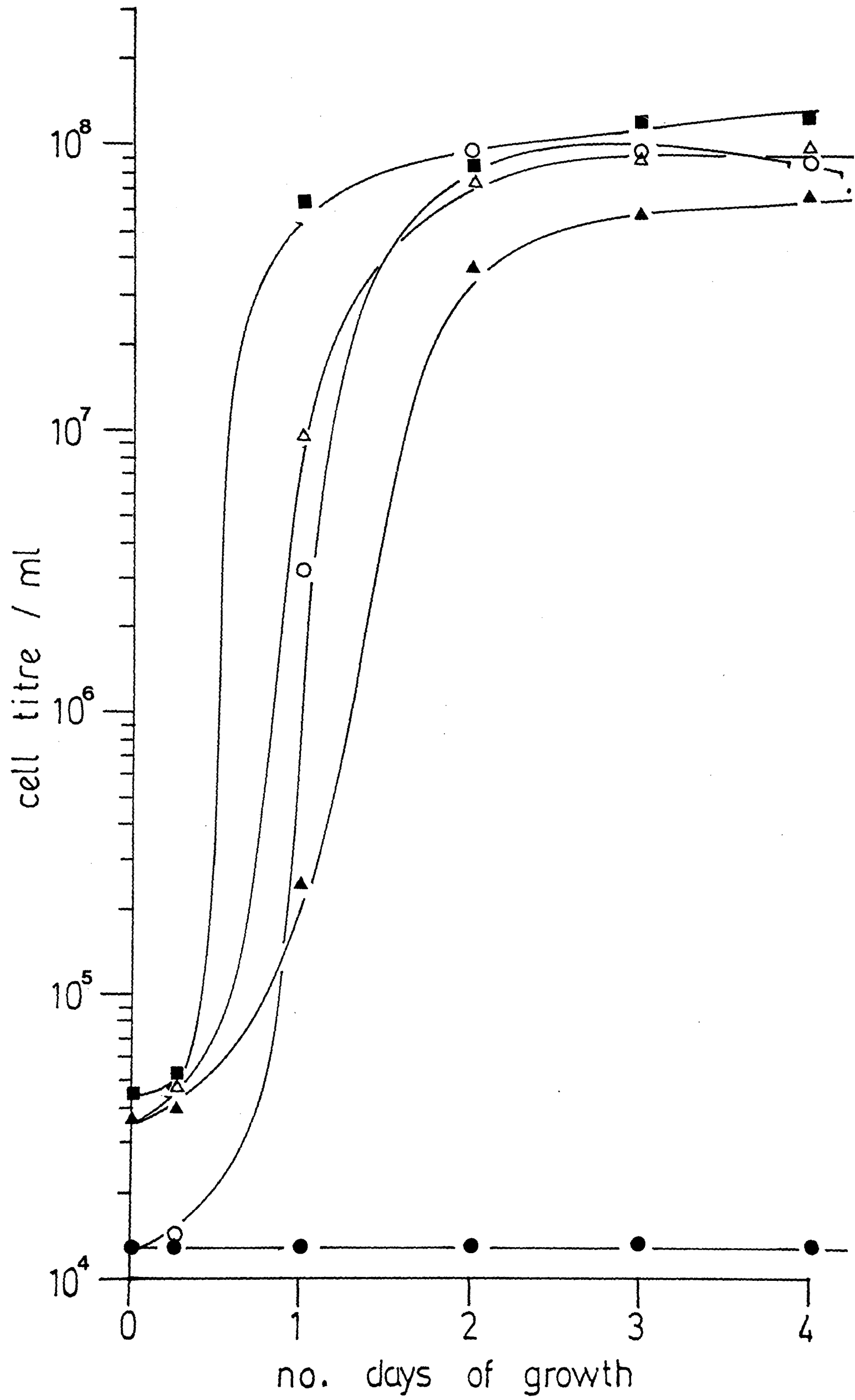
CYCLOHEXIMIDE CONCENTRATION ($\mu\text{g/ml}$)	TREATMENT	FREQ. OF cyh^r MUTANTS / 10^5 CELLS PLATED	NUMBER OF MUTANTS ISOLATED	MUTANT ANALYSED
0.50	UV	88.3	113	MTC39
1.0	UV	10.9	14	MTC2
2.00	NONE	0.19	14	-

FIGURE 8.3 Growth of cycloheximide-resistant mutants in YC
with or without 1 $\mu\text{g/ml}$ cycloheximide.

- ($\circ$) 210-1/4c CYH^+ control
- ($\bullet$) 210-1/4c CYH^+ + 1 $\mu\text{g/ml}$ cycloheximide

- ($\triangle$) MTC2 cyh^r control
- ($\blacktriangle$) MTC2 cyh^r + 1 $\mu\text{g/ml}$ cycloheximide

- ($\blacksquare$) D6/1d cyh^r + 1 $\mu\text{g/ml}$ cycloheximide



507-4/2b had a resistance level of 0.03 µg/ml. Thus in MTC2 and MTC39 cyh^r appeared to be semi-dominant. In each case cyh^r was found to be coded for by at least 2 genes because in tetrads dissected from the crosses to 507-4/2b an overall $1cyh^r : 3CYH^S$ spore segregation was found (Table 8.11). Such a digenic system of cyh^r was first observed by Wilkie & Lee (1965). In both mutants, MTC2 and MTC39, neither resistance component had any effect on the efficiency of SUQ5 to suppress ade2.1, each giving 2 suppressed (SUQ5) and 2 non-suppressed (SUQ5⁺) spores in the tetrads dissected (Table 8.11).

507-4/2b

The parent psi⁺ and a methanol-induced psi⁻ derivative of 507-4/2b (507 Me psi⁻) were used. Stationary phase cells were plated onto YC + various concentrations of CHX, at a density of $c.1-5 \times 10^7$ cells per plate. The number of cyh^r colonies was scored after 10 days growth at 28°C (Table 8.12). The psi⁻ strain showed a higher spontaneous mutation frequency at all 5 CHX concentrations used.

All 174 psi⁺ cyh^r mutants were white ADE⁺ HIS⁺ and all 801 psi⁺ cyh^r mutants were red ade⁻ his⁻. 7/116 (6.0%) of the psi⁺ mutants resistant to 0.25µg/ml CHX were petite compared to 3/3831 (0.1%) in the control plating of 507-4/2b psi⁺ onto YC. Growth on 0.25µg/ml therefore had no effect on the psi⁺ phenotype but appeared to either select, or induce, petite mutants (see Chapter 5).

To determine whether any of the spontaneous psi⁺ mutants resistant to 2µg/ml had any effect on nonsense suppression 4 mutants (C1-C4) were subjected to genetic analysis by crossing each to:

465/2a a SUQ5⁺ ade2.1 his5.2 can1.100 ura3.1 psi⁺ and

483/1b a SUQ5 ade2.1 his5.2 can1.100 lys1.1 PNM1 psi⁻

TABLE 8.11 Genetic analysis of two cycloheximide-resistant mutants of 210-1/4c; x 507-4/2b (SUQ5 ade2.1 psi⁺).

MUTANT	NO. TETRADS DISSECTED ^{*1}	SPORE VIABILITY %	SEGREGATION IN TETRADS			
			S:NS ^{*2}	ura3.1 URA	cyh ^r : CYH ^s	can ^r :CAN ^s
210-1/4c	20(8)	80.00	8x2:2	7x2:2 1x3:1	8x0:4	2x2:2 5x3:1 1x4:0
MTC 2	19(6)	72.37	6x2:2	6x2:2	6x1:3	-
MTC 39	10(5)	90.00	5x2:2	5x2:2	4x1:3 1x2:2	-

*1 the figure in brackets is the number complete non-abberrant tetrads obtained and analysed.

*2 S = ADE⁺ HIS⁺ NS = ade⁻ his⁻.
 WHITE RED

TABLE 8.12 The isolation of spontaneous mutants resistant to different concentrations of cycloheximide in an isogenic psi⁺ and psi⁻ (507-4/2b and 507 Me3 psi⁻).

CYCLOHEXIMIDE CONCENTRATION ($\mu\text{g/ml}$)	<u>psi</u> ⁺		<u>psi</u> ⁻	
	NUMBER OF MUTANTS	cyh ^r /10 ⁶ CELLS PLATED	NUMBER OF MUTANTS	cyh ^r /10 ⁶ CELLS PLATED
0.25	116	18.61	141	20.92
0.50	19	5.24	399	15.10
1.00	19	1.49	137	5.75
1.50	12	0.87	95	3.51
2.00	8	0.48	29	0.79

The diploids from these crosses failed to grow on YC + 2µg/ml CHX, indicating that the cyh^r mutations were recessive. All the crosses tested for CHX resistance gave viable spores of the genotype:

<u>SUQ5</u>	<u>psi</u> ⁻	cyh^r	
<u>SUQ5</u>	<u>psi</u> ⁻	<u>PNM1</u>	cyh^r
<u>SUQ5</u> ⁺	<u>psi</u> ⁺	cyh^r	(Table 8.13)
<u>SUQ5</u>	<u>psi</u> ⁺	cyh^r	

All had the predicted phenotypes with respect to suppression of the ade2.1 marker.

Table 8.13 also shows that the cyh^r phenotype, in two of the mutants (C2 and C3), was inherited cytoplasmically in crosses to the CYH^+ strains. To see if the resistance phenotype was associated with the psi⁺ phenotype 4 cyh^r psi⁺ spores from the C3 cross, and 2 cyh^r psi⁺ spores from the C2 cross, were subcultured on YC + 6% DMSO and psi⁻ derivatives of each obtained (see Chapter 6). Of these spores 2 (from C3) had altered levels of CHX-resistance being sensitive to 1.5µg/ml and only weakly resistant to 1µg/ml. The remaining 4 were still resistant to 2µg/ml. As a control 8 psi⁺ spores carrying the nuclear cyh^r mutations (4 from C1 x 465/2a and 4 from C4 x 465/2a) were converted to psi⁻ by subculturing on the DMSO medium. In all cases the psi⁻ derivatives were still resistant to 2 µg/ml. It was concluded from this that C2 and C3 carried cytoplasmically inherited cyh^r mutations, probably of the class recently reported by Guerineau et al (1974), and were not associated with the psi⁺ phenotype.

None of the 4 cyh^r mutations isolated (C1-C4) had any effect on nonsense suppression. 105 trichodermin resistant mutants of 507-4/2b psi⁺ were also picked from the 50µg/ml trichodermin well tests (see Section 8.2). All were suppressed ADE^+ HIS^+ . 8 out of the 15 tested were resistant to > 10µg/ml TRD.

TABLE 8.13 A genetic analysis of 4 mutants of 507-4/2b psi⁺ resistant to 2 µg/ml cycloheximide .

MUTANT	CROSSED TO	DIPLOID*1	SPORE VIABILITY	NO. OF COMPLETE TETRAADS	SEGREGATION IN TETRADS			
					S:NS	cyh ^r : CYH ^s (3 DAYS)	cyh ^r : CYH ^s (7 DAYS)	
C1	465/2a	S	97.50	9	8x2:2 1x1:3	9x2:2	9x2:2	
	483/1b	NS	95.00	8	5x0:4 3x1:3	-	-	
C2	465/2a	S	97.73	10	10x2:2	-	-	
	483/1b	NS	100.00	10	8x0:4 2x1:3	1x2:2 1x3:1 8x4:0	10x4:0	
C3	465/2a	S	97.50	9	7x2:2 2x1:3	1x2:2 2x3:1 6x4:0	1x2:2 8x4:0	
	483/1b	NS	92.50	8	6x0:4 2x1:3	-	-	
C4	465/2a	S	95.00	8	8x2:2	7x2:2 1x3:1	7x2:2 1x3:1	
	483/1b	NS	97.50	9	5x0:4 2x1:3 2x2:2	-	-	

*1 S = suppressed NS = non-suppressed .

8.4 Discussion

Several classes of mutation which might affect translational fidelity in yeast have been isolated in our laboratory on the basis of their influence on the activity of the ochre nonsense suppressor SUQ5. Such changes in translational fidelity could arise by alterations in one of several macromolecular elements which make up the translational machinery ie. aminoacyl tRNA synthetases, tRNA, ribosomes or termination factors. In E.coli mutations have also been isolated which affect the efficiency of translational nonsense and missense suppressors. The majority are in genes coding for ribosomal proteins (Gorini 1974, Bollen et al 1975, Piepersberg et al 1975). Thus the structural integrity of the ribosome is essential in maintaining the fidelity of translation in prokaryotes.

The component(s) of the translational machinery altered by sal, asu or psi mutations is not yet known, but one explanation for their effects, drawing analogy with the findings in E.coli, would be that they cause some alteration or modification of the ribosome structure such that recognition of ochre codons by the SUQ5 coded mutant tRNA is improved (sal, psi⁺) or reduced (asu, psi⁻). Waldron and Cox (1978) have shown that the ribosomal protein patterns of these mutants are indistinguishable from those of wild type strains, but failure to pick up difference cannot rule out the ribosome because the ribosomal protein electrophoretograms of drug resistant mutants, known to have altered 80S ribosomes, are also identical to those of wild type strains (C.S. McLaughlin, personal communication, C. Waldron, personal communication).

Evidence, presented in Section 8.3 of this Chapter, has shown that known ribosomal mutations do not mimic the phenotypes of any of the mutations which modify the efficiency of SUQ5.

However the cyh1 mutant of the fission yeast Schizosaccharomyces pombe, which is resistant to 100µg/ml cycloheximide (Ibrahim and

Coddington 1976), has been shown to have an electrophoretically different ribosomal protein compared to that of a CYH^+ strain (Coddington and Fluri 1977). Two cyh1 alleles tested had an antisuppressor effect when combined with the ochre/amber suppressor sup1-35 although they had no such effect when combined with ochre-specific suppressors sup3-18 and sup8-1 or with the amber-specific suppressors sup3-84 and sup8-4 (Thuriaux et al 1975). None of the 34 antisuppressor mutants isolated by Thuriaux et al (1975), against the ochre-specific and amber-specific suppressors, showed allelism with cyh1 nor did any of the 23 antisuppressor mutants isolated against sup1-35. Because sup1-35 suppresses both UAA and UAG codons it may be analogous to the omnipotent suppressors of Saccharomyces cerevisiae which are believed to code for proteins (possible ribosomal) rather than tRNA (Gerlach 1976, Hawthorne & Leupold 1974). It would therefore be of interest to see if ribosomal drug resistant mutations of S.cerevisiae have any antisuppressor activity in combination with the omnipotent suppressors. It is evident from the results presented in Section 8.3 that they have no detectable effect, when combined with the suppressor SUQ5, which is known to code for a mutant tRNA (Capecci et al 1975).

'In vivo' resistance to ribosomal drugs of the mutant strains carrying mutations affecting the efficiency of SUQ5 would add weight to the hypothesis that these mutations are expressed at the level of the ribosome. In E.coli the strA mutation confers resistance to streptomycin and the ramA and ramC mutations confer supersensitivity to this drug (Gorini 1974). In eukaryotes, apart from the cyh1 antisuppressor already mentioned, an anti-suppressor mutation (AS3) in Podospora anserina shows both in vivo and in vitro resistance to cycloheximide (Coppin-Raynal 1977) although it should be noted that the difference was not large

and the level of resistance was significantly less than that conferred by an unlinked cycloheximide-resistant mutation cyh1-1 whose ribosomes showed in vitro resistance to cycloheximide (Perrot quoted in Coppin-Raynal 1977). Two alleles of the AS4 antisuppressor mutation also conferred hypersensitivity to trichodermin (Picard-Bennoun and Cohen, quoted in Picard-Bennoun 1976).

None of the 8 asu mutations tested, nor any of the 14 allo-suppressor mutations tested, conferred any significant resistance or supersensitivity to either cycloheximide or trichodermin. This implies that these mutations probably do not code for altered ribosomal components but, until an efficient in vitro assay system is developed, this cannot be directly confirmed. The recent developement of an in vitro protein synthesising system composed entirely of yeast components (C. McLaughlin, personal communication) opens the way for developing such a system to assay for translational components altered in these and in /psi/ mutants.

The genetic approach to the problem of in vitro effects of ribosomal drugs and ribosomal mutations on SUQ5 suppressor efficiency is relatively crude picking up only "all or nothing" cahnges in suppression. A more accurate determination of any minor effects on translational fidelity could be made by using one of two methods:

- a) the system of Liebman et al (1975a) in which the efficiency of nonsense suppression is determined by measuring the levels of iso-i-cytochrome C in the ochre mutants cyc1-2 and cyc1-72.
- b) the cell-free assay system of Gesteland et al (1976) to translate Q β RNA which carries a natural UAA termination signal at the end of the synthesase gene. Adding the S100 fraction from a yeast strain, carrying an ochre suppressor, results in the

formation of a "read-through" protein, (Gesteland et al 1976). Efficiency of suppression is expressed as the amount of "read-through" product made.

The hypothesis that the $[\bar{\text{psi}}]$ gene product is a component of the 80S ribosome has not been confirmed in this study, but, until more accurate in vitro analyses have been developed, this hypothesis cannot be ruled out.

8.5 Summary

Protein synthesis inhibitors which act at the level of the 80S ribosome, and mutations which alter 80S ribosomes, were found to have no effect on the efficiency of nonsense suppression in strains with the general genotypes : SUQ5 psi⁺, SUQ5⁺ psi⁺ and SUQ5 psi⁻. No difference in sensitivity to 4 different ribosomal drugs was observed between isogenic psi⁺ and psi⁻ strains. asu and sal mutants had no associated ribosomal drug resistant phenotype.

CHAPTER 9

GENERAL DISCUSSION

9.1 The molecular nature of $\overline{\text{psi}}$

Nuclear genetic material, DNA, carries information about the amino acid sequences of proteins and the nucleotide sequences of RNAs such as tRNA and rRNA. The genetic role of organelle DNA appears to be similar; carrying information for organelle-specific proteins and RNA. RNA genomes can also carry information about the amino acid sequences of proteins. The most thoroughly studied RNA genome is that of the "male" phages of E.coli such as $Q\beta$ and R17. The $Q\beta$ genome codes for three principal proteins; maturation, coat and replicase, with the genome acting directly as a template for protein synthesis (see Lewin 1977 for a summary).

One of the major objectives of this thesis was to determine whether the cytoplasmically inherited $\overline{\text{psi}}$ factor has a nucleic acid genome which codes for the psi^+ phenotype and, in particular, to demonstrate that it was DNA. No physical evidence for an extrachromosomal nucleic acid species associated with the $\overline{\text{psi}}$ determinant has been obtained despite extensive searches in several laboratories using a number of techniques. Analysis of the response to mutagens has been one of the most powerful methods of studying the nature of genetic material at the cellular level. This was the approach taken here.

Several mutagens have known reactivity with DNA with the induced alterations being expressed as alterations in phenotype. Chapter 3 describes studies which demonstrated that psi^+ could be mutated to psi^- by two such mutagens, ultraviolet light (UV) and nitrosoguanidine (NTG) with an alteration in phenotype from suppressed to non-suppressed. This observation is entirely consistent with $\overline{\text{psi}}$ having a nucleic acid genome though not necessarily DNA. NTG, for example, induces mutation in Tobacco Mosaic Virus, a plant

virus with a RNA genome (Singer et al 1968).

Evidence for $\overline{\text{psi}}$ having a DNA genome was obtained by looking at the repair of UV-induced genetic damage to the $\overline{\text{psi}}$ genome (Chapter 4). UV-induced premutational lesions, leading to the psi^- mutant phenotype, were shown to be photoreactivable by white light. The photoreactivating enzyme has a strict requirement for pyrimidine dimers as a substrate. Pyrimidine dimers are therefore implicated as being at least part of the UV-induced damage to the $\overline{\text{psi}}$ genome responsible for the psi^+ to psi^- mutation. As discussed in Chapter 4 little is known about photoreactivation of UV-induced pyrimidine dimers, in RNA. Those studies demonstrating in vivo photoreactivation of UV-induced dimers in RNA were carried out under artificial photosensitising conditions (Jackle and Kalthoff 1978). Under normal conditions photoreactivation was absent presumably because either dimers were not formed in the RNA. UV-induced damage to yeast RNA in vivo has yet to be investigated.

Further evidence for $\overline{\text{psi}}$ having a DNA genome was obtained by looking at the dark repair of UV-induced genetic damage. The data presented (Chapter 4) clearly demonstrates that the repair, or expression, of UV-induced damage to the $\overline{\text{psi}}$ genome is entirely under the control of nuclear-coded systems known to repair UV and chemical mutagenic damage to nuclear DNA. The RAD3 excision-repair, error-free pathway and the RAD6 error-prone pathway are implicated in repair of the $\overline{\text{psi}}$ genome.

In summary the evidence presented in Chapter 3 and Chapter 4 points to $\overline{\text{psi}}$ having a DNA genome which codes for a gene product responsible for the psi^+ phenotype.

9.2 The number of $\overline{\text{psi}}$ genetic determinants

A recent study of the kinetics of elimination of the psi^+ phenotype from a PNM⁻/pnm⁺ diploid demonstrated that,

on the basis of a model of [psi] as an autonomous genetic determinant, approximately 58 [psi] "particles" are present in a haploid psi^+ strain (McCready et al 1977). Assuming one " [psi]^+ " particle only is required for the psi^+ phenotype to be expressed the induction of the psi^- mutation by UV would require simultaneous mutation in all 58 molecules. The kinetics of UV-induction of the psi^- mutation were shown to be linear ($n=1$) when plotted as log UV dose against log mutation frequency (Chapter 3). This suggests only one "target" molecule need be hit to induce the psi^- mutation.

The UV-induction kinetic data for the psi^- mutation can be accounted for in a number of ways:

- (1) only one [psi] genome exists per haploid cell which must be replicated once every cell cycle to ensure its information is passed on to the daughter cell, or
- (2) there is a direct interaction between many [psi] genomes present in the cell and that mutation in one molecule may be spread or amplified such that it affects all others. Such a "reference-copy" hypothesis has been proposed to account for the induction of the cytoplasmic petite mutation (Oliver 1977). An initial mutation in a single mitochondrial DNA molecule may be amplified by a process which is normally used by the cell to ensure the genetic identity of the cell's complement of mitochondrial DNA molecules (Oliver 1977). A haploid yeast cell characteristically contains about 50 mitochondrial DNA molecules (Williamson 1970), or
- (3) there is a single master-copy of the [psi] genome which gives rise to a number of "slave" [psi] genomes per cell, or
- (4) by an indirect action where a mutation in a single [psi] genome might result in the production of an altered repressor which would prevent the expression of nuclear genes whose

products are required for replication of the $[\underline{\text{psi}}^-]$ genome. Such a model has been proposed by Williamson (1970) to account for the petite mutation.

9.3 The nature of the psi^+ to psi^- mutation

The psi^+ phenotype can be lost i.e. mutated to psi^- by three, apparently different, processes; non-specific nuclear mutagens at a low frequency, specific chemical and physical agents at a very high frequency and by nuclear mutations (namely PNM^- and pnm^-) at a frequency up to 100%. These will be discussed separately.

(a) nuclear mutagens: UV and NTG were shown to induce psi^- mutants at a frequency up to 5×10^{-3} . For UV mutagenesis this non-specific induction of the psi^- mutation showed the characteristics of UV-induced forward nuclear gene mutation; mutation frequency compared to the mutation of, for example ASU^+ to asu ; the kinetics of induction; the effect on position in the cell cycle of the cell on mutation frequency; the occurrence of mutants as both pure and sectorized mutant clones. This suggests that the type of premutational lesion in the $[\underline{\text{psi}}^-]$ genome, induced by UV, may undergo a process of fixation and expression in the same manner as premutational lesions induced in nuclear DNA. The involvement of nuclear DNA repair processes in pure mutant clone formation has been implicated (Hannan et al 1976, Eckardt and Haynes 1977b) and was shown, in Chapter 4, to be the case for the psi^- mutation.

The occurrence of psi^- mutants as sectorized mutant clones suggests that the $[\underline{\text{psi}}^-]$ genome is at least a two strand molecule, presumably DNA. Since only one strand of such a molecule should be altered at any one site, the UV-induced premutational lesion, in the absence of heteroduplex repair, should be transmitted to only one of the two daughter genes following mutagenesis. This should give rise to $\frac{1}{2}$ sectorized colonies or $\frac{1}{4}$ sectorized colonies

if the DNA had replicated in preparation for the next nuclear division at the time of treatment. For psi⁻ mutants this was not always the case with some sectored colonies showing "hairline" or "striped" (alternate red and white components) psi⁻ sectors or colonies with $\frac{3}{4}$ mutant sectors. However some nuclear mutations arose in such irregular clones as well. Similar observations have been made for UV-induced mutation in a different forward nuclear gene mutation test system in yeast (Eckardt and Haynes 1977b). It is unclear what the molecular mechanisms responsible for the "hairline" or "striped" clones are. For the psi⁻ mutants they could be explained in terms of "diluting out" of psi⁺ "particles" due to an induced defect in transmission and /or replication of the [psi] genome.

(b) curing agents: the induction, at very high frequencies (up to 100%), of psi⁻ mutants by a number of chemical and physical agents is described in Chapter 6 (summarised in Table 6.22). Such a "curing" phenomenon is incompatible with the normal process of gene mutation described above. It probably represents a gross physical change, or even loss, of the [psi] genome to give a psi⁰ cell. Plasmid curing agents in bacteria (eg. Hirota 1960) and agents that cure yeast cells of their grande phenotype (see Nagley et al 1977 for review) cause such physical loss of the cytoplasmic genome. As discussed in Chapter 6 these [psi] curing agents may prevent the [psi] genome from replicating by destroying membrane sites required for successful duplication of the genome. Only a reversion analysis of "cured" strains will reveal whether genetic information is lost.

(c) PNM⁻ and pnm⁻ : Young & Cox (1971) demonstrated that PNM⁻psi⁻ x pnm⁺psi⁺ diploids are psi⁻ and segregate PNM⁻psi⁻ and both pnm⁺psi⁺ and pnm⁺psi⁻ spores. The proportion of pnm⁺psi⁺ spores decreased with the age of the zygote ie. with

increasing number of cell divisions.

The experiments reported in Chapter 7 demonstrate that $\text{pnm}^+ \text{psi}^-$ progeny, from such a diploid, have not lost the ability to revert back to psi^+ which can occur either spontaneously or be induced by UV. This implies that $\text{pnm}^+ \text{psi}^-$ segregants do not represent those spores which failed to receive $[\text{psi}]$ determinants during meiosis i.e. they are not psi^0 . An inability to revert to psi^+ would indicate loss of the genome.

The action of PNM^- is immediate with respect to the expression of the psi^+ phenotype because all $\text{PNM}^- \text{psi}^- \times \text{pnm}^+ \text{psi}^+$ diploids are homogenously non-suppressed. It was shown in Chapter 7 that such a diploid can revert to psi^+ only by the loss of the PNM^- allele through ^amitotic crossover event i.e. $\text{PNM}^- / \text{pnm}^+ \text{psi}^- \rightarrow \text{pnm}^+ / \text{pnm}^+ \text{psi}^+$. This again demonstrates that there is no loss of psi^+ information in the diploid.

These mutation studies have demonstrated that the molecular events leading to the psi^- mutation are probably different to those events ^eleading to the petite mutation. The mutagenic events differ in a number of ways:

(i) spontaneous mutation frequency; for psi^- the frequency is less than 10^{-4} whilst petite mutations were found to occur at frequencies between 0.1% and 50% depending on the strain. The spontaneous frequency of psi^- mutants was not enhanced in a cdc21 strain whilst such strains gave at least a tenfold increase in petite mutation.

(ii) the frequency of psi^- mutation induced by non-specific agents such as UV and NTG was a factor of at least 10^{-2} lower than observed for the petite mutation, in the same strains and experiments.

(iii) agents, for example thymidylate starvation, Mn^{2+} and 5FU, that induced the petite mutation at frequencies up to 100%, had no such drastic effect on the induction of psi^- mutants. Similarly agents that induced the psi^- mutation at a high frequency,

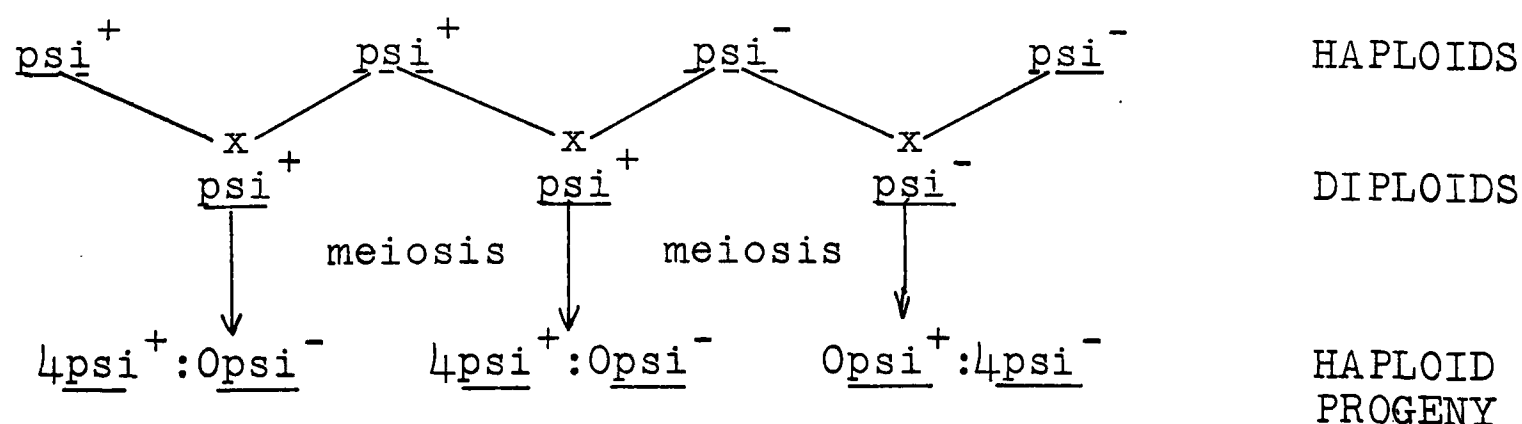
for example DMSO, MeOH and hypertonic media, had little effect on petite mutation. One agent, GuHCl, was mutagenic for both the $\overline{[\rho]}$ and $\overline{[\psi]}$ genomes.

(iv) the mechanisms responsible for the repair and expression of UV-induced premutational^{lesions} is different for the two cytoplasmic genomes; the $\overline{[\psi]}$ genome is exclusively under the control of the processes acting upon nuclear DNA damage whilst the $\overline{[\rho]}$ genome appears to be, at least in part, under the control of mitochondrial DNA-specific processes.

(v) ψ^- mutants can be reverted to ψ^+ whilst generally petite mutants cannot revert to the grande phenotype (Ephrussi et al 1949). There is a major exception to this rule, namely the mit⁻ mutants recently isolated by Tzagaloff et al (1975). These are point mutations on the mitochondrial genome which give rise to the petite respiration-deficient phenotype.

9.4 The extrachromosomal location of $\overline{[\psi]}$ and it's relationship to other extrachromosomal elements of yeast

Evidence for the extrachromosomal location of the $\overline{[\psi]}$ genome comes from a number of experiments. Cox (1965) demonstrated that crosses between a ψ^+ and a ψ^- strain/diploid^{gave a ψ^+} with almost exclusively ψ^+ spores segregating from such a diploid. This was found to be the rule in all such crosses carried out in this thesis. The inheritance of the ψ^+ and ψ^- phenotypes can be summarised as follows:



The heterokaryon test, described in Appendix 3, conclusively demonstrates that the psi⁺ phenotype is inherited independently of the nucleus with only SUQ5 psi⁺ heteroplasmons segregating from a SUQ5⁺ ade2.1 kar1 psi⁺ x SUQ5 ade2.1 KAR⁺ psi⁻ zygote

A characteristic of extranuclear genes located on a particular cytoplasmic determinant is mitotic segregation. If a small number of particles were present in the cytoplasm, and were randomly distributed, one would expect occasional mitotic segregants that fail to receive any of the particles. Mitotic segregation is associated with cytoplasmically inherited determinants in yeast; namely /rho (see Nagley et al., 1977) and /killer (Bevan and Somers 1969). However psi⁺ x psi⁻ diploids are stable for their psi⁺ phenotype (Young and Cox 1972; Conde and Fink 1977). Psi⁻ diploids segregate very rarely. This could reflect either:

- i) a very large number of /psi determinants present in the cytoplasm,
- or ii) only a small number of determinants which are not randomly distributed during mitosis.

In E. coli the association of the bacterial genome with the cell membrane ensures even segregation of replicated chromosomes to all daughter cells. Possibly the /psi genome(s) is attached to the yeast cell membrane.

Young (1969) reported some rare instances of psi⁻ spores obtained from psi⁺ x psi⁻ diploids. This suggests that distribution of /psi at meiosis may be random or that the mechanism responsible for even segregation at mitosis is less efficient during meiosis. No evidence for such random meiotic distribution was obtained in this thesis.

The finding that certain chemical and physical agents

specifically induce the loss of the psi⁺ phenotype at a very high frequency (Chapter 6) is further evidence for the cytoplasmic location of /p̄si/. "Curing" is a characteristic of both the grande and K⁺ phenotypes of yeast. /p̄si/-specific agents, with the exception of GuHCl, had no significant ability to cure these two phenotypes.

Studies by Young and Cox (1972) demonstrated that there was no linkage between the /p̄si/ and /r̄ho/ determinants. Cells lacking all detectable mitochondrial DNA (r̄ho⁰) have been found that maintain the psi⁺ phenotype (B. Cox personal communication). /P̄si/ must therefore represent a different extrachromosomal genome. Experiments with 5FU (Chapter 5) demonstrate that a cell could lose the K⁺ phenotype whilst retaining the psi⁺ phenotype. 5FU is known to induce loss of either the P2 dsRNA or both the P1 and P2 dsRNA (Mitchell et al., 1976b). The dsRNA contents of none of the 5FU-induced K⁻psi⁺ mutants have been determined. However the /p̄si/-genotypes of seven strains known to lack all dsRNA were determined (Appendix 1). All were psi⁻. The finding that mak mutants and the petS mutant are able to maintain the psi⁺ phenotype (Leibowitz and Wickner 1977; B. Cox personal communication, Appendix 1) demonstrates conclusively that /p̄si/ is not associated with the P2 dsRNA. The mak and petS mutants unable to maintain the P2 dsRNA (Wickner and Leibowitz 1976; Mitchell et al., 1976a). To date no psi⁺ mutant lacking the P1 dsRNA has been reported. Until such a mutant is found the possibility that /p̄si/ is determined by the P1 dsRNA cannot be ruled out. The mutation studies described in this thesis (Chapter 3 and 4) however demonstrate that

$\bar{\psi}$ is probably a DNA determinant.

Studies by McCready and McLaughlin (1977) have demonstrated that ψ^+ and ψ^- mutants do not show any difference in numbers or sizes of $\bar{\mu}$ DNA. The $\bar{\mu}$ plasmid remains the likeliest candidate for the $\bar{\psi}$ determinant but, until an easily detectable phenotype associated with the $\bar{\mu}$ plasmid is found, no linkage studies can be performed. A restriction enzyme analysis of $\bar{\mu}$ DNA from both ψ^+ and ψ^- strains might reveal any differences in overall structure of this plasmid associated with one or other of the $\bar{\psi}$ phenotypes. An analysis of the gene products of $\bar{\mu}$ circles isolated from isogenic ψ^+ and ψ^- strains, using the *E. coli* minicell system (see Hollenberg et al., 1977 for details), might prove rewarding.

No other phenotypes, apart from its effect on nonsense suppression, have been associated with the $\bar{\psi}$ determinant.

9.5 Models for the $\bar{\psi}$ phenomena

One can only speculate on possible models for $\bar{\psi}$ using the wide range of genetic data now available. A number of possible models are plausible and two will be discussed here in the light of data obtained in this thesis and of previously reported data.

(1) A DNA plasmid

This is the most plausible model where the $\bar{\psi}$ determinant would be an autonomous nucleic acid molecule analogous to the plasmids of certain bacteria.

The target theory data, discussed in Section 9.2, suggests that only one molecule per cell is present in a ψ^+ strain. To ensure that its genetic information is passed on to all mitotic

progeny a mechanism must be present to ensure even segregation of the replicated genome. This could be achieved by attachment to the cell membrane. Such a theory is supported by the observation that agents with known effects on cell membranes can cure cells of their psi⁺ phenotype (Chapter 6). They would destroy cell attachment sites for the /psi genome necessary for successful replication of the molecule and prevent its segregation to daughter cells. Cell membranes have been implicated in initiating new cycles of replication and in ensuring even segregation of replicated chromosomes to daughter cells in bacteria (Lewin 1975). There is both genetic and biochemical evidence for membrane involvement in the replication of yeast mitochondrial DNA (reviewed by Nagley et al., 1977). However if the psi⁺ curing agents reacted chemically with either the protein or lipid moiety of the cell membrane they should be effective in non-growth conditions. This was not found for DMSO, GuHCl or MeOH all of which require growth for their effect.

To ensure even segregation of a single /psi genome the DNA would have to be replicated, once per cell cycle, in step with nuclear DNA. A possible experimental approach to answering this question would be to look at NTG mutagenesis of /psi in a synchronous cell culture. NTG induces mutation at the replicating fork of E. coli DNA (Cerdá-Olmedo et al., 1968). If the /psi genome replicated once during the cell cycle one would predict that the psi⁺ to psi⁻ mutation would occur, at a discrete point, during the nuclear S phase. This has been observed for nuclear gene mutation induced by NTG in synchronous cultures of yeast (Dawes and Carter 1974; Kee and Haber 1975; Burke and

Fangman 1975). If the $\underline{\text{psi}}^+$ to $\underline{\text{psi}}^-$ mutation occurred continuously through the cell cycle this would indicate that $\underline{\text{psi}}^-$ replication was not co-ordinated with nuclear DNA.

The $\underline{\text{pnm}}^+$ loci could represent nuclear genes involved in the replication of the $\underline{\text{psi}}^-$ genome. The $\underline{\text{pnm}}^-$ mutation(s) could occur in genes coding for components of the membrane attachment sites. This would be analogous to the $\underline{\text{dnaA}}$ and $\underline{\text{dnaB}}$ mutants of *E. coli* both of which are temperature sensitive DNA replication mutants which show alterations in membrane proteins (Shapiro *et al.*, 1970; Siccaldi *et al.*, 1971). The $\underline{\text{PNM}}^-$ mutants must have some other effect on $\underline{\text{psi}}^-$ replication because they are dominant. It has been postulated that in the presence of $\underline{\text{PNM}}^-$ the $\underline{\text{psi}}^-$ genome either stops replicating or replicates more slowly. This would result in loss or diluting out of $\underline{\text{psi}}^-$ determinants (Young and Cox 1971; McCready *et al.*, 1977). Young and Cox (1971) proposed that $\underline{\text{PNM}}^-$ coded for an altered repressor of $\underline{\text{psi}}^-$ replication such that it was not recognised by an 'apo-repressor' present in the cell.

The $\underline{\text{psi}}^+$ to $\underline{\text{psi}}^-$ mutation, induced by NTG or UV, would represent a point mutation on the $\underline{\text{psi}}^-$ genome such that it now coded for an altered gene product unable to function in translation. The $\underline{\text{psi}}^-$ genome as a single, double-stranded DNA molecule would explain the occurrence of $\underline{\text{psi}}^-$ mutants as both whole and sectorised mutant clones as discussed in Section 9.3

(2) A self-perpetuating metabolic system

It is possible that the $\underline{\text{psi}}^-$ phenomenon represents a self-perpetuating cytoplasmic state without invoking the presence of information, of the type carried in DNA, in the cytoplasm. If there existed a $\underline{\text{psi}}^-$ nuclear gene its transcription could be controlled by a promoter-operator system as described, for

example, in the lac operon of E. coli (see Lewin 1975 for a review). The gene product of the $\bar{\psi}$ gene would not only give rise to the ψ^+ phenotype but be involved in a chain of reactions which result in the synthesis of an inducer molecule (I). A repressor molecule, whose normal function would be to bind to the operator region to prevent transcription of the $\bar{\psi}$ gene, would be inactivated by I. Thus one would get a possible feed back mechanism giving rise to a self-perpetuating cytoplasmic state.

The ψ^- state could be envisaged to occur in one of two ways:

a) if the $\bar{\psi}$ gene itself was hit giving a mutant gene product unable to function in translation. This state would be stable in the haploid cell. To explain the observation that, in a $\psi^+ \times \psi^-$ cross, only ψ^+ spores are segregated one could postulate, as have McCready et al., (1977), that the $\bar{\psi}$ gene is in fact a ribosomal RNA (rRNA) gene. As discussed by McCready et al., (1977) there must be some rectification mechanism which prevents random mutations in rRNA genes from accumulating so that rRNA sequences do not diverge. In a $\psi^+ \times \psi^-$ diploid the mutant ψ^- rRNA gene would be rectified to non-mutant ψ^+ by such a mechanism. This would explain the non-Mendelian inheritance of ψ^+ .

b) if the I molecule was in some way lost or inactivated. If it were subject to metabolic breakdown it could easily be ineffective below a certain concentration resulting in a reduction in the synthesis of the $\bar{\psi}$ gene product. This would further reduce the concentration of I molecules. Above the critical

level the $\bar{\psi}$ gene would be transcribed resulting in a build up of I molecules. This could occur very rapidly giving the effect of a sudden switch-on or mutation.

Any change in nuclear genotype, which altered the level of the inducing agent, would influence the stability of the two cytoplasmic states. One could envisage the pnm^- mutation as having such an effect.

The switch from one state to the other depends upon the effect of I: curing agents could have a direct effect on the I molecule by breakdown chemically of the molecule causing loss of the ψ^+ phenotype due to binding of the repressor molecule to the operator of the $\bar{\psi}$ gene. When such a "cured" strain is crossed to a ψ^+ strain the inducer would be reintroduced with subsequent build up of I molecules by the positive feedback mechanism described above.

One could also postulate that the $\bar{\psi}$ curing agents interfere with the protein-DNA binding (repressor-operator binding). Friend *et al.*, (1974) have postulated that DMSO can have secondary effects on transcription by inhibiting binding of repressor molecules to DNA.

The PNM^- genes could code for an altered inducer or repressor such that the $\bar{\psi}$ gene is not transcribed. This predicts that the cell would still have the potential of reverting to ψ^+ if a mutation modifying the PNM^- gene product, to restore its activity, were present, or if the mutant PNM^- allele was lost by meiotic or mitotic segregation. Both predictions were confirmed (see Chapter 7). The progressive elimination of the

psi⁺ phenotype (Young and Cox 1971), or at least the failure by PNM⁻ to eliminate totally the psi⁺ phenotype of all progeny from a PNM⁻ x pnm⁺ diploid (Chapter 7), would suggest that some active I or repressor molecules remain giving a small but significant level of transcription of the /p̄si/ gene. The level of the /p̄si/ gene product would not be high enough for the psi⁺ phenotype to be expressed.

These two models for the /p̄si/ phenomena may have loopholes but can provide the background for further genetic analysis of /p̄si/. One possible way to distinguish between a piece of genetic material governing its own replication by virtue of its specific macromolecular pattern (base sequence of DNA or RNA) and a self-maintaining cycle of metabolic reactions, would be genetic transformation. That is by isolating cytoplasmic DNA or RNA/^{free} of other materials and demonstrating its genetic properties by transforming psi⁻ cells to psi⁺. The phenomena of genetic transformation in yeast has recently been reported (Hinnen *et al.*, 1978). They demonstrated that, by mixing treated sphaeroplasts of a non-revertible leu2 mutant with a colE1 plasmid carrying an integrated yeast LEU2⁺ gene, stable LEU⁺ transformants could be obtained. This has recently been adapted using the yeast /2μ/ plasmid as a vector with very high frequencies of transformation being obtained (J. Beggs personal communication). One possible approach would be to transform psi⁻ strains, which are leu2, with a colE1 - /2μ/ - LEU2⁺ hybrid plasmid using /2μ/ DNA from either a psi⁺ or psi⁻ strain.

Appendix I

Association of $\overline{\text{Psi}}$ with other extrachromosomal elements

In an attempt to demonstrate the non-identity of $\overline{\text{psi}}$ with other extrachromosomal elements found in yeast several yeast strains were obtained from a number of research workers. These strains lacked one or more of the known extrachromosomal elements (Table A1.1). Finding a psi^+ strain lacking in one or other of these elements would be confirmation of their non-identity with $\overline{\text{psi}}$. psi^+ strains lacking detectable mitochondrial DNA have been found (B.S. COX personal communication), but to date strains known to lack dsRNA or $\overline{2u}$ DNA have not been characterised with respect to their psi phenotype.

The $\overline{\text{psi}}$ phenotype of the strains was determined by crossing each to a standard SUQ5 ade2.1 psi^- tester strain (either 468/1a or 468/1d). Table A1.2 gives the predicted segregation of suppressed:non-suppressed spores in tetrads derived from such a cross together with data obtained during the course of this Thesis with mutants of known $\overline{\text{psi}}$ phenotype. In all cases the predicted segregation was confirmed.

a) R93a

This mutant gave the spore segregation pattern of an ADE⁺ psi^- strain (Table A1.3). This was confirmed by crossing 12 of the red ade^- spores to either 468/1a or 468/1d (SUQ5 ade2.1 psi^-). No complementation was seen as predicted for a psi^- mutant.

R93a was found to be defective in a nuclear gene essential for maintenance of the killer (K^+) phenotype. This mutation was found to be recessive and presumably belongs to the class of mak mutations that have been described

TABLE A1.1 Yeast strains used in this study lacking
known extrachromosomal elements.

STRAIN	NUCLEAR GENOTYPE	CYTOPLASMIC GENOTYPE	SOURCE
R93a	α arg9	ds RNA ⁻	A.J.Herring
Y379-5D	α	ds RNA ⁻ circ ^o	C.P.Hollenberg ^{1,5} (via H.Tabak)
CB11	α ade1 MAL6	ds RNA ⁻ circ ^o 2OS ⁻	B.S.Cox ⁴
FM11 - F	a ade2.1 lys1.1		G.R.Fink ²
FM11 - V	met8.1 his 4.580 SUP4.3	K ⁺	M.Vodkin ³
JM1		ds RNA ⁻ 2OS ⁻	
JM6	derived from	ds RNA ⁻ 2OS ⁻	
JM7	FM11 - V by EMS mutagenesis	ds RNA ⁻	M.Vodkin ^{3,4}
JM15		ds RNA ⁻ 2OS ⁻	

* ds RNA⁻ = lacks all ds RNA species ; circ^o = lacks free or
integrated $\left[\overline{2\mu} \right]$ DNA ; 2OS⁻ = unable to synthesise 2OS RNA .

REFERENCES

1. Livingston (1977)
2. Rasse - Messenguy and Fink (1973)
3. Vodkin (1977b)
4. Garvik and Haber (1978)
5. Tabak (1977)

TABLE A1.2 Segregation of suppressed:non-suppressed spores in tetrads derived from crosses between
an SUQ5 ade2.1 psi⁻ strain (468/1a or 468/1d) and mutants of known [psi⁻] phenotype.

MUTANT GENOTYPE ^{*1}	DIPLOID NUMBERS	DIPLOID ^{*2} PHENOTYPE	NO. OF TETRADS	SUPPRESSED:NON-SUPPRESSED						
				OBSERVED						
				PREDICTED	4:0	3:1	2:2	1:3	0:4	
SUQ5 ade2.1 psi ⁺	MT166, MT167	S	15	4:0	15	0	0	0	0	0
SUQ5 ade2.1 psi ⁻	MT214	NS	14	0:4	0	0	0	0	0	14
+ ade2.1 psi ⁺	MT158, MT163, MT225	S	19	2:2	0	0	18	1	0	0
+ ade2.1 psi ⁻	MT141, MT188	NS	9	0:4	0	0	0	0	0	9
+ + psi ⁺	MT145, MT195	S	19	3:1	0	16	3	0	0	0
+ + psi ⁻	MT217	S	10	2:2	0	1	9	0	0	0

^{*1} only the relevant genotype is given.

^{*2} S= white ADE⁺, NS=red ade⁻.

TABLE A1.3 Genetic analysis of crosses between mutants lacking one or more extrachromosomal elements and an SUQ5 ade2.1 psi⁻ tester (468/1a).

MUTANT	CROSSED TO	DIPLOID NUMBER	DIPLOID PHENOTYPE	TETRAD ANALYSIS			SPORE SEGREGATION IN COMPLETE TETRAIDS		SPORE * TOTAL S:NS
				NO.	SPORE VIABILITY	NO. WHOLE TETRAIDS	S:NS	K ⁺ :K ⁻	
R93a	468/1a	MT207	S	20	82.5	10	10x2:2	9x2:2 1x1:3	32:34
R93a	466/4a	MT215	S	10	100	10	2x4:0 7x3:1 1x2:2	10x2:2	31:9
Y379-5D	468/1a	MT229	S	20	28.8	0	-	-	19:4
CB11	468/1a	MT245	S	10	62.5	3	3x1:3	3x4:0	6:19

* including data from incomplete tetrads.

(Somers and Bevan 1968, Wickner 1976, 1978). To see if the mak gene in R93a was required for maintenance of the psi⁺ phenotype R93a was crossed to a standard SUQ5 ade2.1 psi⁺ strain (466/4a). The spore segregation patterns from the tetrads derived from this cross (Table A1.3, cross no. MT215) indicated that the psi⁺ phenotype was maintained in both mak and MAK⁺ spore progeny.

The preliminary tetrad data obtained also indicated that this mak gene was linked to both arg9 (PD=5, NPD=0, TT=4) and ura3 (PD=4, NPD=0, TT=5) but not to ade2 (PD=2, NPD=1, TT=6). Because arg9 and ura3 are closely linked to the centromere of chromosome V it was tentatively concluded that this mak gene also lies on chromosome V and is presumably the m (mak10) gene (Somers and Bevan 1968) which also lies on chromosome V (Wickner and Leibowitz 1976). R93a therefore had the genotype α arg9 mak10 psi⁻ K⁻.

b) CB11

This mutant has been reported to lack all detectable dsRNA, and $\sqrt{2u}$ DNA as well as lacking the dominant cytoplasmic genetic element that controls 20S RNA synthesis during sporulation (Garvik and Haber 1978). The spore segregation data obtained (Table A1.3, cross no. MT245) was obscured by the presence of an ade1 mutation in CB11. Spores carrying this mutation accumulate red pigment even when psi⁺ and SUQ5 are present.

13 red spores, derived from MT245, were tested and 4 were found to complement a known ade1 carrying strain (and were, therefore, ade2.1 ADE1⁺). By the standard complementation test it was found that 3 of these 4 had the genotype SUQ5 ade2.1 psi⁻ the other being SUQ5⁺ ade2.1 psi⁻. It was

concluded that CB11 was psi⁻.

c) Y379-5D

The cross with this mutant gave very poor spore viability. Of 23 spore clones obtained, 19 (83%) were suppressed (ade⁺), suggesting that Y379-5D may be psi⁺. To confirm this the 4 red spore clones obtained were crossed to both standard SUQ5 ade2.1 psi⁻ and SUQ5⁺ ade2.1 psi⁺ strains and red diploid clones, which failed to sporulate, were obtained in all cases. The red spores were therefore SUQ5⁺ ade2.1 psi⁻. It was concluded that Y379-5D was psi⁻.

d) FM11

A series of K⁻ mutants lacking all detectable dsRNA (dsRNA⁻) have been recently isolated by treating the psi⁺ K⁺ strain FM11 with EMS (Vodkin 1977b). Four of these dsRNA⁻ mutants (JM1, JM6, JM7 and JM15) were checked for their [psi] phenotype.

JM6 and JM7 were found to carry new adenine-requiring mutations (adex), which presumably blocked the adenine pathway before the ade2 block, giving white ade⁻ rather than red ade⁻ mutants. The psi phenotypes of red ade⁻ spores derived from the crosses between JM6 and JM7 and the SUQ5 ade2.1 psi⁻ tester 468/1d were determined (MT236 and MT247 respectively). 8 spores from MT236 and 6 spores from MT247 all failed to complement the standard SUQ5 ade2.1 psi⁻ strains. JM6 and JM7 were therefore psi⁻. JM1 and JM15 gave the predicted spore segregation patterns for ade2.1 psi⁻ mutants (Table A1.4, cross no. MT235 and MT248 respectively).

In a control cross between the FM11 parent strain (FM11-F- obtained from Dr. G. Fink via Dr. C. Waldron) and 468/1d the spore segregation pattern obtained indicated that FM11

TABLE A1.4 Genetic analysis of FM11 and strains derived from FM11 that lack all dsRNA.

MUTANT	CROSSED TO	DIPLOID NO.	DIPLOID PHENOTYPE	TETRAD ANALYSIS			SPORE SEGREGATION IN COMPLETE TETRADS			* SPORE TOTALS S:NS
				NO. DISSECTED	SPORE % VIABILITY	NO. WHOLE TETRADS	S:NS K ⁺ :K ⁻			
FM11-F	468/1d	MT233	NS	12	91.7	8	8x0:4	8x4:0	0:32	
	507-4/2b	MT243	NS	12	100	12	3x0:4 8x1:3 1x2:2	10x4:0 2x3:1	10:38	
FM11-V	507-4/2b	MT246	NS	10	100	10	10x2:2	10x4:0	20:20	
JM1 (red)	468/1d	MT235	NS	12	93.8	9	9x0:4	9x4:0	0:36	
JM6 (white)	468/1d	MT236	NS	13	69.2	0	—	—	26:9	
JM7 (white)	468/1d	MT247	NS	9	100	9	9x2:2	9x4:0	18:18	
JM15 (red)	468/1d	MT248	NS	10	87.5	7	7x0:4	7x4:0	0:28	
JM15 (red)	182/8d	MT249	NS	10	97.5	9	1x0:4 5x1:3 3x2:2	NT	11:25	
JM15 (white)	168/1d	MT237	NS	11	95.4	9	1x0:4 3x1:3 5x2:2	2x4:0 3x3:1 4x2:2	23:13	

NT = not tested.
 NS = non-suppressed;red.
 * including data from incomplete tetrads.

was psi⁻ NOT psi⁺ (Table A1.4, cross no. MT233). When FM11-F was crossed to an SUQ5 ade2.1 psi⁺ strain predominantly non-suppressed spores were obtained from a non-suppressed diploid (Table A1.4, cross no. MT243). Thus FM11-F appeared to carry a dominant mutation in a gene required for maintenance of the psi⁺ phenotype ie. a PNM⁻ mutation (Young and Cox 1971). An identical result was also obtained when the JM15 dsRNA⁻ mutant was crossed to a second SUQ5 ade2.1 psi⁺ strain (MT182/8d); again predominantly non-suppressed spores were obtained, confirming the presence of a PNM mutation in the JM15 mutant.

The original FM11 isolate used to obtain the JM mutant series (designated here as FM11-V) was obtained from Dr. Vodkin (University of South Carolina). When crossed to 507-4/2b (SUQ5 ade2.1 psi⁺) a non-suppressed diploid was obtained which gave rise to tetrads that had two suppressed and two non-suppressed spores. The non-suppressed spores all failed to complement the standard SUQ5 ade2.1 psi⁻ strains although the segregation pattern indicated that no PNM mutation was present. FM11-V possibly carried a weak PNM mutation which was closely linked to the SUQ5 locus on chromosome XVI.

e) Summary and Conclusion

A total of 7 mutants lacking any detectable dsRNA (R93a, Y379-5D, CB11, JM1, JM6, JM7 and JM15), 4 of which also lacked the cytoplasmic genetic element required for production of the sporulation-specific 20S RNA (ie. CB11, JM1, JM6 and JM15), were characterised with respect to their psi phenotypes. All were psi⁻ although in the case of the JM mutants this appeared to be a result of the parent strain,

FM11, carrying a mutation in a psi⁺ maintenance gene which had no effect on [killer] maintenance. Two mutants (Y379-5D and CB11), lacking any [2u] DNA, were found to be psi⁻.

Until a psi⁺ strain is found that lacks one or other of the known cytoplasmic genetic elements the physical non-identity of these elements and [psi] cannot be confirmed. To date only psi⁺ rho⁰ strains have been found (B.S. Cox personal communication).

Appendix 2

The Genetic Activity of Benomyl in Yeast

Over the past 15 years several benzimidazole compounds have been introduced as systemic fungicides in agriculture, the most prominent of these being benomyl (methyl-1-(butyl-carbamoyl)-2-benzimidazole carbamate). In Aspergillus nidulans, although it does not cause point mutation (Hastie 1970), benomyl has been found to increase somatic segregation in heterozygous diploid strains probably as a result of induced chromosome non-disjunction (Hastie 1970, Kappas et al 1974). This antimitotic effect of benomyl (or its commercial form BENLATE) is possibly due to its direct breakdown product (methyl benzimidazol-2-yl carbamate, MBC) binding to tubulin thus disrupting spindle formation in dividing nuclei (Davidse and Flach 1977).

To date no chemical agent has been shown to induce haploidisation in the yeast Saccharomyces cerevisiae at high frequencies although p-fluorophenylalanine (PFA) is known to induce segregation in diploids of many other fungi (but not S.cerevisiae). The availability of such a haploidising agent for S.cerevisiae would be of great help in genetic analysis of the organism eg. demonstration of gene linkage. The genetic activity of benomyl on S.cerevisiae was therefore investigated to see if it induced haploidisation. Pure benomyl (as opposed to BENLATE which is 50% benomyl) was kindly supplied gratis by Du Pont (UK) Ltd..

The effects of benomyl and its commercial form BENLATE on 2 diploid strains D5 and D6, specially constructed to monitor various induced genetic events, were examined.

(a) D5

Constructed by Dr. F.K. Zimmermann; this strain allows for rapid visual screening for mitotic crossing-over on solid medium (Zimmermann 1973). It has the genotype:

<u>ade2.40</u>	<u>leu1</u>	<u>trp1</u>	<u>MAL1</u>	<u>MAL4</u>
ade2.119	+	+	+	+

The two ade2 alleles complement to give a white diploid but ade2.40/ade2.40 homozygous diploids give dark red colonies and ade2.119/ade2.119 homozygous diploids give pale pink colonies. Mitotic crossing-over generates 2 reciprocal products which show up together in colonies as red and pink sectors (Zimmermann 1973). Chromosome non-disjunction (haploidisation) would generate either whole pink or whole red colonies, as would mitotic gene conversion, or mitotic crossing-over with a lethal segregating with one of the 2 reciprocal products.

Stationary phase cells of D5 were plated onto YNB + leu + trp containing a reduced adenine concentration (5µg/ml instead of 20µg/ml) to allow better pigment formation. Benomyl or BENLATE were added to this medium, after autoclaving, from 10mg/ml stock DMSO solutions. After cells were plated the plates were incubated for 5-7 days at 28°C before the phenotypes of the colonies arising were scored (Table A2.1).

The results presented demonstrate that benomyl (25µg/ml or 50µg/ml) and BENLATE (50µg/ml) had significant effects on cell viability and caused a high number of surviving colonies (up to 25%) to show aberrant morphology. For 25µg/ml benomyl, 54% (182/337) of the aberrant colonies

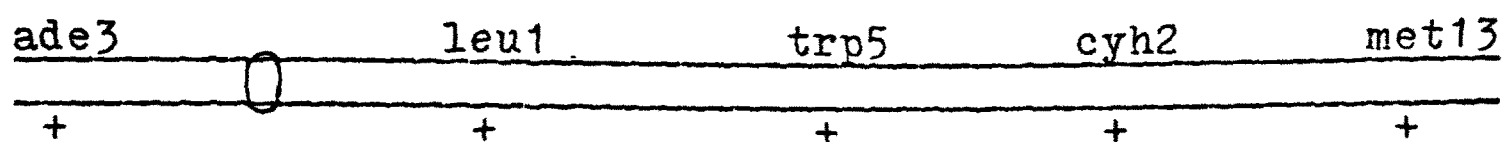
TABLE A2.1 Phenotypes of colonies arising from cells of D5 plated onto YNB + trp + leu + ade +
benomyl or BENLATE.

TREATMENT	% SURVIVAL	NO. OF COLONIES SCORED	NO. OF INDEPENDANT EXPERIMENTS	MUTANT COLONY PHENOTYPES *3						TOTAL ABBERRANT *2	
				RED	PINK	RED/ PINK	RED/ WHITE	PINK/ WHITE	HAIRLINE *1		
CONTROL	100	4488	5	3	1	0	3	2	0	1	10 (0.2)
benomyl (25µg/ml)	23.3	1342	1	100	82	14	40	30	7	64	337 (25.1)
benomyl (50µg/ml)	2.9	31	1	3	0	0	3	1	0	0	7 (22.6)
BENLATE (25µg/ml)	10.1	1049	2	5	5	2	12	4	1	9	38 (3.6)
BENLATE (50µg/ml)	0.9	1408	3	63	64	19	83	45	6	12	292 (20.7)

*1 defined as colonies with very small sectors the colour of which could not be determined visually.

*2 percentage of total colonies scored in parentheses.

*3 number of colonies scored.



CHROMOSOME VII

Unmasking of all the markers on Chromosome VII gives white (the *ade3* block precedes the *ade2.40* block in the adenine biosynthetic pathway) *leu⁻trp⁻met⁻cyh^r* colonies. By plating cells onto YC + cycloheximide (2µg/ml) plates one can detect the frequency of cells in a population that have become monosomic for chromosome VII (Parry and Zimmermann 1976).

100µl aliquots of a stationary phase culture of D6 were used to inoculate 10ml of liquid YC containing either 25µg/ml or 50µg/ml benomyl or 50µg/ml BENLATE, or no additions. The cultures were grown at 28°C for 72 hours with constant shaking. Samples were taken after various incubation periods and plated onto YC, to estimate the viable cell titre, and onto YC + cycloheximide (2µg/ml). A sample was taken at time 0 from each culture immediately after inoculation. The colonies were counted after 7 days incubation at 28°C (Table A2.2).

Both benomyl and BENLATE inhibited growth of D6. After 72 hours growth both the benomyl and BENLATE cultures showed significantly higher frequencies of white *cyh^r* colonies than the YC control culture. The genotypes of some of the white *cyh^r* colonies was determined (Table A2.3) and at least 70% of them were also *leu⁻trp⁻met⁻* indicating they were monosomic for chromosome VII.

It is possible that *cyh^r* cells are selected for during growth in the presence of benomyl or BENLATE. However the

TABLE A2.2 Cell viability and frequency of white cyh^r colonies during growth of D6 in the presence of benomyl or BENLATE.

INCUBATION TIME (hours)	CONTROL		BENLATE (50µg/ml)		BENOMYL (25µg/ml)		BENOMYL (50µg/ml)	
	VIABLE CELLS/ml	WHITE cyh ^r /10 ⁵ *1	VIABLE CELLS/ml	WHITE cyh ^r /10 ⁵ *1	VIABLE CELLS/ml	WHITE cyh ^r /10 ⁵ *1	VIABLE CELLS/ml	WHITE cyh ^r /10 ⁵ *1
0	2.46 x10 ⁵	2.8 (7)	2.46 x10 ⁵	11.0 (27)	2.46 x10 ⁵	11.4 (28)	2.46 x10 ⁵	4.1 (10)
1.5	4.9 x10 ⁵	NT	2.4 x10 ⁵	11.5 (28)	2.5 x10 ⁵	11.6 (29)	NT	NT
3	7.9 x10 ⁵	NT	2.2 x10 ⁵	12.4 (27)	2.5 x10 ⁵	9.2 (23)	NT	NT
6	6.0 x10 ⁶	2.5 (3)	7.0 x10 ⁵	75.7 (14)	NT	NT	NT	NT
72	2.5 x10 ⁸	4.0 (3)	1.3 x10 ⁷	128.2 (50)	4.4 x10 ⁷	26.7 (39)	2.1 x10 ⁷	50.2 (31)

NT = not tested.

*1 figure in parentheses is the number of white cyh^r colonies scored.

TABLE A2.3 Genotypes of white *cyh^r* colonies of D6 after
various treatments with benomyl or BENILATE.

GENOTYPE	NUMBER OF COLONIES			
	CONTROL	50µg/ml BENILATE	25µg/ml BENOMYL	50µg/ml BENOMYL
TOTAL SCORED	7	45	39	31
met ⁻ trp ⁻ leu ⁻	7	32	32	24
met ⁻ trp ⁻ +	0	0	0	0
met ⁻ + +	0	2	1	0
+ + +	0	11	6	7

observation of a sevenfold increase in the frequency of white cyh^r colonies from 0 to 6 hours in the 50 μ g/ml BENLATE culture, a time period during which the viable cell titre only increased by a factor of X3, would suggest that induction of non-disjunction was occurring.

In summary, the results obtained with the D5 and D6 diploid strains show that benomyl, or its readily available commercial product BENLATE, can be used to induce haploidisation in S.cerevisiae with a high frequency. The observation that these two compounds also cause other, undefined, genetic events in D5 indicate that, both in the laboratory and in the field, caution should be taken when handling them. It has recently been reported in fact that MBC, the direct breakdown product of benomyl, interferes with nuclear division in mammalian cells both in vitro and in vivo (Seiler 1975).

Appendix 3

Heterokaryon Studies

The recent finding of a mutant of yeast that is defective in fusion of nuclei after conjugation (Conde and Fink 1976) has provided new tools, the heterokaryon and heteroplasmons, for the genetic analysis of yeast. This mutant (kar1) was used to:

- (a) confirm that the psi⁺ phenotype is inherited independently of the nucleus, and
- (b) to look for any nuclear restriction of the SUQ5-coded ochre suppressor tRNA.

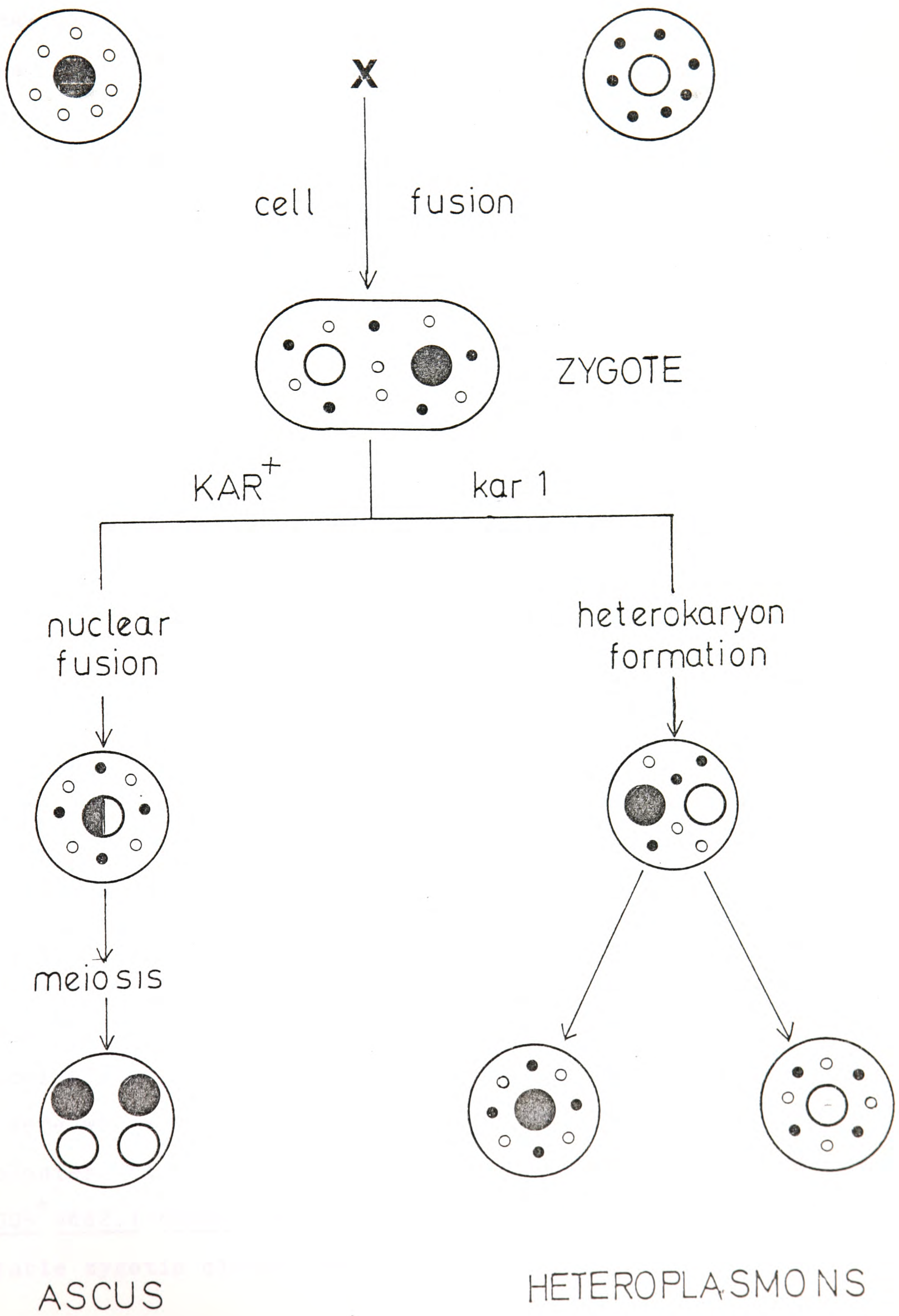
All kar1 strains used in these studies were derived from JC8 (a leu1 kar1 psi⁺) obtained from Dr. G. Fink (Cornell, USA).

(1) Cytoplasmic Inheritance of the psi⁺ phenotype

Direct confirmation of the cytoplasmic location of a putative extrachromosomal element has been obtained in several fungi by the 'heterokaryon test' (Jinks 1958). This involves combining two parent strains, one of which carries the putative extrachromosomal marker, in a heterokaryon so the two types of nuclei are present in a common cytoplasm. The heterokaryon is then resolved into its component homokaryons (= heteroplasmons) which are identifiable by differences in nuclear gene markers. If the heteroplasmons still show the original differences of the two parent strains this demonstrates that the putative mutant extrachromosomal phenotype is coded for by determinants which are bound to, or inside, the nuclei. If, on the other hand, the two heteroplasmons are alike, except for their nuclear gene markers, this demonstrates that the

FIGURE A3.1 A schematic representation of the normal sexual cycle of yeast ($\underline{\text{KAR}}^+$) and the consequence of the kar1 mutation being present in one or both of the haploid parents.

- (●) haploid nucleus, α mating type.
- (○) haploid nucleus, a mating type
- (◐) diploid nucleus, α/a
- (●,○) cytoplasmic components from one or other of the haploid parents.



difference lay in the cytoplasm of the 2 parents (Figure A3.1).

During normal mating between α and a mating type cells occasional transient heterokaryons are formed which rapidly break down into their haploid components with a mixed cytoplasm (= cytoductants). This abortive mating process, termed 'cytoduction' (Zakharov et al 1969), occurs at a very low frequency ($< 1\%$) but has been used to demonstrate the cytoplasmic location of the rho factor and associated antibiotic resistance markers (Wright and Lederberg 1957, Zakharov and Stepanova 1977, Zakharov and Yarovoy 1977) and the $[\overline{\text{killer}}]$ factor (Spondina and Kozhina 1977).

A strain carrying the kar1 mutation is defective in karyogamy (nuclear fusion during conjugation) and forms heterokaryons at a very high frequency (Conde and Fink 1976) which breakdown, under non-selective conditions, to give heteroplasmons. It therefore shows a high frequency of "cytoduction". A strain carrying the kar1 mutation was used to demonstrate that the psi⁺ phenotype was inherited independantly of the nucleus by the classical 'heterokaryon test' of Jinks (1958) (Figure A3.1).

A kar1 strain MT152/1d (α ade2.1 his5.2 can1.100 leu1 kar1 psi⁺ K⁻) was constructed (see Chapter 7 Section 7.4 for details) and crossed to the strain 468/1a (a SUQ5 ade2.1 his5.2 ura3.1 psi⁻ K⁺) . 16 viable zygotic clones were isolated (out of 20 picked from mating-mix); 3 were red, 5 were white and 8 were a mixture of red, pink and white colonies. A control cross between 468/1a and 465/2a (α SUQ5⁺ ade2.1 his5.2 can1.100 ura3.1 psi⁺ KAR⁺) yielded 12 viable zygotic clones out of 12 zygotes isolated by micro-

manipulation. All were stable, suppressed clones.

Two of the unstable zygotic clones, the three red zygotic clones and 1 of the 5 white zygotic clones from the MT152/1d x 468/1a cross, were each suspended in saline and replated onto YC (Table A3.1).

(i) Red Zygote Clones

They both gave only stable red colonies. 20 individual colonies arising from each of the clones were crossed to an a ade1 tester strain. All 40 showed very weak complementation. All 40 colonies were found to be $ade^-his^-leu^-URA^+$ suggesting each clone contained only cells of the MT152/1d strain. 2 red colonies from each clone were crossed to 468/1a and individual zygotes picked- all 4 gave at least one red/pink/white zygotic colony confirming the presence of the kar1 mutation in these strains.

(ii) White Zygote Clones

All 20 colonies from 1 white zygotic clone replated were stable for the suppressed phenotype and were $ADE^+HIS^+LEU^+URA^+$. The one colony tested sporulated. This appeared, therefore, to be a true diploid clone. 3 of the original 5 white zygotic colonies were unable to sporulate and were ura^- . They were, therefore, 468/1a heteroplasmons that were psi⁺.

(iii) Red/White/Pink Zygote Clones

All white colonies scored from the 2 zygote clones replated were stable for the suppressed phenotype. 20 and 31 colonies, respectively, were tested and found to be $ADE^+HIS^+ura^-LEU^+$ and of mating type a. They were all, therefore psi⁺ 468/1a heteroplasmons. This was confirmed by crossing 8 of these heteroplasmons (4 from each clone) to 468/1d

TABLE A3.1 Phenotypes of cells present in zygotic clones, arising from the cross
468/1a and MT152/1d (kar1 x KAR⁺), replated onto YC.

PHENOTYPE OF ZYGOTIC CLONE	TOTAL COLONIES SCORED	NO. OF COLONIES WITH THE			% WHITE COLONIES
		GIVEN PHENOTYPES			
		RED	WHITE	MIXTURE RED/PINK/WHITE	
RED - 1	619	619	0	0	0
RED - 2	677	677	0	0	0
WHITE - 1	20	0	20	0	100
RED/PINK/WHITE - 1	281	200	50	31	17.8
RED/PINK/WHITE - 2	338	245	64	29	18.9

($\propto$ SUQ5 ade2.1 psi⁻) and to 465/4d ($\propto$ SUQ5⁺ ade2.1 psi⁺).

All gave white suppressed diploids with both testers. 6-10 tetrads were dissected from crosses to two of the 468/1a heteroplasmons (one from each clone). In crosses to 468/1a both diploids segregated only suppressed spore progeny (totals of 29 suppressed: 0 non-suppressed and 22 suppressed: 0 non-suppressed spores respectively). In crosses to 465/4d only tetrads with 2 suppressed and 2 non-suppressed spores were obtained. Totals were 20:18 and 19:20 respectively.

One of the 468/1a psi⁺ heteroplasmons was also crossed to 483/1a ($\propto$ SUQ5 ade2.1 PNM psi⁻ strain) and, from the non-suppressed diploids obtained, 10 tetrads were dissected (92.5% spore viability) all containing only non-suppressed spores. Thus the 468/1a heteroplasmons, from the two mixed zygotic clones tested, had a normal psi⁺ phenotype ie. psi⁺ was dominant in crosses to a psi⁻ strain and efficiently eliminated in the presence of PNM1.

The finding that all 468/1a heteroplasmons identified were psi⁺ confirms that the psi⁺ phenotype is inherited independantly of the nucleus. This has recently been confirmed in a similar study with a kar1 mutant (Fink and Conde 1977).

Only one of the two parent strains (468/1a) was K⁺. 15 out of the 16 zygotic colonies obtained in crosses to MT152/1d were also K⁺. The one K⁻ clone was found to contain cells of only the kar1 K⁻ parent. The other two kar1 parent "zygotic" colonies obtained were K⁺ indicating that mixing of cytoplasm must have occurred. 19, of the 20 MT152/1d heteroplasmons examined from one zygote clone, were K⁺ confirming that the killer factor is also inherited indep-

endantly of the nucleus. This was also confirmed by Fink and Conde (1977) using the kar1 mutant. The zone of killing of the MT152/1d heteroplasmons was much larger than observed for either the 468/1a parent or the 20 468/1a heteroplasmons tested. This suggests that the efficiency with which K^+ cells kill sensitive cells is influenced by nuclear genetic background.

The heterokaryon colonies from the 468/1a x MT152/1d cross were pink in colour, showed weak suppression of ade2.1, but were $HIS^+LEU^+URA^+$. They were very unstable during growth on YC rapidly breaking down into heteroplasmons. 15 out of 19 heterokaryon colonies tested showed < 10% asci when incubated on FMA for 5-7 days at 28°C. 10 complete tetrads dissected from one such colony showed a 2 suppressed: 2 non-suppressed spore segregation and showed independent assortment of the leu1 and ura3.1 markers (PD=1, NPD=3, TT=6). If the heterokaryon had sporulated one would predict that no such reassortment would take place. These heterokaryons must therefore have contained a high proportion of diploid cells.

(2) Compartmentation of the Suppressor tRNA?

It has been demonstrated that the gene products of 3 super-suppressor loci (ssu1-ssu3) in Neurospora crassa cannot enter the transcription/translation system coded by the other component nucleus in a heterokaryon (Griffiths 1976). Although not conclusively demonstrated, it is reasonable to assume that the ssu loci code for mutant tRNA's, as do yeast 'super suppressors'. Using kar1 mutants 'nuclear restriction' of the SUQ5 gene product, which is known to code for a mutant tRNA (Capecci et al 1975), was looked for.

The following two strains were constructed:

MT177/4c α ade2.1 his5.2 + ura3.1 + kar1 psi⁺

MT160/4a a ade2.40 + leu1 + SUQ5 kar1 psi⁺

If the SUQ5-coded tRNA is 'nuclear restricted' in a heterokaryon the ade2.1, in a different nucleus from SUQ5, should remain unsuppressed giving a red ade⁻HIS⁺LEU⁺URA⁺ heterokaryon because the ade2.40 is a non-suppressible allele of the ADE2⁺ gene and fails to complement ade2.1. If no nuclear restriction occurred then the heterokaryon should be suppressed (ADE⁺HIS⁺LEU⁺URA⁺).

Log phase cells of both strains were mixed together on YC and, after 24 hours incubation at 28°C to allow mating to occur, a saline suspension of the mating-mix was prepared and plated onto YC, to determine the viable cell titre, and onto YNB, YNB + ade, YNB + his and YNB + ade + his. Colonies were counted for the YC plates after 3 days incubation and for the YNB plates after 14 days incubation at 28°C (Table A3.2). No colonies were found on the YNB with no supplements, nor YNB + his, even though 10⁵ cells were plated. This indicated that no suppressed heterokaryons were present in the mating-mix. However any diploids formed should grow on all YNB plates. kar x kar crosses rarely give diploid cells (Conde and Fink 1976). No suppressed colonies were found on the YC plates out of 1882 colonies screened. All 18 of the LEU⁺URA⁺ colonies from this experiment were found to be red on YC and ade⁻HIS⁺LEU⁺URA⁺ with all having many white papillae present. All sporulated well and failed to show any complementation with an a or α ade1 strain. It appeared therefore that MT177/4c x MT160/4a diploid was ade⁻.

6 individual zygotes were also isolated from this cross

TABLE A3.2 Frequency of $\text{URA}^+ \text{LEU}^+$ colonies arising on different selective media from

MT177/4c x MT160/4a.

CELLS PLATED	YNB	YNB + ade	YNB + his	YNB + ade + his
MASS MATING MIX				
No. viable cells plated	2.24×10^5	2.24×10^5	1.12×10^5	2.24×10^5
No. colonies	0	11	0	7
Revertants / 10^5	0	4.91	0	3.12
ZYGOTIC CLONE 1				
No. viable cells plated	14.43×10^5	14.43×10^5	14.43×10^5	14.43×10^5
No. colonies	0	668	0	679
Revertants / 10^5	0	46.29	0	47.05
ZYGOTIC CLONE 2				
No. viable cells plated	1.68×10^5	1.68×10^5	1.68×10^5	1.68×10^5
No. colonies	0	472	0	541
Revertants / 10^5	0	280.95	0	322.02

by micromanipulation, all giving rise to red $\text{ade}^- \text{HIS}^+ \text{LEU}^+$ clones. 2 of the clones were suspended in saline and plated onto YC and selective media as for the mass-mating experiment (see above). Similar results to the mass-mating experiment were obtained (Table A3.2). A total of 94 $\text{LEU}^+ \text{URA}^+$ colonies were picked, all of which were red on YC, many containing large numbers of white papillae. 11 failed to mate with an α or a ade1 tester strain. Only 2 showed any asci following growth on FMA for 7 days at 28°C .

From these results it was apparent that no suppressed heterokaryons were present, but failure to find any suppressed diploids prompted $\text{KAR}^+ \times \text{KAR}^+$ controls to be conducted. The following strains were mated:

D6/3C	<u>a</u>	<u>ade2.40</u>	<u>cyh2</u>	<u>+</u>	<u>+</u>	<u>+</u>	<u>psi</u> ⁻
x							
465/1b	<u>α</u>	<u>ade2.1</u>	<u>+</u>	<u>his5.2</u>	<u>ura3.1</u>	<u>SUQ5</u>	<u>psi</u> ⁺

3 zygotes were isolated by micromanipulation. All gave rise to non-suppressed $\text{ade}^- \text{HIS}^+$ clones with many white papillae (approximately 5-20 per clone) present. By restreaking one of these diploid clones onto YC, a stable white $\text{ADE}^+ \text{HIS}^+$ colony was isolated and, together with a stable red colony, were induced to sporulate. Tetrads were dissected from both haploids (Table A3.3). The red diploid gave overall spore segregation ratios of 3 red: 1 white spores as would be predicted for a SUQ5/+ ade2.1/ade2.40 diploid. The white diploid gave tetrads with an excess of white spores. A second cross:

D6/6d	<u>a</u>	<u>+</u>	<u>ade2.40</u>	<u>his4</u>	<u>+</u>	<u>+</u>	<u>psi</u> ⁻
x							
507/4-2b	<u>α</u>	<u>SUQ5</u>	<u>ade2.1</u>	<u>his5.2</u>	<u>can1.100</u>	<u>ura3.1</u>	<u>psi</u> ⁺

gave a diploid with the red ade^- phenotype and both a red and a white ADE^+ revertant diploid were subjected to

TABLE A3.3 Tetrad analysis of SUQ5/+ ade2.1/ade2.40 diploids and ADE⁺ revertant diploids.

CROSS	DIPLOID PHENOTYPE	TETRAD ANALYSIS		NO. COMPLETE TETRADS	SUPPRESSED:NON-SUPPRESSED					SPORE TOTALS S:NS
		NO. TETRADS DISSECTED	% SPORE VIABILITY		4:0	3:1	2:2	1:3	0:4	
D6/3c	RED									
x 465/1b	ade ⁻ psi ⁺	33	84.8	23	0	0	4	17	2	29:82
D6/3c	WHITE									
x 465/1b	ADE ⁺ psi ⁺	12	89.6	9	1	6	1	1	0	30:13
D6/6d	RED									
x 507-4/2b	ade ⁻ psi ⁺	20	85.0	11	0	0	2	7	0	18:50
D6/6d	WHITE									
x 507-4/2b	ADE ⁺ psi ⁺	9	88.9	5	0	0	5	0	0	15:17
483/6c	RED									
x D6/1d	ade ⁻ psi ⁻	10	97.2	9	0	0	0	0	9	0:35

tetrad analysis with similar results to the previous cross, except the white diploid gave only 2 suppressed: 2 non-suppressed spores in each tetrad (Table A3.3). This confirms that the effects observed were not strain specific.

To see if the psi⁺ phenotype of the diploid had an effect the following cross was made:

483/6c	α	<u>SUQ5</u>	<u>ade2.1</u>	<u>lys1.1</u>	<u>can1.100</u>	<u>psi</u> ⁻
x						
D6/1d	a	±	<u>ade2.40</u>	±	<u>his4</u> <u>cyh2</u>	<u>psi</u> ⁻

All the resulting diploids were red, ade⁻ and showed many white papillae. The red diploid gave only red, ade⁻ spores (Table A3.3) confirming the diploid to be psi⁻.

From this work it is concluded that a diploid of genotype $\frac{\text{SUQ5}}{+} \frac{\text{ade2.1}}{\text{ade2.40}}$ psi⁺ or psi⁻, or a heterokaryon with SUQ5 ade2.40 and SUQ5⁺ ade2.1 nuclei are ade⁻. ade2.1 and ade2.40 are non-complementing alleles of the ade2 locus eg. D6/1d (a ade2.40 his4 cyh2 psi⁻) x 468/4d (α ade2.1 lys1.1 psi⁻) or D6/1d x 518/1d (α ade2.1 his5.2 can1.100 ura3.1 psi⁺) gave only red diploids that were ade⁻ and showed several white papillae. The ade2.40 mutation is recessive in an ade2.40/± diploid. This was demonstrated by crossing D6/3c (α ade2.40 cyh2 psi⁻) to 4 different ADE2⁺ psi⁻ strains (MT215/1c, 5d, 7d, 9c) and 2 different ADE2⁺ psi⁺ strains (MT192/7a, MT192/9b). 2-4 individual zygotes were micro-manipulated from each cross and all gave rise to white ADE⁺ diploids.

The ADE2⁺ gene codes for a multimeric enzyme, phosphoribosylaminoimidazole carboxylase (Silver and Eaton 1969), and interallelic complementation is known to occur between different ade2 alleles confirming this (Woods and Bevan 1966). The failure of the SUQ5/± ade2.1/ade2.40 diploids (or heter-

okaryons) to grow in the absence of exogenously supplied adenine indicates that the ade2.1 suppressed gene product, although showing normal enzyme activity (an SUQ5/+ ade2.1/ade2.1 psi⁺ diploid will grow in the absence of adenine), the gene product must still be 'mutant' in being unable to form a wild type hybrid enzyme in an SUQ5/+ ade2.1/ade2.40 diploid. Because ade2.40 is recessive (see above) the wild type x ade2.40 hybrid enzyme must be a 'wild type' as determined on the basis of growth criteria. One is thus getting dominance of a more defective allele over a less defective, but not wild type, one. When hybrid enzymes are formed they need not always be active even if one monomer would give an^{active} enzyme by itself (Zimmermann et al 1969).

SUQ5 is known to be recessive in certain diploids (F. Sherman, personal communication to B.S. Cox) but when MT160/4a (a SUQ5 ade2.40 kar1 leu1 psi⁺) was crossed to 507-4/2b (α SUQ5 ade2.1 his5.2 can1.100 ura3.1 psi⁺) all 3 zygotic colonies were found to contain only red ade⁻ cells ie. even when SUQ5 is homozygous in an ade2.1/ade2.40 psi⁺ diploid, it has no effect on adenine requirement.

The white papillae observed on colonies of all ade2.1/ade2.40 diploids were due to mitotic recombination occurring between the ade2.1 and ade2.40 at the 2 strand (D6/6d x 507-4/2b) or 4 strand (D6/3c x 465/1b) stage (see Table A3.3).

These experiments therefore demonstrate that a different non-suppressible allele of ade2, to ade2.40, must be used to study the compartmentation of the SUQ5 gene product in the heterokaryon system first described in N.crassa by Griffiths(1976).

(3) Summary and Conclusion

The inheritance of the psi⁺ phenotype has been shown to be truly cytoplasmic by the classical 'heterokaryon test' first described by Jinks (1958). This was made possible in yeast, a fungus that does not have heterokaryosis as part of its normal life cycle, by the kar1 mutant isolated by Conde and Fink (1976). When a kar1 strain is crossed to a kar1 or KAR⁺ strain the zygote gives rise to both heterokaryons and heteroplasmons at a high frequency. For the strains used in this study the efficiency with which the psi⁺ phenotype was found in heteroplasmons was 100%. Livingston (1977) has recently reported that [2μ] DNA does not transfer with 100% efficiency to heteroplasmons distinguishing it from psi and from the [killer] -associated dsRNA which transfer with 100% efficiency (this study, Fink and Conde 1977). The psi⁺ phenotype of heteroplasmons was found to be normal with respect to its elimination by the PNM1 mutation and its dominance in crosses to psi⁻ strains.

A heterokaryon test, first described by Griffiths (1976) for looking at compartmentation of the super suppressor loci(ssu) gene products in N.crassa was set up, using a kar1 x kar1 cross, to test for nuclear restriction of the SUQ5-coded suppressor tRNA. Although preliminary results indicated that the tRNA might be nuclear restricted, the construction of control diploid strains (KAR⁺ x KAR⁺) demonstrated that the suppressed ade2.1 gene product fails to give a wild type 'hybrid' enzyme when paired with the ade2.40 gene product in a diploid. The failure of an SUQ5 ade2.40/SUQ5⁺ ade2.1 heterokaryon to grow in the absence of adenine was presumed to be a consequence of this. The [psi] phenotype was shown to have no effect on this phenomenon in diploids.

Another, non-suppressible allele of ade2 (eg. ade2.119) will be required to look for compartmentation of suppressor tRNA in yeast. The SUQ5 mutation was found to be dominant in heterokaryons with the genotype SUQ5 ade2.1/SUQ5⁺ ade2.1.

CORRECTIONS

- p2, line27. mitechondrien
- p8, line19. indirectly
- p50, line25. glucose
- p80, line23. should
- p129, line27. was
- p157, line15. induction
- p174, line18. acriflavine
- p196, line18. add "determined" to end of sentence
- p218, line25. phenotype
- p219, line22. drug" should be "agent"
- p219, line24. should read "induction of mutants in media
in which...."
- p239, last line. phenotype should read genotype
- p275, line10. breakdown should read breakthrough
- p374, line22. changes

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