A STUDY OF REPLICATING INSTABILITIES IN *SCHIZOSACCHAROMYCES POMBE*

BY

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DECLARATION

This thesis was composed by myself. The work described is my own, except where otherwise stated in the Aknowledgements

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This work is dedicated, with love and gratitude, to my family.

ABSTRACT

In this study the first physical evidence as to the nature of the products of a replicating instability in *Schizosaccharomyces pombe* has been obtained. It was shown that there is significant restriction fragment heterology between mutant and non-mutant derivatives of a replicating instability giving repeated mutation at the *ade1* locus. A number of ideas as to how these results could have been obtained were discussed and ways of testing them suggested. Strictly speaking the suggestions are pertinant only to the *ade1* replicating instability isolated in this study. To discover if these ideas are applicable to replicating instabilities in general it would be necessary to isolate and characterise a number of other replicating instabilities. Such analysis would have, initially, to focus on the *ade1* locus as this is the best characterised gene in the adenine biosynthetic pathway of *S. pombe*. Eventually, however, studies should be extended to other loci.

This study began with an analysis of the efficiency with which replating identified *bona fide* replicating instabilities. It was concluded that replating primary mosaics over-estimated the frequency of secondary mosaics, and that the technique did not distinguish between genuine and spurious mosaics with sufficient clarity to warrant the molecular analysis of apparently unstable strains isolated in this manner. As much of the work published in the past regarding the nature of replicating instabilities in *S. pombe* has been based on identifying replicating instabilities by replating mosaic colonies, it is perhaps not surprising that the conclusions reached seem frequently to have been contradictory and confusing.

Single cell isolation was used to isolate genuine replicating instabilities, the overall frequency of secondary mosaicism observed was 2.2×10^{-3} (11/5,109) this agrees with the frequency reported by Nasim and James (1971) of 1.7×10^{-3} (10/5,816). However, unlike Nasim and James (1971) who found that 6/8 mosaics tested gave rise to subsequent mosaic colonies it was found here that only 6/24 primary mosaics gave rise to secondary mosaics. In terms of the number of secondary mosaics observed, however, both studies are small scale. It would be necessary to carry out a more extensive analysis before the proportion of primary mosaics which are, in fact, replicating instabilities could be predicted confidently.

Three of the replicating instabilities isolated were tested for diploidy by comparing the levels of azygotic asci observed in crosses using derivatives of the unstable strain with those seen in control crosses. No evidence was found that the unstable lines exhibited a significantly elevated level of azygotic asci compared to the controls. Thus the trivial explanation of heteroallelic diploidy for the unstable phenotype could be ruled out.

This was subsequently confirmed for one of the three replicating instabilities when preliminary molecular analysis revealed evidence of complex changes at the *ade1* locus. The analysis was carried out by Southern hybridisation to restriction digests of total genomic DNA samples from 6 derivative colonies of the original *ade1* replicating instability (two red, two white *ade1* mutants and two mosaics). The probe used contained the coding sequence of the *ade1* gene. Southern analysis revealed significant restriction fragment heterology between the DNA samples. The *ade1* mutants appeared to give essentially identical restriction patterns but the samples of DNA from the stable red and mosaic colonies revealed complex differences making interpretation the results ambiguous. Southern hybridisation revealed no evidence for a duplication of the coding sequence of the *ade1* gene, thus this class of molecular explanation can be ruled out as the explanation in this case.

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

Since the discovery of the mutagenic properties of some physical (Muller, 1930) and chemical (Auerbach, 1943; Auerbach and Robson, 1944) agents, one of the most intriguing puzzles has been the ability of some mutagens to produce delayed mutation (Timofeeff-Ressovsky, 1931). Data obtained from the UV irradiation of pollen grains (Stadler, 1939) and the mustard gas treatment of *Drosophila* (Auerbach, 1946; Auerbach and Robson, 1946) showed that a high proportion of mosaics were obtained after mutagenesis, implying a delayed effect of the treatment. This effect was not due to carry over of the mutagen (Auerbach and Robson, 1946). The generation of mosaics by mutagens is now known to be largely attributable to misreplication or misrepair of the primary DNA lesions (Auerbach and Kilbey, 1971; Auerbach, 1976; Kilbey, 1984).

However there is a class of delayed mutation for which the molecular basis remains obscure; these mutations are observed to have an induced tendency for repeated delayed mutation at a specific locus or at least in a very narrow region of the chromosome over several generations (Auerbach, 1947). A central observation about these mutants is that the tendency for delayed mutation is heritable, in other words individuals carrying this type of mutation can pass them on to more than one of their progeny. Thus the premutational lesion must be capable of replicating as such as well as giving rise to stable mutant and non-mutant derivatives (Auerbach, 1947). To underline the heritability of the propensity for delayed mutation, mutants falling into this class have been termed "replicating instabilities" (Nasim, 1967).

A replicating instability can be viewed as an induced form of genetic instability. Spontaneous genetic instabilities are, of course, well known, the first to be observed being controlling elements in maize (McClintock, 1956; Fedoroff, 1983). Another early observation of genetic instability was hybrid dysgenesis in *Drosophila*, where some matings between strains were found to result in low offspring viability, a high spontaneous mutation rate and a tendency for chromosome breakage in germ-line cells (reviewed by Bregliano and Kidwell, 1983). Spontaneous unstable mutations in both prokaryotes and eukaryotes have often been attributable to transposable elements (Roeder *et al*, 1980); these elements have been well characterised and the mechanism by which they can generate unstable mutations is well understood. All aspects

relating to transposable elements such as mechanisms of transposition etc. have been extensively reviewed (Iida *et al*, 1983; Kleckner, 1981, 1983; Roeder and Fink, 1983; Rubin, 1983).

Chromosomal aberrations including aneuploidy and translocations have also been shown to give rise to genetic instabilities. Aneuploids of *Aspergillus nidulans*, probably produced by non-disjunction during diploid mitosis, tend spontaneously to give sectors of more vigorous growth, analysis of these sectors showed that euploidy had been restored (Kafer, 1960). Similarly, duplicated chromosome segments have been shown to cause genetic instability in which loss of one of the duplicates during mitosis gives rise to sectors of improved growth and near normal morphology (the duplication being characterised by abnormal morphology and poor colonial growth). Duplications of this kind have been observed in *A. nidulans* (Bainbridge and Roper, 1966) and *Neurospora crassa* (Turner, 1977; Newmeyer and Taylor, 1967) and it is well established that they are due to chromosomal rearrangements.

The examples of unstable behaviour listed above are either the results of unequal exchanges or non-disjunction at mitosis or meiosis or are the result of specific sequences of DNA. There are also examples where the unstable behaviour of segments of DNA represent a switching mechanism by which differentiation can be achieved (reviewed by Fincham, 1983 chapter 17). Examples in this category include host-range switching by G-segment inversion in bacteriophage Mu (van de Putte *et al*, 1980), host-range switching in T-even phages by recombination between homologous genes (Riede, 1986), and phase variation in *Salmonella* by segmental inversion (reviewed by Silverman and Simon, 1983). Eukaryotic cellular differentiation has also been shown in some cases to be due to genetic instability, the best characterised example is probably mating-type switching in yeast due to copy transposition of mating type information from storage loci to an expression locus (Haber, 1983; Egel and Gutz, 1981). Also well documented are the genomic rearrangements involved in the generation of antibody diversity (Lewin, 1986).

In summary, there are many well documented examples of genetic instability some of which are both locus specific and heritable (for example reversion in mutations caused by transposable elements). Replicating instabilities can be distinguished from all the above by the fact that the tendency for locus specific mutation is induced rather than being spontaneous.

This does not, of itself, preclude replicating instabilities from arising spontaneously but underlines the fact that mutatagenic treatment greatly enhances the frequency with which they occur.

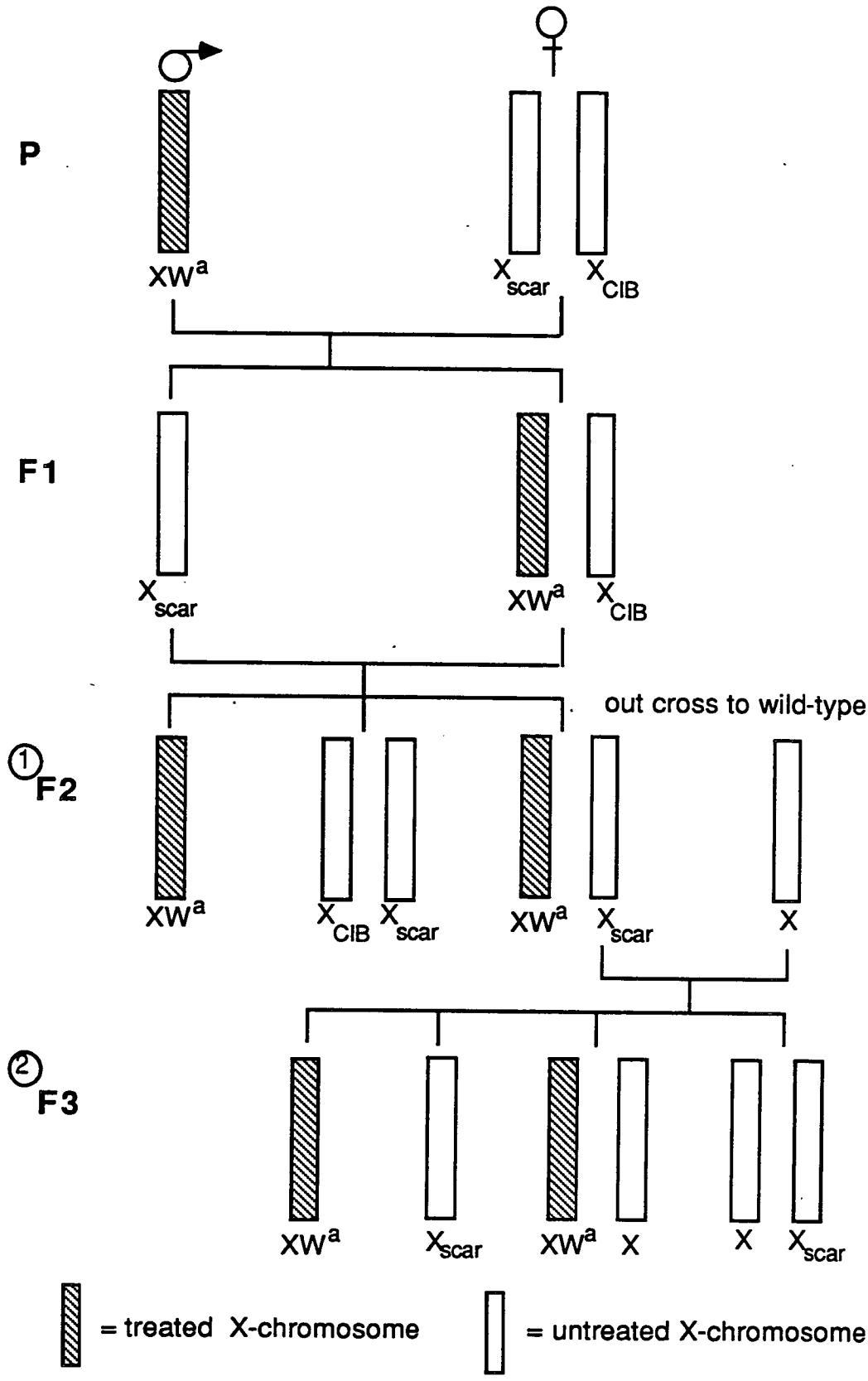
A class of genetic instability which can be mutagenically induced are the so called "mutator genes", these can enhance mutation frequencies by up to 10^5, depending on the gene affected. The term "mutator gene" covers a number of different categories of mutations including mutations in DNA polymerases leading to decreased fidelity of replication, mutations at other loci which also act at replication to decrease the overall fidelity of replication and mutations in enzymes normally involved in the error free repair of DNA lesions. Mutator genes gave been described in a number of systems including yeasts (Munz, 1975; Phipps *et al*, 1985) but are best charaterized in *Escherichia coli* (Cox, 1976). Although mutator genes can be specific for the type of mutation induced for example *MutT* causes mainly AT to GC transversions, they are not locus specific. Because replicating instabilities are reported to be highly locus specific (Auerbach, 1947; Nasim and Grant, 1973) it seems unlikely that they can be caused by mutator genes.

It appears, therefore, that replicating instabilities do indeed represent a distinct class of mutational event which cannot be easily included in any of the categories considered above for which a molecular explanation exists. Although there are some analogies between replicating instabilities and some of the other classes of gene instability listed above none of them fully described the observed phenotype. Compared to many other areas the phenomenon of replicating instability has been rather neglected, having only been studied in any depth in two organisms, *Drosophila* (Auerbach, 1947) and *Schizosaccharomyces pombe* (Nasim, 1967).

1.1 OBSERVATIONS OF REPLICATING INSTABILITIES IN *DROSOPHILA*

Replicating instabilities were first observed in *Drosophila* by Auerbach (1947); in this classic paper several observations were made which are central to theories on the molecular nature of replicating instabilities and so it is worth considering this paper in some detail.

The paper covers two areas both of relevance to the present study, in the



Notes ①: females chosen from cultures containing some W^a males

② : a lethal arising in the F1 becomes manifest as an absence of W^a males in the F3.

Figure 1.1. Mating scheme used to test for mutagenesis arising in the germ track of daughters or treated male *Drosophila* (After Auerbach, 1947.)

first part of the paper the frequency with which sex-linked lethals arise in the F_3 of mutagen treated males, this has bearing on the question of the delayed action of mutagen treatments. The mutagen used by Auerbach was mustard gas. The experimental procedure used was as follows (see also figure 1.1), males carrying X-chromosomes, marked with w^a amongst other markers, were subjected to mutagenic treatment and mass mated to females with individually marked X-chromosomes (X_{CIB}/X_{Scar}). The X_{CIB} carries a long inversion covering virtually the whole of the X-chromosome and so in females receiving this chromosome from their mothers crossing-over is prevented and they will pass the treated X-chromosome on to the next generation intact. F_1 females of the genotype X^*/X_{CIB} (where X^* is the treated X-chromosome) were crossed in pair-wise matings to brother X_{Scar} . Females from F_2 cultures where there were at least some males carrying the treated X-chromosome (distinguished by w^a eye colour) were mated to wild-type males and the frequency of sex-linked lethals in the F_3 scored as an absence of w^a males. It was found that the frequency of sex-linked lethals in the F_3 of mustard gas treated males was more than 10X higher than the controls. This excess of sex-linked lethals in the F_3 is proof of a delayed effect of the mutagenic treatment, the very earliest that these mutations could have arisen was in the germ track of the F_1 (the F_1 female must have been a gonadic mosaic for the lethal trait as she had some sons with the treated X-chromosome). The high mutation frequency in the F_3 could be traced back to the presence of a few F_1 females which were gonadic mosaics for the lethal trait, this effectively eliminates carry over of the mutagen as being the explanation for the observed results as this would be expected to give rise to a high level of mosaics in the F_1 .

The second part of the paper (Auerbach, 1947) deals with gonadic mosaics as a basis for spurious "semi-lethality" and it is here that the first observations on replicating instabilities are reported. A sex-linked semi-lethal is a recessive mutation which so drastically impairs the viability of males carrying the mutated X-chromosome that only a few emerge as adult flies. However, as Auerbach points out, a female which is a gonadic mosaic for a full lethal will appear to be carrying a semi-lethal, since only some of her male progeny will receive the normal X-chromosome the remainder will have the full lethal and die. A gonadic mosaic for a trait will be heterozygous with respect to the trait in part of her ovary and homozygous either for the trait or its normal allele in the rest. It is possible to distinguish between the two situations, semi-lethality

TABLE 1.1[1]

The frequency of gonadic mosaics among daughters of gonadic mosaics
(The mothers were all daughters of chemically treated males)

F_1 Female	Number of daughters examined	Genotypes of daughters (F_2)			Genotypes of daughters of F_2 mosaics (F_3)	
		Heterozygous for lethal	No Lethal	Gonadic mosaic for lethal	Heterozygous for lethal	No Lethal
A	33	29	4	0	-	-
B	18	15	2	1	1	1
C	21	17	4	0	-	-
D	9	4	5	0	-	-
E	18	14	3	1	8	2
F	9	6	3	0	-	-
G	18	10	8	0	-	-
H	16	14	2	0	-	-
I	18	6	10	2	32	4
J	16	10	4	2	8	3
K	16	14	2	0	-	-
TOTALS	192	139	47	6	49	10

[1] Taken from Auerbach, 1947.

and mosaicism, in the F_2 of such females as any surviving sons from a female mosaic for a full lethal will carry a normal X-chromosome and so will give a normal sex ratio when mated to attached X females. However, male progeny from a semi-lethal will give rise to a low sex ratio when mated to attached X females. Using this analysis Auerbach showed that 9 out of 20 apparent semi-lethals which arose from mustard gas treatment were in fact gonadic mosaics for full lethals. This figure compared to 1 in 15 X-ray induced "semi-lethals".

A female which is a gonadic mosaic is in general expected to have two types of daughter either without a lethal, that is having a normal number of sons carrying the paternally derived, treated X-chromosome or heterozygous for a lethal, having no sons with the treated chromosome. It was observed, however, that four of the females found to be gonadic mosaics produced one or more daughters which also behaved as gonadic mosaics. The rest of the daughters were true breeding. As Auerbach points out;

> "The crucial point for the interpretation of this apparent transmission of mosaicism is the following; were mother and daughter gonadic mosaics for the same mutation, or was mosaicism in the daughter due to spontaneous origin of a new mutation?".

This could only be tested for three of the four F_2 mosaics and in each case it was found that the mutation was locus specific. These results are summarized in table 1.1 (taken from Auerbach, 1947).

In summary:

1. Analysis of sex-linked lethals in the F_3 of treated males revealed that the mutation frequency was at least 10X higher than the spontaneous mutation frequency confirming a delayed effect of the mutagenic treatment. This high frequency of sex-linked lethals was shown not to be due to carry over of the mutagen.
2. An analysis of apparent semi-lethals showed that for chemical treatment about half of the semi-lethals could be attributed to mosaics for full lethals.
3. Of the females carrying semi-lethals shown to be gonadic mosaics four gave rise to one or more daughters which were also gonadic mosaics.
4. Where these could be tested, the daughters were found to be mosaics for the same trait as their mothers. This test shows that the mosaics were replicating instabilities rather than spontaneously arising mutants due to mutator genes.
5. Once a lethal had established itself in the sister or daughter of a female carrying a mosaic, then it is stable in subsequent generations of the same line.

After Auerbach's initial observations some subsequent work on replicating

instabilities was carried out in *Drosophila*. This included tests on the effects of CEMS (chloroethyl methanesulphonate) on the production of sex-linked lethals, in every case mosaic females in the F_1 were found to give mosaics amongst their daughters. The lethals appearing in the F_3 females were mapped and traced back to the F_1 from which they had arisen, in every case the F_3 lethals from an F_1 mosaic were clustered in the same region of the X-chromosome. Different F_1 mosaics were found to have lethals in completely different sites (Mathew, 1964).

Mosaics were also produced by feeding males with calf-thymus DNA. When these were tested it was found that both F_2 and F_3 mosaics were found; in this experiment the autosomes as well as X chromosomes were examined for lethals. Lethals of chromosome II were subjected to location tests and a high incidence of allelism was found. In this work the pattern of allelism was suggestive of a set of over lapping deficiencies rather than being identical mutations (Mathew, 1965). Heliotrine also induced mosaic lethals in the F_1, out of 20 mosaics tested 7 produced further mosaics (Brink, 1966).

These findings confirmed and extended Auerbach's initial findings, demonstrating that replicating instabilities are not a peculiarity of mustard gas but applied more generally to alkylating agents as well as calf-thymus DNA.

1.2 REPLICATING INSTABILITIES IN OTHER SYSTEMS

Following on the work carried out in *Drosophila* there were a number of reports of replicating instabilities or a similiar phenomenon in a variety of organisms. Some of these reports such as the reversion instability observed in *gal* mutants of *E. coli* (Morse and Pollock, 1969) and the spontaneous and UV-induced unstable revertants from tryptophan auxtrophy in *E. coli* (Hill, 1963) were probably not genuine replicating instabilities but may well be explained by other events such as transposition. However at least two reports of unstable behaviour do appear to have all the hallmarks of a replicating instability. It was reported (Mathew, 1965a) that a high proportion of recessive lethal mutants observed in *Neurospora* using the Atwood recessive lethal test and mating after treating conidia with the alkylating agent CEMS were mosaics for lethal traits. Plating conidia from apparently non-lethal G2 heterokaryon culture revealed mosaics for lethals in the G3, in many cases a mosaic colony

generated several mosaic progeny. However the lethals could not be located so the identity of the mutations in the successive generations could not be established. A report of gene unstabilization in *E. coli* using either heat or nitrous acid (Zamenhof, 1961) also appears to have the hallmarks of a replicating instability. In this report unstable *lac*- mutants were isolated after treatment by plating on tetrazolium-lactose agar, where the mutant phenotype is is visualised by red colouration due to reduction of the tetrazolium dye. It was found that restreaking these red sectors gave one of two results either all the colonies were red (the mutant was stable) or that a mixture of colourless (stable wild-type), red and mosaic colonies were obtained. It was found that when sectored colonies were cultured in broth that eliminated *lac*- cells and then replated on tetrazolium-agar, sectored colonies were still seen. Thus the mutagenic treatment had induced in the *lac+* cells the potential to become *lac*- at a high frequency.

1.3 REPLICATING INSTABILITIES IN *SCHIZOSACCHAROMYCES POMBE*

Most of the work on replicating instabilities has been done in *S. pombe*. This organism is particularly suited to the study of replicating instabilities for two reasons: firstly, the classical and more recently the molecular genetics are well developed and secondly, mutations at some of the loci in the adenine biosynthetic pathway give rise to pigmented colonies which enables mutations in this pathway to be selected by inspection.

1.3.1 *S. POMBE* GENETICS

The fission yeast *Schizosaccharomyces pombe* Lidner has a haplontic life cycle, vegetative cells are haploid. When cells of compatible mating types are grown together, strong sexual agglutination occurs at the end of vegetative growth. The resulting zygotes give rise to four haploid ascospores which form within the cell wall of the original zygote. The *S. pombe* strains used in genetic research were isolated from a culture of *S. pombe* Lidner strain *liquiefaciens* (Leupold, 1950). Heterothallic strains representing two opposite mating types, *h+* and *h*- and a homothallic, self-fertile strain were isolated. The homothallic strain, *h90* is cross-fertile with the heterothallic + and - strains (Gutz *et al*, 1974). The life cycle of *S. pombe* is depicted in figure 1.2.

The life cycle, known gene loci, chromosome maps, processes of mutation and repair and

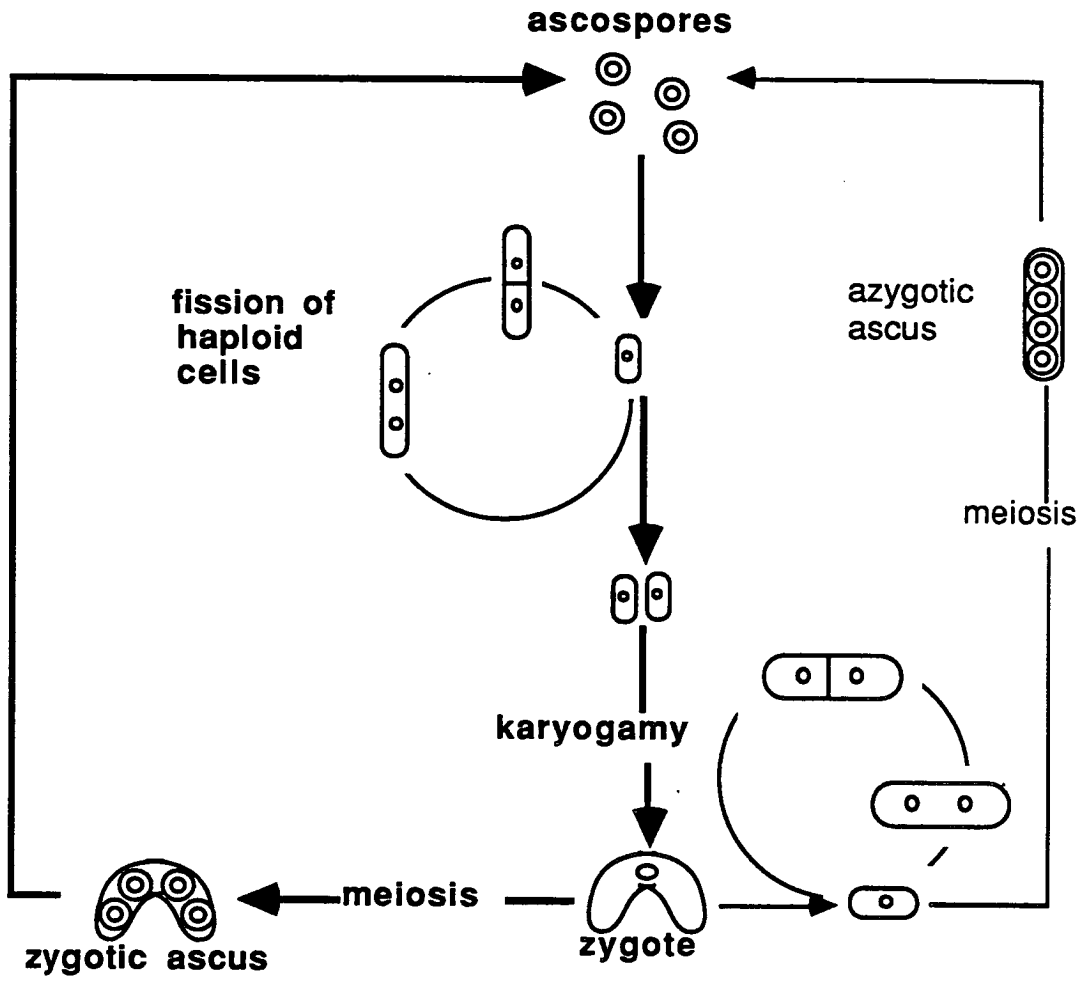
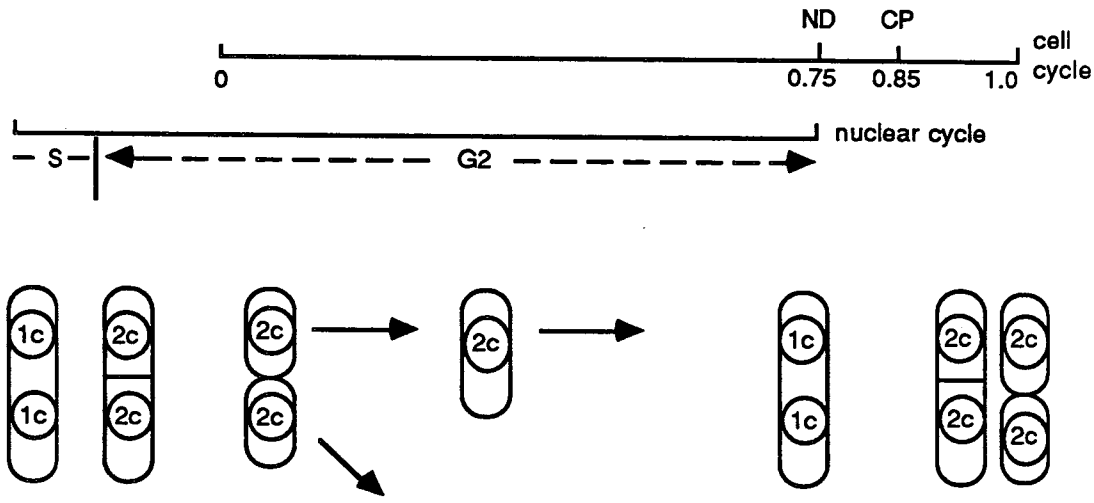


Figure 1.2. Life cycle of *S. pombe* . (From Gutz *et al.* , 1974)



(ND) Nuclear division; (CP) the first appearance of the cell plate.

Figure 1.3. The cell and nuclear cycles of *S. pombe.*
(From Mitchison, 1970)

the biochemical genetics of *S. pombe* have been reviewed by Gutz *et al.* (1974). An up-dated review of the genetics of *S. pombe* including mating types, mitotic cell cycle, informational supression and tRNAs, recombination and mitochondrial genetics was presented by Egel *et al.* (1980). The chromosome map of *S. pombe* has been revised by Gygax and Thuriaux (1984) and recovery with a recent minor amendment by Lehmann and Munz (1987), repair and mutagenesis have been reviewed by Phipps *et al.* (1985).

Amongst recent advances in *S. pombe* genetics one of the most notable is a detailed understanding of mating type switching, which occurs by a mechanism broadly analogous to that in *Saccharomyces cerevisiae* (Hicks *et al*, 1979; Klar *et al*, 1980). Switching is achieved by copy transposition of DNA from two silent storage loci *Mat2-P* and *Mat3-M* to an expression locus, *Mat1* (Beach, 1983; Egel and Gutz, 1981; Egel, 1984).

The *S. pombe* cell cycle is considered to be more typically eukaryotic than that of *S. cerevisiae*, having a distinct G2 phase and visible condensation of the three chromosomes during mitosis (Russel and Nurse, 1986). Because of this a great deal of attention has been focused on the the control of the cell cycle and the stages at which it becomes committed to carry out a particular programme, for example mitosis as opposed to sexual agglutination and meiosis. Work in this field is well advanced and many of the genes involved in the cell cycle have been identified and are currently being characterized (Nurse, 1985; Aves *et al*, 1985; Hindley and Phear, 1984; Gordon and Fantes, 1986; Fantes, 1983; Beach *et al*, 1982).

The nuclear cycle of *S. pombe* is unusual in having a very short G1 phase with DNA synthesis following rapidly after mitosis in exponentially growing cells, as a result the nucleus spends 90% of the time with a 2C quantity of DNA (Mitchison, 1970). The cell and nuclear cycles are shown schematically in figure 1.3.

Genetic and biochemical evidence suggests only a weak relationship between *S. pombe* and *S. cerevisiae*, the sequence of cytochrome c and the organisation of the pathway for tryptophan biosynthesis indicating a closer relationship to *Neurospora crassa* than to *S. cerevisiae* (Gutz *et al*, 1980).

OPPOSITE FIGURE 1.4

THE ADENINE BIOSYNTHETIC PATHWAY OF *S. POMBE*

●	= ribose phosphate
PRPP	= 5-phosphoribosyl-1-pyrophosphate
PRA	= 5-phosphoribosyl-1-amine
GAR	= glycinamide ribotide
FGAR	= N-formlyglycinamide ribotide
FGAM	= N-formlyglycinamidine ribotide
AIR	= 5-aminoimidazole ribotide
CAIR	= 5-amino-4-imidazole-carboxylic acid ribotide
SACAIR	= 5-aminoimidazole-4-N-succinocarboxamide ribotide
AICAIR	= 5-aminoimidazole-4-carboxamide ribotide
FAICAIR	= 5-formaminoimidazole-4-carboxamide ribotide
IMP	= inosinic acid
SAMP	= adenylosuccinic acid
AMP	= adenylic acid
+/-	= points of feedback control
+	= PRPP and glutamine (cooperative saturation kinetics)
-	= IMP, GMP, (AMP)

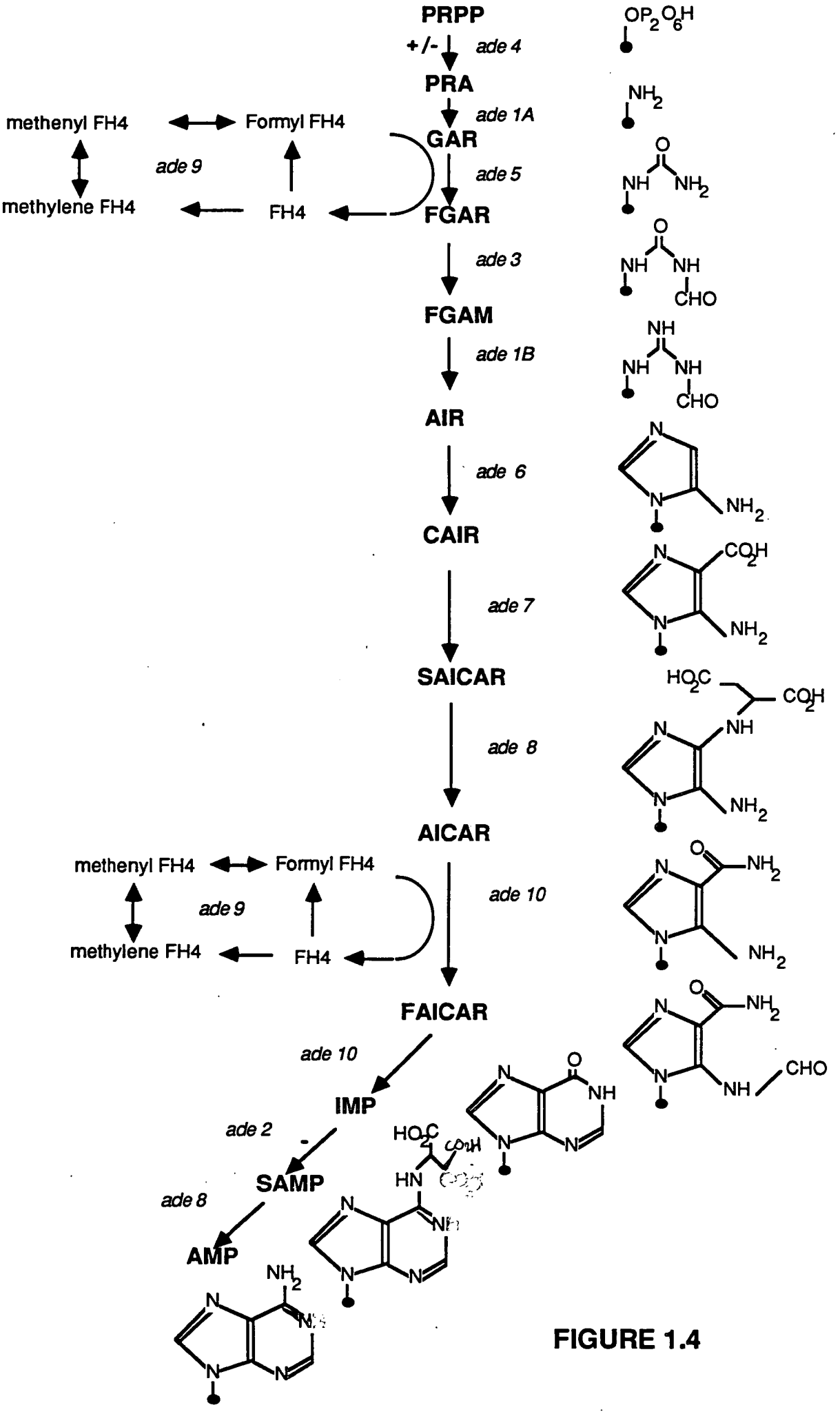
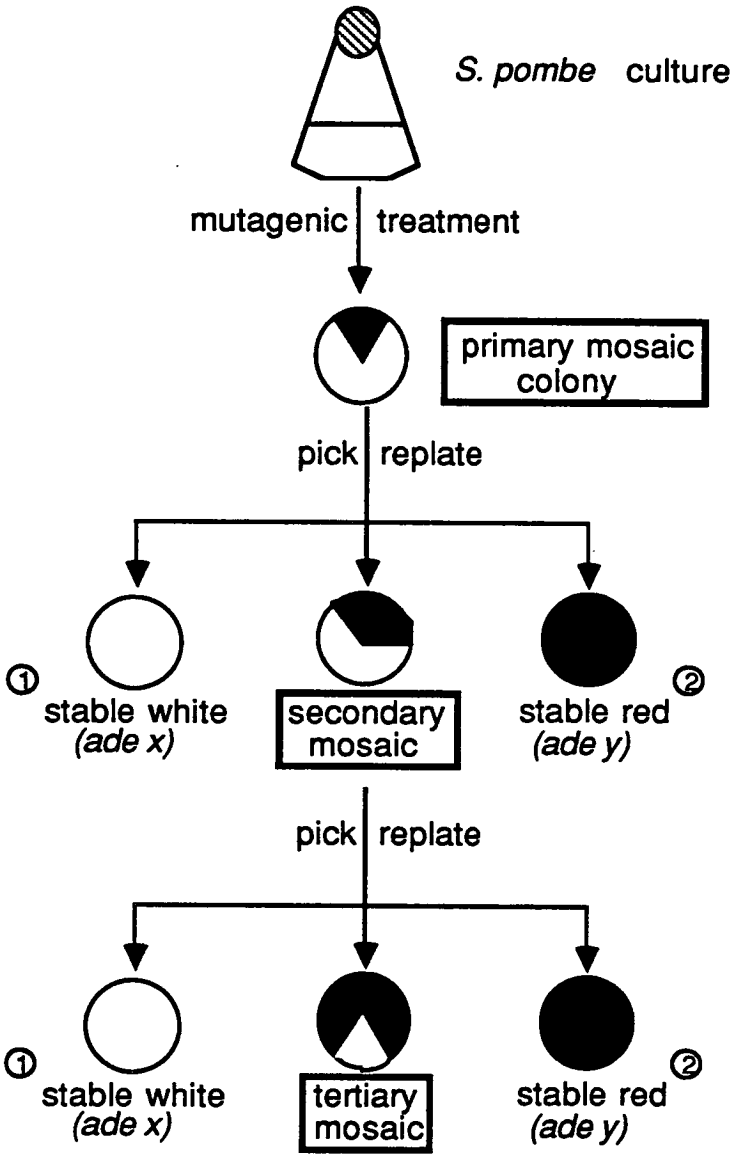

PRPP
+/-
ade 4
PRA
ade 1A
GAR
ade 5
FGAR
ade 3
FGAM
ade 1B
AIR
ade 6
CAIR
ade 7
SAICAR
ade 8
AICAR
ade 10
FAICAR
ade 10
IMP
ade 2
SAMP
ade 8
AMP
methenyl FH4
Formyl FH4
ade 9
methylene FH4
FH4
methenyl FH4
Formyl FH4
ade 9
methylene FH4
FH4

FIGURE 1.4

1.3.2 THE ADENINE BIOSYNTHETIC PATHWAY

In *S. pombe* the *de novo* pathway for purine biosynthesis appears to be identical to that described for all other organisms (Henikoff, 1986). Twelve loci have been identified in total, *ade1-10* and *gua1* and *gua2*. Mutants of the constitution *ade1-8* require adenine for growth. Mutants of *ade9* and *ade10* require adenine and histidine, *gua1* and *gua2* require guanine (Gutz *et al*, 1980). The adenine biosynthetic pathway is depicted in figure 1.4. Mutants in *ade1A* and *ade1B* are defective in different enzyme functions but map at opposite ends of the same gene, thus the *Ade1* gene codes for a bifunctional polypeptide possessing two enzyme activities. The pathway is regulated by feed-back inhibition at three points (Gutz *et al*, 1974). On a growth medium containing limiting amounts of adenine, such as YEA, mutants of *ade6* and *ade7* form red colonies due to a pigmented polymer derived from the metabolic intermediate formed by the previous step in the pathway. On adenine limiting medium the growth of *ade6* and *ade7* mutants is slow, double mutants of the type *ade6/7 adex* (where *x* =1,3,4,5 or 9) have a normal white phenotype and show improved, but not wild-type, growth rates.

It is the *de novo* adenine biosynthetic pathway that forms the basis for visual scoring of replicating instabilities in *S. pombe*. Replicating instabilities can be scored either by forward mutation of wild-type strains to adenine dependence at the *ade6* and *ade7* loci, or by forward mutation of *ade6* or *ade7* mutants to double mutants of the constitution described above. A delayed mutation using this system will be seen as a red/white mosaic or sectored colony. As *S. pombe* spends much of its time with a 2C DNA content it might be expected to give a high yield of mosaics after mutagenesis, this has indeed been shown to be the case (Nasim and Clarke, 1965; Loprieno *et al*, 1966). If a primary mosaic colony is picked and replated then the appearance of secondary mosaic colonies should be indicative of a replicating instability, providing that the delayed mutation is at the same locus as the original mutation which can be easily determined by complementation analysis (see figure 1.5).



Notes. ① : stable white either wild type or *ade 6/7 ade x (x = ade 1,3,4,5 or 9)* depending on forward mutation system used.

② : stable red *ade 6* or *ade 7*

Figure 1.5. Replicating instabilities in *S. pombe*

1.3.3 EXPERIMENTAL OBSERVATIONS ON REPLICATING INSTABILITIES IN *S. POMBE*

1.3.3.1 REPLICATING INSTABILITIES IN THE *ADE6/7* TO *ADE6/7, ADEX* SYSTEM

The bulk of the work of replicating instabilities in *S. pombe* has used the mutation system from stable *ade6* and *ade7* mutants (red) to double mutants of the constitution *ade6/7 adex* (*x* =1,3,4,5 or 9) which are phenotypically white. The first report of mosaics arising from mutagenic treatment in this system was by Nasim and Clarke (1965); they used nitrous acid (NA) to carry out forward mutation experiments in the strain *ade7.407h-*. It was found that at varying levels of survival the proportion of mosaic to complete mutants observed was between 2.5-1:1, however no secondary mosaics were observed in 40 colonies examined by replating.

The first report of replicating instability in this system was published by Nasim (1967): in this paper the effects of the five mutagens ethyl-methanesuphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), hydroxylamine (HA), nitrous acid (NA) and UV light on the induction of replicating instabilities were reported. It appeared that the mutagens tested fell into two distinct groups with respect to their ability to induce replicating instabilities, in the first group came the two alkylating agents EMS and MNNG and UV light which gave rise to secondary mosaics with a frequency of 0.97%, 0.87% and 0.73% respectively. The second group consisted of NA and HA which induced 0 and 0.05% secondary mosaics respectively, very much less frequently than mutagens of the other group. In this paper a number of control experiments designed to estimate the frequency at which spurious or false mosaics arise were described. Twenty red and white colonies were picked, resuspended, mixed and replated without mutagenic treatment. Any colonies arising on these plates should be spurious or false mosaics, it being known that the frequency of spontaneous mutation at the five loci in question is in the region of 10^{-6} (Friis *et al*, 1971). The observed frequency of secondary mosaics was reported as 0.08% using this technique.

A second and even more persuasive control reported in this paper (Nasim, 1967) is the result of mixing and replating mosaic colonies of genotype *ade7 adex* with a second complete double mutant of the constitution *ade7 adey*. It was argued that under these circumstances secondary mosaics of the

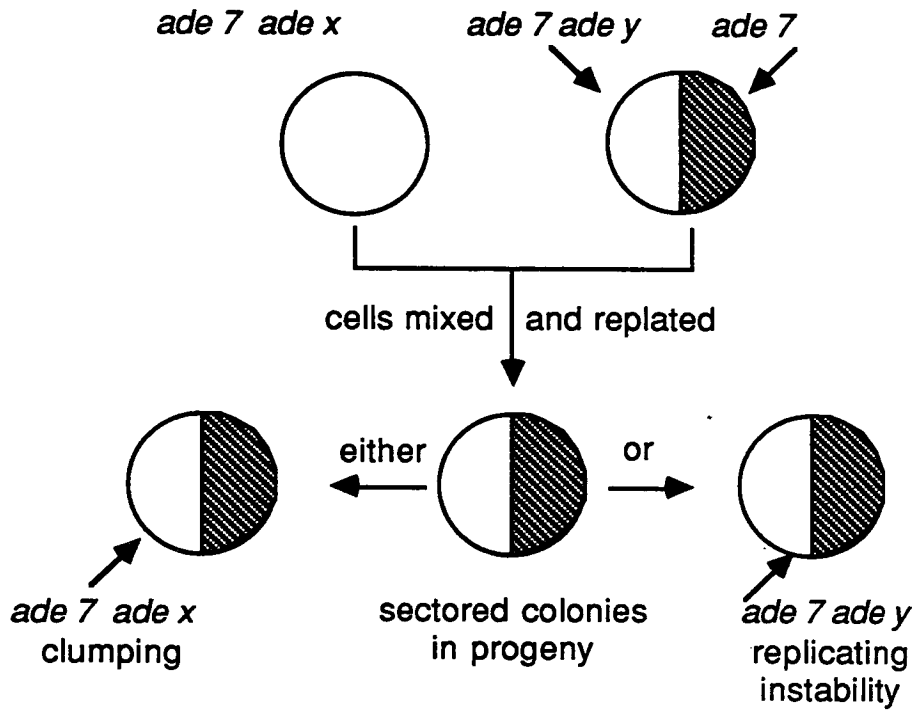


Figure 1.6. Control experiment to measure the frequency with which clumping causes spurious secondary mosaics. (From Nas[illegible], 1967)

constitution *ade7 adex* would be the result of genuine delayed mutation, whereas mosaic colonies having the genotype *ade7 adey* would be an indication of the frequency with which spurious mosaics arose due to cell clumping (see figure 1.6). It was found that 60 out of 64 secondary observed had the constitution *ade7 adex* while none were *ade7 adey*. The genotype of the remaining four mosaics was not reported. On the basis of the results of these experiments Nasim arrived at the following conclusions regarding the nature of replicating instabilities in *S. pombe* :

1. replicating instabilities can be generated by treatment with either alkylating agents or UV; NA and HA seemed to be ineffective in producing delayed mutation.
2. The frequency of secondary mosaicism observed was about 0.8%.
3. Of the primary mosaics tested 80% gave rise to at least one secondary mosaic (32/40 for EMS, 13/15 for MNNG, 30/37 for UV).
4. Two different control experiments indicated that the frequency with which spurious or false secondary mosaics arose as a result of cell clumping was so low as to be negligble.

The question of spurious mosaicism due to cell clumping continued, however, to be a source of criticism of work regarding the occurence of replicating instabilities in *S. pombe*. As a consequence single cell isolation of cells from secondary mosaics was carried out by Nasim and James (1971). It was found that 6 out of 8 secondary mosaics, derived by replating primary EMS-generated mosaic colonies, gave rise to one or more tertiary mosaics. The mean frequency at which tertiary colonies arose was 1.7×10^{-3} (10/5,816). Two of the mosaics tested were also replated and the mean frequency with which tertiary mosaics arose was again found to be 1.7×10^{-3} . As a control synthetic mosaics were constructed by deliberately placing "red" and "white" cells at the same place on an agar plate; these synthetic mosaics were picked and replated, the frequency with which the spurious tertiary mosaics was observed being in this case 0.04%. The results of these experiments seem to be in good agreement with those published by Nasim (1967), namely 75% of secondary mosaics gave rise to tertiary mosaics (6/8), the level of spurious mosaicism observed was estimated at 0.04% as compared to Nasim's original estimate of 0.08% (1967). This again seemed to confirm that replating is a good technique for identifying genuine replicating instabilities. There is one point on which the two papers do differ substantially, that is the frequency with which secondary mosaics are observed. The original report made by Nasim (1967) estimated the frequency of secondary mosaicism to be 0.8%, the later report had an estimated frequency of only 0.17% (Nasim and James, 1971).

This difference goes unremarked by the authors even though it must throw doubt on the conclusion of the authors that most of the secondary mosaics observed in the first paper were genuine.

Do the unstable cells in a mosaic exist as a distinct class, or are all the cells in a mosaic colony equally likely to be unstable? To answer this question James *et al.* (1972) carried out the following experiment. Wholly red and wholly white colonies which were the product of a mosaic were picked, replated and scored for frequencies of mosaics amongst the resultant colonies. The observed frequencies of secondary mosaics arising from red and white colonies was 0.01% and 0.00063% respectively, thus they concluded that unstable cells do indeed represent a separate class from the majority of cells in a mosaic colony which appear to have become stable. However it should be noted that the observed mutation frequency if both mosaics and completes are considered is 1.94×10^{-4} and 3.45×10^{-5} for the red and white colonies respectively. These figures are 100- and 10-fold higher than the spontaneous mutation frequency at all five of the loci preceding *ade6*, as has been measured by Friis *et al.* (1971). This would seem to imply that either some of the colonies scored as completes in fact had tiny mosaic sectors not visible to the naked eye or that the complete colonies picked were in some cases unstable.

Attempts have been made to estimate the rate at which the unstable state becomes resolved or stabilised by mutation to stable red or stable white cells (James *et al*, 1972). It was argued that as the frequency at which secondary mosaicism was observed was 0.8% then this figure is an indication of the level of unstable cells in a mosaic colony. Using this assumption the calculated mutation rate from unstable to stable was 0.2 or 0.4 per cell per generation depending on the model used for the calculation. The figure of 0.2 is arrived at using a model which assumes that when a cell carrying the unstable state resolves both progeny are stablized. The figure of 0.4 is arrived at by using a model in which the stabilization of an replicating instability in one daughter cell does not necessarily mean that the other daughter cell has also become stable. Alternative explanations for the low frequency at which secondary mosaics are observed have not been considered in this paper (James *et al*, 1972). For example it could also be argued that instead of having a few cells in an unstable colony carrying an instability which resolves at a high rate in fact many of the cells in a replicating instability carry an instability which has a lower frequency of resolution. This second alternative would also give the

observed results and would have the advantage of explaining why replicating instabilities can be inherited through so many generations. However it would not accommodate the observations made in *Drosophila* by Auerbach (1947) that the majority of the progeny of a mosaic are stable with respect to the trait in question (see table 1.1).

If the proportion of unstable cells in a mosaic colony is low then it would be helpful in predicting the nature of the unstable state if the following two questions could be answered;

1. Are the unstable cells located in a specific part of the colony? If for example they could be shown to be more closely associated with the centre than the periphery of a colony this would lend support to the conclusions of James *et al.* (1972) that the rate of mutation from instability to stability is high. (The cells at the periphery of a colony are presumably dividing faster and therefore have gone through more divisions than those in the centre, thus they are more likely to have stabilized according to the model of James *et al.* (1972).)
2. Can the unstable state be associated with one of the phenotypes? If it was shown that the unstable state was associated with either the "red" or "white" phenotype then models could be constructed which took account of whether or not the unstable state caused gene inactivation.

To answer the first of these questions Nasim and James (1972) carried out both plating and single cell isolation from the peripheries and the centres of mosaic colonies. It was found that by replating the frequency of secondary mosaics from the periphery was 0.28% as opposed to 0.48% for the centre of a mosaic colony. By single cell isolation it was found that 0.02% of peripheral cells gave secondary mosaics as compared to 0.21% of central cells. It was therefore concluded that the unstable cells in a colony are located towards the centre of the colony. It should be noted that on replating the ratio of secondary mosaics from peripheral : central areas was 1:2 whilst single cell isolation gave a ratio of 1:10, possibly indicating that replating overestimates genuine secondary mosaics.

In answer to the second question posed above a comparison of the frequency with which secondary mosaics arise when red and white sectors of a mosaic colony are replated would not be satisfactory as it could not be established that the sample did not contain a mixture of genotypes. However the problem could be approached by analysing the relative numbers of "red" and "white" cells in a mosaic colony and comparing this to the frequency at which secondary mosaics arose when the whole colony was picked and replated. An analysis of the relationship between the relative numbers of "red"

and "white" cells in a primary mosaic and the freqency at which secondary mosaics are produced when a colony is picked and replated was carried out (Nasim and James, 1972). It was found that a plot of proportion of secondary mosaics against proportion of "red" cells gave a regression line with a slope not significantly different from 0, thus the authors concluded that secondary mosaics could not be shown to be more closely associated with the red or the white sectors of a mosaic.

Tests carried out on replicating instabilities derived from EMS-mutagenesis showed that over three plating generations the mutations were site specific for five different primary mosaics analysed (Nasim and Grant, 1973). Tests were carried out using both interallelic complementation and intragenic recombination for two of the five strains. It was found that in one case only 1 *ade+* revertant was found in 6.86 X 10^9 spores analysed, in the other 8 revertants were observed in 2.25 X 10^9 spores analysed (Nasim and Grant, 1973). The ability of UV to induce replicating instabilities in different UV-sensitive strains was investigated (Nasim, 1974). The strains used were *rad1.1*, *rad5.158* and *rad10.198* all of which had been induced in an *ade7.407h-* background, the control strain with normal, wild-type response to UV was *ade7.407h-*. Mutants of the constitution *rad1.1* are deficient in recombinational repair and show enhanced sensitivity to both UV and ionizing radiation along with a 100-fold reduction in induced mutation frequency (Nasim and Smith, 1974; Gentner *et al*, 1978). The *rad5.158* mutation shows enhanced sensitivity to UV only, survival after mutagenic treatment is sensitive to caffeine in the plating medium (Nasim and Smith, 1974). The forward mutation frequency of *rad5.158* strains after UV mutagenesis is roughly the same as that of a repair proficient strain, however spontaneous mutation frequency is greatly enhanced (Schupbach, 1971; Grossenbacher-Grunder and Thuriaux, 1981). The *rad10.198* mutation has likewise been shown to be a strong mutator strain (Loprieno, 1973). It was found that the different mutants had apparently no significant effect on the frequency with which replicating instabilities were induced as a proportion of the total mutants, consequently it was concluded that changes in the repair capacity of treated cells did not influence the induction of replicating instabilities. The recombinational repair pathway, controlled by *rad1.1*, is specifically inhibited by caffeine (Fabre, 1971; Nasim and Smith, 1974). The effect of caffeine on the frequency of secondary mosaics was tested by adding caffeine to the plating medium but no significant effect was observed (Nasim,

1974). The finding by Nasim (1974) that radiation sensitivity does not influence the production of replicating instabilities was confirmed by Dubinin *et al.* (1972) who also found that the frequency of induction of replicating instabilities was equivalent in *ade7.407h-* and *ade7.407/rad1.1h-* strains. However these authors report a 10-fold decrease in the observed levels of secondary mosaicism when replicating instabilities were replated on caffeine containing medium. Dubinin *et al.* (1972) concluded that the *rad1.1* repair pathway was implicated in the production of secondary mosaics. However the level of secondary mosaicism observed by Dubinin *et al.* (1972) ranged up to 50%, far in excess of the 0.2 to 0.8% consistently reported by other authors. It would seem likely therefore that the instabilities observed in the two cases have different causes.

1.3.3.2 REPLICATING INSTABILITIES IN THE WILD-TYPE TO *ADE6/7* SYSTEM

Replicating instabilities have also been induced in the alternative forward mutation system described earlier, that is wild-type (white) to *ade6/7* (red). It was reported (Abbondandolo *et al*, 1967) that replicating instabilities could be induced in this system using the mutagens EMS, MMS, NA and HA. Secondary mosaics were observed in four successive plating generations for all the mutagens listed above. All stable mutants from each replicating instability were observed to have the same genotype, that is each was either *ade6* or *ade7*, and had the same additional phenotype, leaky, temperature-sensitive etc. Reciprocal crosses between mutant clones yielded no wild-type recombinants amongst the spores analysed (data not given). It was concluded therefore that the replicating instabilities induced in this system were also locus specific. Using the same foward mutation system (wild-type to *ade6/7*) Loprieno *et al.* (1968) produced replicating instabilities by NA mutagenesis, replating for up to five generations consistently gave rise to secondary mosaics. The locus specificity of the mutants was tested using interallelic complementation and in each case mutations arose at the same locus for successive generations.

In summary there appears to be a good body of evidence to show that replicating instabilities can be induced in *S. pombe* by mutagenic treatment. Replicating instabilities have been induced at all loci tested in the adenine biosynthetic pathway (Nasim and Grant, 1973; Loprieno *et al*, 1968). The evidence presented by Nasim and James (1971, 1972) where tertiary mosaic colonies were observed after micromanipulation of secondary mosaics is especially strong proof for the existence of replicating instabilities in *S. pombe*

as there can be no question of these results being artefactual.

The data discussed above indicates that alkylating agents, such as EMS and MNNG, and UV light are the most effective mutagens for inducing replicating instabilities. It appears that the unstable cells in a colony exist as a distinct class as compared to stable "red" and "white" cells and that this unstable class of cells is concentrated towards the centre of a mosaic colony (Nasim and James, 1972). Calculations on the rate of mutation from the unstable to the stable state indicate that it may be as high as 0.2 to 0.4 per cell per generation (James *et al*, 1972). However attempts to pin-point the phenotype of the unstable state were unsuccessful (James *et al*, 1972).

There are several issues on which different groups have presented apparently contradictory data, for example Nasim (1967) found NA and HA ineffective in producing replicating instabilities, whilst Abbondandolo *et al*, (1968) found both these mutagens to be highly effective. It is true however that the two groups were using different mutational systems but in the same organism. Nasim (1974) could find no evidence for a correlation between cellular repair process and the frequency at which replicating instabilities could be induced, and Dubinin *et al.* (1972) found significant inhibition of secondary mosaicism in cells blocked in recombinational repair by plating on caffeine containing medium (although caffeine may inhibit a variety of cellular functions). Discrepancies also exist between various reports as to the frequency with which secondary mosaics arise. Between groups working in this field estimates have ranged from as low as 0.17% to as high as 50% (Nasim and James, 1971; Dubinin *et al*, 1972). Estimates also vary within groups so that in some cases the estimate of the frequency of secondary mosaicism seen in one paper may be no higher than the frequency with which spurious or false mosaics were observed in another (Nasim, 1967; Nasim and James, 1971). Any study to be undertaken on the nature of replicating instabilities in *S. pombe* would therefore have to begin by trying to resolve the issue of how effective replating is as a technique for identifying genuine replicating instabilities. This is particularly important if molecular studies are to be subsequently undertaken.

1.4 THE NATURE OF THE UNSTABLE STATE

One of the difficulties in understanding replicating instabilities is that there has always been a paucity of plausible models to explain how the unstable state is generated. Indeed the difficulty in envisaging a replicating instability at the molecular level has been commented on many times (Mathew, 1964; Nasim, 1967; Auerbach, 1976). Early suggestions such as the inheritance of apurinic gaps or modified bases have largely been discarded as the molecular understanding of the mechanisms of DNA replication and repair has grown. Cytological analysis of the salivary gland chromosomes of the larvae of treated *Drosophila* revealed that alklyating agents produce an excess of small duplications in comparison to X-rays (Slizynska, 1963). However it was found that formaldehyde food which produces even more duplications than alkylating agents (Slizynska, 1956, 1963a) does not produce replicating instabilities in *Drosophila* (Ratnayke, 1970). In any case explanations at the supragenic level lost credibility when studies on fission yeast showed that the mutations produced by a replicating instability are site specific (Nasim and Grant, 1973; Abbondandolo *et al*, 1967).

Other suggestions have included the involvement of transposable elements (Nasim, 1967; Auerbach, 1976) or the production of palindromic or quasi-palindromic sequences which could serve as precursors for an increased rate of spontaneous mutation at a specific locus (Todd and Glickman, 1982; Ripley, 1982). These suggestions are, however, purely speculative lacking any experimental evidence to back them.

It seems that there are two reasons why attempts to build a cohesive model should have been frustrated in this way, the first being the elusive nature of the phenomenon. If, as suggested by James *et al.* (1972), the rate of mutation from instability to stability is as high as 0.2 to 0.4 per cell per generation this implies that a very low proportion of the cells in a mosaic colony will be unstable, making them virtually impossible to isolate. The inability to isolate the unstable state even to the extent of deciding it's phenotype (James *et al*, 1972) has in the past frustrated thoughts of a detailed biochemical analysis of replicating instabilities.

In view of the fact that replicating instabilities are induced one would intuitively think that repair and recovery from mutagenesis and the production

of replicating instabilities in *S. pombe* are intimately linked, despite reports that the frequency at which replicating instabilities occur is unaffected by the few repair deficient mutants tested (Nasim, 1974; Dubinin *et al*, 1972). As a consequence of this expectation, the other major difficulty in the way of understanding replicating instabilities is that the mechanisms of the repair processes in *S. pombe* are far from being fully elucidated, virtually none of the radiation sensitive mutants have even been assigned to a particular enzyme deficiency. Indeed the number and relative importance of the different repair pathways in *S. pombe* is still very much a matter for debate (Phipps *et al*, 1985). The current state of knowledge with regard to mutagenesis and repair has been dicussed in detail by Phipps *et al.* (1985) and a brief summary will be presented here.

In common with other organisms, *S. pombe* seems to have more than one pathway for the repair of DNA lesions. This was shown by the synergistic action of independently isolated radiosensitive mutants (Fabre, 1971; Nasim and Smith, 1975). Wild-type *S. pombe* shows considerable radiation resistance being twice as resistant to UV as *S. cerevisiae* and three times more resistant than E. coli (Nasim and James, 1978). It is also very resistant to ionizing radiation being over 20X more resistant than *S. cerevisiae* (Nasim and James, 1978). An unusual feature of *S. pombe* and one where it differs from *S. cerevisiae* is that there is no photorepair (Fabre, 1971). It has been reported that *S. cerevisiae* lacks the adaptive response to alkylating agents (Maga and McEntee, 1985) which is common to many other organisms (Yarosh, 1985; Walker, 1984); whether this also applies to *S. pombe* is to my knowledge unknown.

The sensitivity to killing by radiation depends on the stage of the cell cycle at which cells are treated (Fabre, 1970; Gentner *et al*, 1978). G2 cells are far more resistant to killing than G1, and it has thus been suggested that a recombinational repair process may be important in *S. pombe*. In this study it has been observed that stationary phase cells are far more resistant to EMS than log phase cells, this may be due to differential uptake of the mutagen (Parry *et al*, 1976) but may again reflect the requirement for a 2C nucleus in repair processes, stationary phase cells arresting in G2 under the growth conditions used in this study (Abbondandolo and Bonatti, 1970).

It is believed that there are at least three repair pathways in *S. pombe* :

these are dimer excision and two different recombinational repair pathways (Gentner, 1977). It has been found that both wild-type and recombination deficient strains are capable of dimer excision (Fabre and Moustacchi, 1973). Excision deficient strains show enhanced sensitivity to UV only (Gentner and Werner, 1975), but mutants totally blocked in dimer excision have not been isolated. Because of this it has been suggested that multiple pathways for dimer excision exist in *S. pombe* (Birnboim and Nasim, 1975).

The presence of a recombinational repair pathway in *S. pombe* was first suggested by the finding that cells containing a 2C genome show enhanced radiation resistance, greater than could be explained by having double the quantity of DNA in the cell (Fabre, 1970, 1973). In *S. pombe* the recombinational repair pathway is missing in *rad1.1* mutants (Gentner *et al*, 1978). The UV sensitive strain *rad1.1* produces fewer mitotic recombinants than normal strains, enhancing the opinion that this repair pathway is recombinational in nature (Fabre, 1972). The properties of this recombinational repair pathway have been summarized by Gentner (1981) and conditions for its functioning are as follows;

1. Cells must be able to recombine.
2. A duplicated genome is required.
3. There is requirement for a growth supporting medium.
4. Protein synthesis is required.
5. The pathway is thought to be responsible for UV induced mutagenesis.

It has been observed that one of the pathways of DNA repair in *S. pombe* is sensitive to inhibition by caffeine (Schupbach, 1971); it is believed that caffeine inhibits the recombinational repair pathway (Nasim and Smith, 1974; Loprieno and Schupbach, 1971; Loprieno *et al*, 1974). However the effect of caffeine and indeed the importance of the recombinational repair pathway to recovery and mutagenesis is still very much a matter of debate (Grossenbacher-Grunder and Thuriaux, 1981; Phipps *et al*, 1985).

The observation that *rad1.1* strains, blocked in the recombinational repair pathway discussed above, are still sensitive to ionizing radiation and show lethal enhancement by caffeine (Nasim and Smith, 1974) has led to the proposal that there exists a second prereplicative repair mechanism in *S. pombe* (Gentner, 1977). However mutants defective in this pathway have not yet been isolated. The characteristics of the known repair pathways in *S. pombe* are summarized in table 1.2, taken from Gentner 1977.

TABLE 1.2[1]

Characteristics of Schizosaccharomyces pombe Repair Pathways

Property	Pathway		
	I	II	III
Requirement for duplicated genome	Yes	Yes	No
Inhibited by caffeine	Yes	Yes	No
Requirement for protein synthesis	Yes	No	(No)
Active on UV-induced damage	Yes	No	Yes
Active on gamma-ray induced damage	Yes	Yes	No
Responsible for radiation-induced mutagenesis	Yes	Yes	No
Defective mutant	rad1	not known	rad13

[1] Taken from Gentner, 1977

The relationship between repair and induced mutation is still very much a matter of debate, it is not known which of the repair pathways discussed above contribute to induced mutagenesis (Phipps *et al*, 1985). However experiments conducted with radiosensitive mutants have revealed a number of salient features:

1. Mutants of constitution *rad1.1* and *rad3* are refractory to UV mutagenesis and are dimer excision proficient (Nasim and Hannan, 1977).
2. UV-induced mitotic crossing over is greatly reduced in *rad1.1* strains (Grossenbacher-Grunder and Thuriaux, 1981). Therefore *rad1.1* is excision proficient and deficient in a repair pathway contributing to UV-induced recombination. The residual ammount of UV-induced mitotic crossing-over seen in *rad1.1* strains is unaffected by caffeine, this could be interpreted as meaning that a second, independent process is involved in genetic exchange (Nasim and Hannan, 1977).
3. Mutations are induced in *rad1.1* and *rad3* mutants in response to chemical treatment demonstrating that besides the *rad1.1* pathway a second error prone pathway exists. It remains to be established which, if any, of the UV sensitive strains are involved in this type of repair.
4. It has been proposed that *S. pombe* has multiple pathways for recombinational repair (Grossenbacher-Grunder and Thuriaux, 1981) and for dimer excision (Birnboim and Nasim, 1975).

It is apparent from the summary above that there is still a great deal of work to be done before the mechanisms of repair and mutagenesis are as clearly understood as they are in *E. coli* (Walker, 1984). In light of these considerations it does not seem surprising that the relationship between replicating instabilities and repair is not clear, hence one possible approach to the analysis of replicating instabilities in *S. pombe* has not been available.

1.5 THE EXPERIMENTAL APPROACH

The objective of the present study was to arrive at reasonable, testable hypotheses for the nature of replicating instabilities and their production by alkylating agents. It was hoped that the powerful new techniques of gene cloning and sequence analysis would enable the work started by others but frustrated by lack of suitable analytical techniques to be completed here.

If, as suggested by James *et al.* (1972), the frequency of mutation from instability to stability is indeed so high that the unstable cells in a mosaic colony represent only a small fraction of the total cells present it would not be reasonable to try to isolate the unstable state. However it seemed likely that much could be learnt by the molecular comparison of the mutant and

non-mutant alleles isolated from a replicating instability. Large changes in the locus as a result of the insertion of a mobile element or a duplication or deletion would be apparent by comparing restriction maps of mutant and normal loci. Smaller changes in the locus such as frameshift mutants, small insertions and deletions would be apparent only by comparing the sequences of the mutants derived from replicating instabilities in successive plating generations and non-mutant loci. By analysing the type of change in the DNA sequence of the stable mutant derivatives of a replicating instability it should be possible to arrive at a testable hypothesis as to the nature of the unstable state. At the very worst it should be possible to reject certain hypotheses.

It may be that there are a number of mechanisms which lead to the observed unstable phenotype, the best way to screen for this would be to analyse a number of different loci carrying replicating instabilities and their normal alleles. However this approach is not feasible due to the time and expense involved in the molecular characterisation of a single locus. As a consequence it was decided to concentrate on just one locus in the adenine biosynthetic pathway. The locus selected for analysis was the *Ade1* locus because it was known that this locus codes for a protein of approximately 40 kDal. molecular weight, large enough to be expected to give rise to replicating instabilities with reasonable frequency but not so large as to be impossible to clone and sequence. The characterisation of the wild-type and mutant replicating instability derived alleles was a large project and so as a consequence was divided into two parts, I was to be responsible for identifying and analysing mutant *ade1* alleles whilst the molecular analysis of the wild-type *Ade1* gene was to be carried out by Rod McKenzie.

The approach for my half of the project was to isolate stable mutant and non-mutant derivatives of replicating instabilities in the *Ade1* gene and then to carry out their molecular analysis. The first stage in a molecular analysis of the mutants would be restriction mapping of the locus, using the wild-type clone as a probe in Southern hybridisation. The mutant genes could be cloned by preparing bacteriophage libraries of mutant DNA and identifying plaques carrying the mutant gene by colony hybridisation. It was obviously very important to be able to identify with a high degree of confidence those mosaic colonies which represented the products of a genuine replicating instability. From the literature published on replicating instabilities in *S. pombe* and discussed above it appeared that replating primary mosaics did indeed

represent a sufficiently accurate test for replicating instabilities, as long as the experimental conditions were properly defined. Accordingly the first step in the project was to determine the conditions to be used.

CHAPTER 2
METHODS

2.1 STOCK SOLUTIONS AND MEDIA

The preparation of stock solutions, buffers and media used in this study is described in Appendix A.

2.2 MAINTENANCE OF STOCK CULTURES

(Gutz *et al.*, 1974)

Stock cultures of *Schizosaccharomyces pombe* were obtained from Anwar Nasim, National Research Council, Ottowa, Canada and Peter Fantes, University of Edinburgh.

2.2.1 LONG-TERM

S. pombe strains remain viable for at least five years when the cells are dried on silica gel. This was done using the following procedure:

1. Bijou bottles were half-filled with white,12-28-mesh silica gel, and were dry sterilized at 180°C for 2 hours without their caps . The caps were autoclaved separately, and once dried replaced on the vials.
2. The yeast strains to be stored were grown on YEA slopes at 30°C for 3 days.
3. The cells of each culture were resuspended in 1ml of sterile 0.5% skimmed milk.
4. The cell suspension was slowly transferred to the silica gel tube, avoiding complete saturation of the silica gel.
5. The tubes were stored for 1 week at room temperature with the caps loosely screwed on.
6. The caps were then tightened and sealed with Parafilm. The stocks were stored at 4°C.
7. To grow up stocks from silica gel a small amount of silica gel is transferred to YEL. The silica gel can be resealed for further storage.

2.2.2 SHORT-TERM

Cultures of *S. pombe* which were in constant use were stored by streaking a colony onto either YEA or MA + supplement. After growing for 3 days at 30°C these plates were stored for up to 4 weeks at 4°C.

2.3 GROWTH OF *S. POMBE* IN LIQUID CULTURE

The growth of *S. pombe* in liquid culture was routinely carried out by the inoculation of sterile, liquid medium with a colony from a plate. The growth of *S. pombe* in liquid culture has been well documented by Mitchison (Mitchison, 1970).

Liquid cultures containing adenine requiring mutants of *S. pombe* were routinely supplemented with 75 µg/ml of adenine sulphate to guard against the positive selection of *ade+* revertants or suppressor mutations that might arise spontaneously in liquid culture.

Cultures of *S. pombe* were often required in stationary phase for use in mutagenesis and plating control experiments. In this case cultures were routinely harvested at a cell density of 4 X 10^7 cells/ml, at which point they would be entering stationary phase. In all experiments where liquid cultures of *S. pombe* were used the cell density of the culture was estimated using a haemocytometer before the cells were harvested, so the phase of growth was known.

2.4 PURIFICATION OF *S. POMBE* STOCKS

(Gutz *et al.*, 1974)

Haploid cultures of *S. pombe* always contain a few diploid cells; a haploid clone can be isolated from a culture by streaking colonies on YEA which has 10µg/l phloxin B. When grown on this medium haploid colonies are pale pink, whereas diploid colonies are dark red. The difference in appearance is due to the fact that phloxin B is taken up by dead cells, diploid colonies have a higher proportion of dead cells than haploid colonies and so appear much darker in colour. Spontaneous mating-type mutations also occur, and of most practical importance in experiments are mutations from *h+* to *h90*. From *h+* strains, pure clones can be reisolated by streaking on MEA. After 4 days incubation at 25°C the plates were treated with iodine vapour (see below); *h+* colonies appear yellow.

2.5 MATING TYPE DETERMINATIONS

(Gutz *et al.*, 1974)

When colonies containing asci are exposed to iodine vapour they will turn black due to the reaction between the iodine and the starch in the asci. Non-sporulating colonies, however, turn yellow. This difference is used as the basis for determining the mating type of unknown cultures. Cultures to be tested were patch-plated onto 2 YEA master plates. When the colonies had developed (3-4 days at 30°C) they were replica plated onto 2 MEA plates one of which had been spread with *975* (*h+*, wild-type) and the other with *972* (*h-*, wild-type).

The plates were incubated for 4 days at 25°C after which time they were treated with iodine vapour (in the fume cupboard). This was done by inverting the bottom of the plate to be tested over a can containing a few iodine crystals. The can was heated with a bunsen burner until vapour was visible. A moderate exposure to iodine vapour (2-4 seconds) kills only some of the cells in a colony, so new cultures could be grown from treated colonies An *h-* strain will give a positive reaction with iodine (i.e. turn black) on the *975* lawn where as a *h+* strain will give a positive reaction on the *972* lawn.

2.6 CROSSES

(Gutz *et al.*, 1974)

Crosses were made by mixing haploid strains of compatible mating-type on MEA. The parental strains needed were grown on YEA slopes for two days at 30°C. The parental strains were resuspended in 4-5ml of sterile 0.85% NaCl and mixed in equal amounts, 0.2ml of the resulting mixture was put on a MEA slope. If, however, a homothallic strain (i.e. *h90*) was to be used as one of the parental strains in a cross the problem of selfing was minimised by the addition of a three-fold excess of the other parent. The slopes were incubated at 25°C, and after 2-3 days they contained many asci.

2.7 COMPLEMENTATION ANALYSIS

(Gutz *et al.*, 1974)

A qualitative genetic classification of auxotrophic mutants blocked in the same biosynthetic pathway was achieved by crossing using the following procedure. Mutant strains of opposite mating-type were streaked in a line across YEA plates. When the streaks had grown, the mutants can be crossed by replica plating on MEA in a "criss-cross" pattern; this easily allows 36 crosses in a 6 X 6 matrix. After 24 hours incubation at 25°C the MEA plates were then replica plated onto MA, these plates were incubated at 30°C for a few days and then scored for prototrophic colonies.

2.8 SONICATION OF CELL SUSPENSIONS

Sonication, using a Soniprobe (type 1130A, Dawe Instruments Ltd., London) with a 4mm diameter probe, was carried out in the following manner. The tip of the probe was sterilized with alcohol. 5ml samples were put in sterilin vials, the probe was lowered into the same position in the liquid each time to ensure a consistent power input. The cell suspensions were sonicated for 2 X 15 seconds at setting 4. The time and the power used had been experimentally shown to give the most efficient cell separation (see Results section).

2.9 DILUTION PLATING OF CULTURES

Cultures of *S. pombe* to be plated out were grown in YEL in a shaking incubator at 30°C to the desired growth phase. The concentration of cells was estimated using a haemocytometer and the cells were harvested in sterilin vials in a bench centrifuge, washed in phosphate buffer and resuspended to 1 X 10^7 cells/ml. The cells were then sonicated before immediate serial dilution in phosphate buffer to an appropriate concentration to be plated in 0.2ml aliquots.

2.10 MUTAGENESIS OF *S. POMBE*

2.10.1 EMS MUTAGENESIS

A culture was grown to stationary phase, harvested and washed with phosphate buffer. The cells were then resuspended to 1 X 10^7 cells/ml and a control sample removed. 3ml of cell suspension was pipetted into a sterilin vial and the desired concentration of EMS added. After incubation at 28°C for sufficient time to give the required survival, the cells were harvested by filtration using a 0.45 µm filter (Millipore) and washed by passing 3 X 50 ml of phosphate buffer through the filter. The treated sample was then resuspended in 3ml of buffer and both samples were serially diluted before plating out at 50-100 cells per plate. Glass and plastic ware were decontaminated by soaking for several days in a 6% solution of sodium thiosulphate (L. Ehrenberg and C. A. Wachtmeister 1984a,b)

2.10.2 UV MUTAGENESIS

UV treatment was carried out in a light proof box in a sterile room. A culture was grown to stationary phase, harvested and washed with phosphate buffer. The cells were resuspended to 1 X 10^7 cells/ml in phosphate buffer. 30ml of suspension was placed in a petri dish with a sterile stirring magnet and the dish and contents were placed in the light proof box on a stirrer. The UV lamp (Hanovia 0504/V unit B12) which had been prewarmed for 5 minutes was adjusted to a height of 27 cm from the bottom of the dish; in this position the flux at the surface of the cell suspension has been estimated to be 35J m^{-2} s^{-1} (G.D.E. Njagi, 1982). A 1ml control sample was removed from the dish, treatment was then commenced and the stirred suspension exposed for the desired period. After treatment the control and the treated sample were diluted in phosphate buffer before plating at 50-100 cells per plate.

2.11 MICROMANIPULATION OF CELLS

Colonies from which individual cells were to be isolated were resuspended in 3ml of YEL in a sterilin vial. A slab of YEA was cut from a plate and placed on a sterile microscope slide, and a loop of cell suspension was then streaked along one side of the YEA slab. The slab was mounted upside-down on a

small plastic box on the stage of a Leitz microscope. Micromanipulation was conducted with the aid of a Beauduin (1145) micromanipulator fitted with a dissecting needle of 15 µm diameter made from pyrex glass tubing in a de Fonbrune microforge. Cells were placed at a separation of 4mm from each other in columns 5mm apart. The YEA slab was then placed on a YEA plate and incubated at 30°C until colonies were sufficiently well developed to be scored.

2.12 DISSECTION OF ASCI

A loop of a sporulating culture containing mature asci was suspended in 1ml of phosphate buffer by vortexing. A slab of SPA was cut from a plate and placed on a sterile microscope slide. A loop of cell suspension was streaked on along one edge of the SPA slab which was mounted on the stage of a Leitz microscope, as described above. Asci containing 4 intact ascospores, were drawn from the edge of the streak using the micromaniplulator. The asci were placed at discrete locations 5mm from the edge of the streak and 5mm from each other. The SPA slab was then placed upside-down on a specially made plastic box, the edge between the slide and the box being sealed with vaseline to prevent the slab from drying out or becoming contaminated. The slab was left at room temperature for 24 hours, after which time most (about 80%) of the asci walls will have broken down. The individual asci were then drawn out to locations 4mm apart in a line using the micromanipulator. The slabs of SPA were then placed on YEA plates and incubated at 30°C until the colonies developed.

2.13 TESTING FOR DIPLOIDY IN CULTURES

Where it was suspected that a culture of cells could contain a significant proportion of diploids, the culture was tested by crossing to a known haploid culture and screened for azygotic asci. Cultures which have undergone autodiploidisation will remain heterothallic (i.e. they will be either *h+/h+* or *h-/h-* diploids) and so cannot be identified by using iodine vapour in the way that *h+* to *h90* mutations can. To identify such a heterothallic diploid, it must be crossed to a haploid of the appropriate mating type, some of the resultant ascospores will be *h+/h-* diploids. These when allowed to germinate and then

patch-plated onto MEA will sporulate spontaneously. Such crosses were carried out in the following manner. The putative diploid was crossed to the appropriate haploid, the asci were dissected as described above. Once colonies had begun to develop on the SPA slab their postions were noted and they were then patch-plated onto YEA plates. After incubation at 30°C for 3-4 days the resulting colonies were replica plated onto MEA. The MEA plates were incubated at 25°C for 2 days and then scored for the azygotic asci. This was done by suspending some cells in phosphate buffer and examining the suspension under the microscope. This method was adapted from that described by Kurennaya and Devin 1985.

2.14 PREPARATION OF *S. POMBE* DNA

(Beach *et al.*,1982)

S. pombe DNA was isolated from 200ml cultures grown to near stationary phase in YEA+75µg/ml adenine. The cells were harvested by centrifugation, washed in 20mM sodium citrate/phosphate buffer pH 5.8 and resuspended in 10ml 50mM sodium citrate/phosphate pH 5.8, 1.2M sorbitol, 50mM 2-mercaptoethanol, 40mM EDTA, 2mg/ml Novo SP234 (an extract of *Trichoderma harzianum*, Novo Enzymes). Incubation at 30°C for 30-60 minutes made the cells osmotically sensitive. Once this had happened the cells were lysed directly by addition of 20ml 50mM Tris pH7.6, 50mM EDTA, 2% SDS at 65°C. After approximately 2 minutes the lysate was extracted with an equal volume of phenol-chloroform (50:50 saturated with 0.5M Tris pH 7.6). The aqueous upper layer was brought to 300mM sodium acetate and the nucleic acids precipitated with a half volume of propan-2-ol. The precipitate was pelleted by centrifugation (12,000g for 20 minutes), resuspended in 6ml T.E., 6.4g of caesium chloride and 0.6ml of 10mg/ml ethidium bromide was added. After 40 hours centrifugation one clear band formed in the centre of the gradient (Maniatis *et al.*, 1982 page 93). This was removed with a syringe, extracted with water saturated butan-1-ol and diluted with 4 volumes of 300mM sodium chloride. The DNA was precipitated with 2 volumes of ethanol, washed with 70% ethanol, and redissolved in 300µl of T.E.

2.15 RESTRICTION DIGESTS OF DNA

(Maniatis *et al.*, 1982)

Restriction digests were carried out as described in Maniatis pages 98-106. Typically 1-1.5µg of DNA was used per digest for analytical gels, for Southern blots 5µg of DNA was used per digest. When the digest had been incubated for the desired length of time a "stop" solution containing gel-loading buffer, glycerol and a marker dye was added. Analysis of digests was carried out by running the DNA on agarose gels, made up to an appropriate concentration in Tris-acetate gel buffer (see Maniatis *et al.*, 1982, pages 150-172 on agarose gels). The DNA was visualised by staining the gel with 2µg/ml ethidium bromide (from a 10mg/ml stock) for about 30 minutes. The gel was then inspected on a transilluminator (Chromato-Vue, transilluminator model C-62, Ultra-violet Products Inc. U.S.A.) permanent records of results were made by photographing gels using a Polaroid camera (Genetic Research Instrumentation Ltd.,U.K.)

2.16 GEL ELECTROPHORESIS

(Maniatis *et al.*, 1982 pages 150-172)

Analytical gels, usually containing 0.8% agarose, were run in a mini-gel system (Cambridge Biotechnology Laboratories) at 60mA using an Atta mini power pack (SJ-1082). After about 90 minutes the DNA should have run sufficiently far through the gel to be visualised. Gels for Southern blots were run on a horizontal gel electrophoresis system (BRL model H1) which has a gel bed of 20 X 25cm. The 0.5% agarose gels were run over-night at 25mA (Atta mini power pack) at 4°C, and the Tris-acetate buffer in the tank was circulated by a pump to maintain mixing.

2.17 SOUTHERN BLOTTING

(Southern 1975, Maniatis *et al.*, 1982 pages 382-386)

The method used for transferring DNA onto nitrocellulose filters was adapted from that described by E.M.Southern (1975). The solutions used were filtered through 0.45µm filters (Millipore) to remove small particles. The 0.5%

agarose gels used were rather fragile and so they were left on the UV transparent gel tray during the preparative stages. The rate of transfer of DNA from a gel onto nitrocellulose paper depends on the size of the DNA fragments. The transfer of small fragments (<5kb) occurs rapidly, being virtually complete in 3-4 hours; however large fragments (>10kb) are inefficiently transferred. As the DNA in question was of high molecular weight the gel was soaked in 0.2M hydrochloric acid for 20 minutes, this causes depurination of DNA and subsequent hydrolysis. The gel was then soaked in 0.5M sodium hydroxide, 1.5M sodium chloride for 2 X 15 minutes to denature the DNA. Finally the gel was transfered to 0.5M Tris pH 7.5, 1.5M NaCl for 2 X 30 minutes to neutralize. The blotting was carried out as follows:

1. A large sheet of polythene was laid on the bench.
2. 4 sheets of 3mm paper (Whatmann Chromatographic) well soaked in 20 X SSC were laid on top.
3. The agarose gel was carefully placed on the paper, avoiding trapping any air bubbles.
4. The whole area was sealed off with strips of cling film leaving a hole over the area of the gel to be transfered.
5. The nitrocellulose filter (0.45µm Schleicher and Schuell, W. Germany) soaked first in distilled water and then dipped in 20 X SSC, was laid carefully over the gel. Great care was taken to avoid trapping air bubbles as once in place the filter cannot be moved.
6. Two sheets of 3mm paper cut to exactly the size of the filter and soaked in 20 X SSC were laid on top of the filter.
7. A pack of soft paper towels were placed on the 3mm paper, a glass plate was placed on top and weighted down with a 2kg weight. The blot was left overnight to transfer, and the filter was then rinsed in 2 X SSC, blotted dry on 3mm paper and placed between sheets of 3mm paper and two sheets of glass. The filter was then baked dry at 80°C for 2 hours under vacuum.

2.18 HYBRIDISATION OF SOUTHERN FILTERS

(Maniatis *et al.*, 1982 pages 387-389,470-471)

For preparation of probes and hybridisation bags see below. Hybridisation was carried out using the following protocol, where more than one filter was to be hybridised to the same probe then two filters could be placed in one hybridisation bag.

1. The filters were wetted with 4 X SSC
2. The filter was soaked in 0.2% Denhardts, 4 X SSC, at 65°C for 30 minutes.
3. The filter was then soaked in prehybridisation fluid (0.2ml per square cm of filter) in a hybridisation bag at 65°C for 30 minutes. The prehybridisation fluid contained 5 X Denhardts, 4 X SSC, 50µg/ml salmon sperm DNA, 0.1% SDS.

4. The filter was transferred to a fresh bag and soaked in the hybridisation fluid at 65°C for 30 minutes. The hybridisation fluid was 5 X Denhardts, 4 X SSC, 0.1% SDS 9% dextran sulphate.
5. The bag was then opened and the probe was added, the bag was resealed and the contents mixed well before incubating overnight at 65°C
6. The filter was washed in :

4 X SSC for 5 minutes at room temperature.
2 X SSC, 0.1% SDS for 30 minutes at room temperature.
2 X SSC, 0.1% SDS for 60 minutes at 65°C
2 X SSC, 0.1% SDS for 30 minutes at 65°C
2 X SSC. 0.1% SDS for 30 minutes at room temperature.

The filter was blotted dry, sealed in a polythene bag and autoradiographed.

2.19 PREPARATION OF PROBES FOR HYBRIDISATION TO SOUTHERN FILTERS

Two probes were used to hybridise to the southern filters, one was the 2.5kb HindIII fragment of the plasmid pPS6. pPS6 is the plasmid in which the *Ade1* gene from S.pombe was originally cloned by Peter Schuchert (Berne, Switzerland). The HindIII fragment of this plasmid contains 1.8kb of the *Ade1* coding sequence and 0.7kb of 3' downstream sequence. The other probe was a combination of three digests of lambda DNA. These digests had been designed to give a suitable range of molecular weight markers on the gel, and aliquots of the digests had been run on the two outside tracks of each gel. Radioactively labelled samples of the lambda digests were hybridised to these markers so that they could be seen on autoradiographs. The probes were nick translated in the presence of ^{32}P dCTP using the Klenow fragment of E. coli DNA polymerase I. The following protocol was used;

COMPONENT	VOLUME/µl
10 X NT Buffer	5
1µg DNA	
2mM dATP	0.5
2mM dGTP	0.5
2mM dTTP	0.5
50µM dCTP	2
^{32}P dCTP (10pCi)	1
DNAase (10^{-4})	0.5
distilled water	to 50
DNA pol I (Klenow frag.)	1

After mixing thoroughly the reaction was incubated ot 16°C for 90 minutes then stopped by the addition of 5µl of 0.2M EDTA. The mixture was then passed through a Sephadex G50 spun column (see Maniatis *et al.*, page 466) to

separate the probes from unincorporated label. The nick translated probes were added together and mixed with 0.5ml salmon sperm DNA and 2.0ml distilled water. The mixture was heated to 100°C for 5 minutes and then immediately cooled on ice. The probe was then ready for use.

2.20 ASSEMBLY OF HYBRIDISATION BAGS

1. Heavy duty polythene sheeting was cut to allow a generous border around the filter and extra length at the top for sealing.
2. The sides of the polythene to be in contact with the filter were washed with alcohol and wiped dry.
3. With cleaned sides together the sheeting was double sealed along one side using a heated bag sealer (Calor 24.03).
4. The filters were placed close to the edge inside the sheets of polythene, enough of a border was allowed to double seal the base.
5. The base and other side were double sealed to form an open topped bag.
6. The hybridisation mix was added and allowed to collect in the bottom of the bag.
7. As much air as possible was excluded from the bag and the bag was sealed across the top.
8. All remaining air bubbles were pushed to the top of the bag, whilst keeping as much of the fluid as possible at the bottom. The remaining air bubbles were sealed in the top of the bag.
9. The bag was then ready for incubation in the water bath.

CHAPTER 3
GROWTH CURVES, KILL CURVES AND MUTATION RATES

3.1 GROWTH OF *S. POMBE* IN LIQUID CULTURE

The growth curves of the three strains of *S. pombe* most frequently used in this study were determined for both YEA and YEA + 75 µg/ml adenine sulphate at 30°C. Determination of the cell density of the culture was done by counting appropriately diluted samples of cultures on a haemocytometer. The results are depicted graphically in figures 3.1 and 3.2.

The addition of adenine to cultures of the wild-type, *972h-*, had no noticable effect on the rate of growth of the culture or on the cell density reached in stationary phase. Whether or not the cultures were supplemented they reached a stationary phase density of 5.5 X 10^7 cells/ml within 36 hours. The addition of adenine sulphate to the medium had little effect on the rate of growth of the adenine requiring mutants *ade6.704h-* and *ade7.50h-* in all cases stationary phase was reached after about 36 hours. The addition of adenine sulphate to the medium did, however, have a marked effect on the final cell density reached by adenine requiring strains. In unsupplemented medium the cell density in stationary phase was found to be about 1 X 10^7 cells/ml whereas in YEA + adenine the final cell densities reached reflected the wild-type value of 5.5 X 10^7 cells/ml. As a precaution against positively selecting for *ade+* revertants or suppressor mutations liquid cultures of adenine requiring mutants of *S. pombe* were routinely supplemented with 75 µg/ml of adenine sulphate.

3.2 EMS KILL CURVES

Stationary phase cultures of *S. cerevisiae* are less sensitive to the lethal effects of EMS, NA and heat-shock than exponentially growing cultures (Parry *et al*, 1976). The sensitivity to killing by EMS displayed by *S. cerevisiae* varies over 1000-fold between stationary and exponential phase cultures, this can only be partly explained by the 7-fold difference in up-take of mutagen observed (Parry *et al*, 1976). To see if this effect was also found in cultures of *S. pombe* an EMS kill curve was constructed by harvesting *ade7.50h-* cultures at different growth phases and subjecting them to a uniform regime of

Figure 3.1

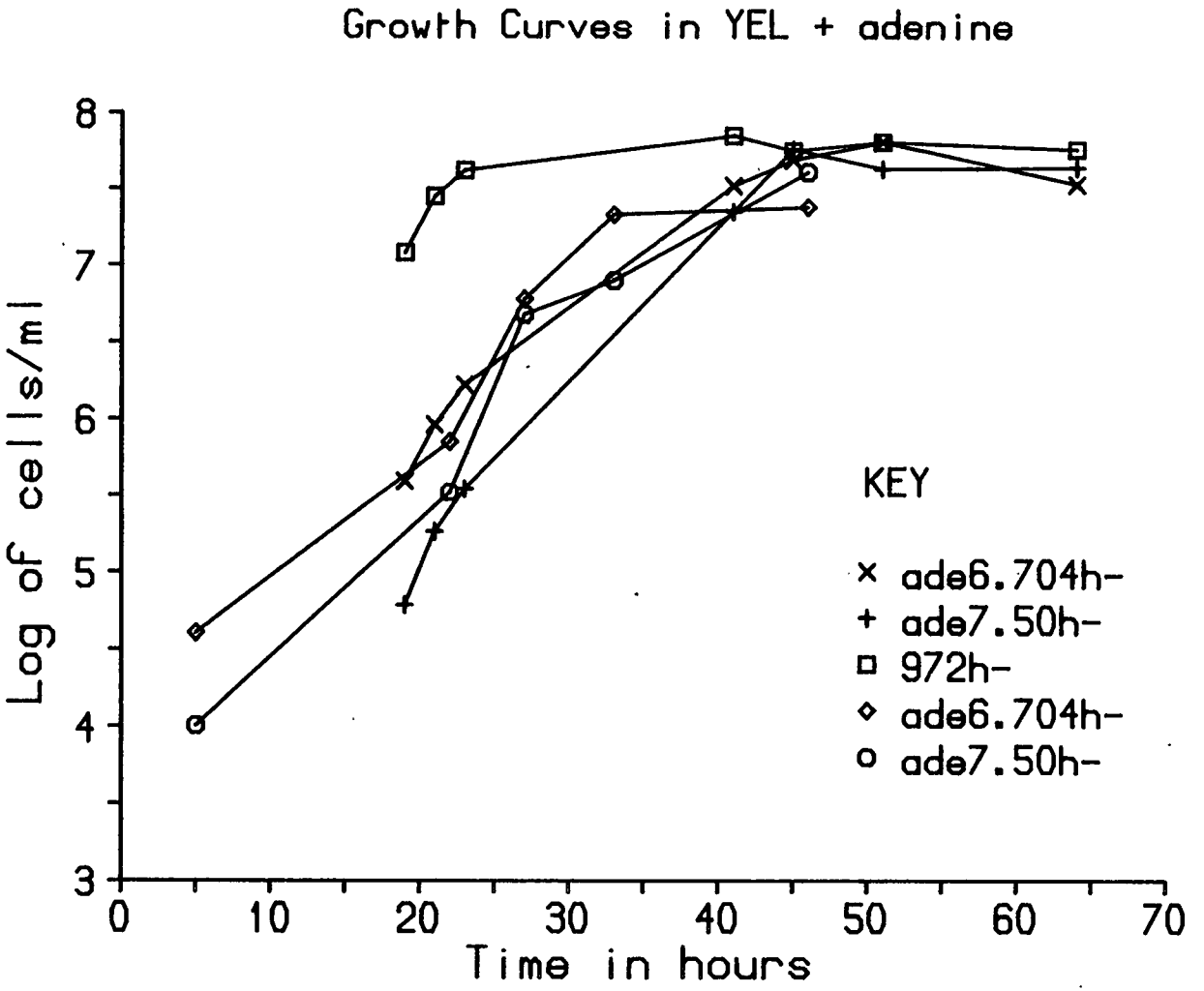

Growth Curves in YEL + adenine
Log of cells/ml
8
7
6
5
4
3
0
10
20
30
40
50
60
70
Time in hours
KEY
ade6.704h-
ade7.50h-
972h-
ade6.704h-
ade7.50h-

Figure 3.2

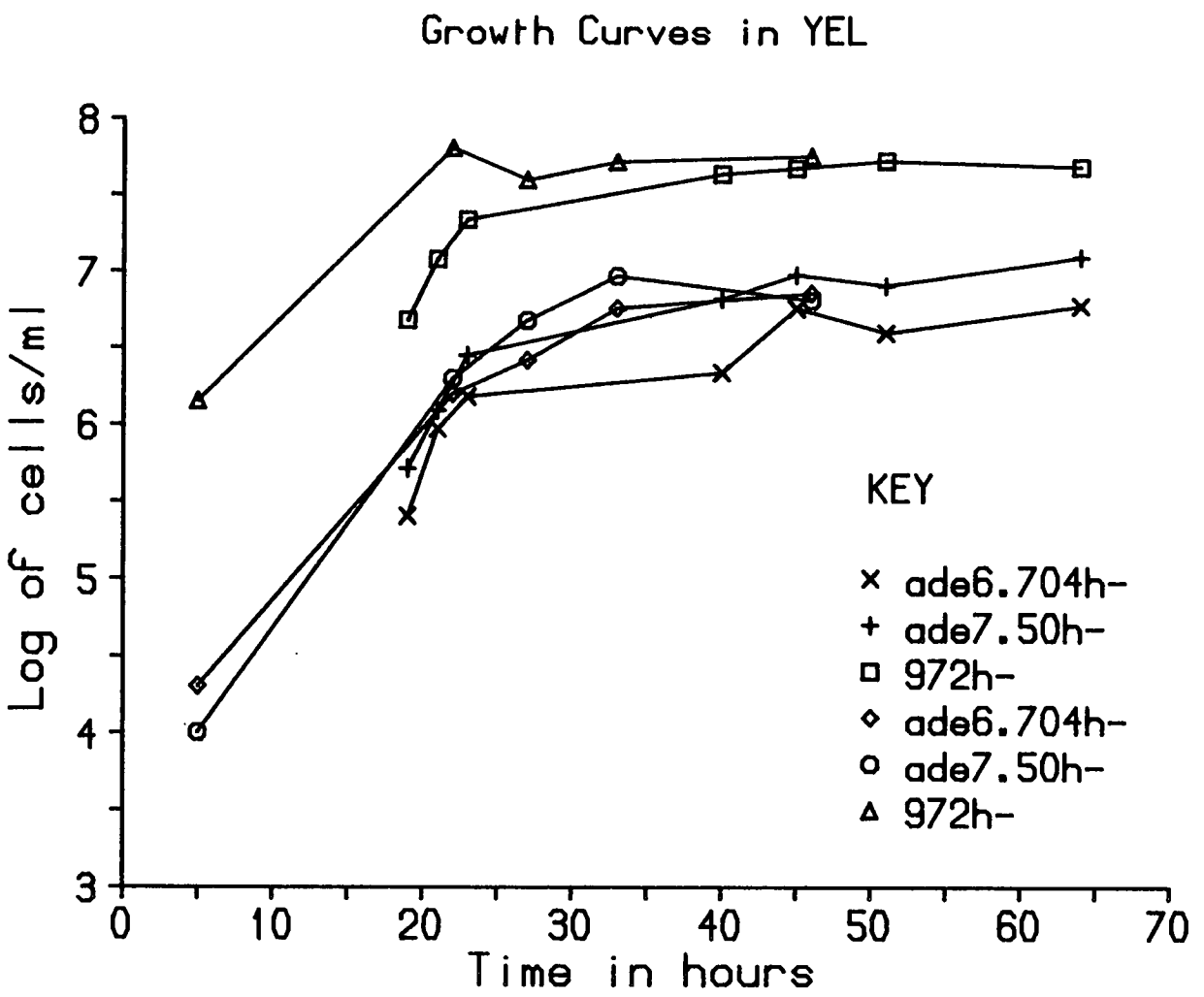

Growth Curves in YEL
Log of cells/ml
8
7
6
5
4
3
0
10
20
30
40
50
60
70
Time in hours
KEY
ade6.704h-
ade7.50h-
972h-
ade6.704h-
ade7.50h-
972h-

Figure 3.3

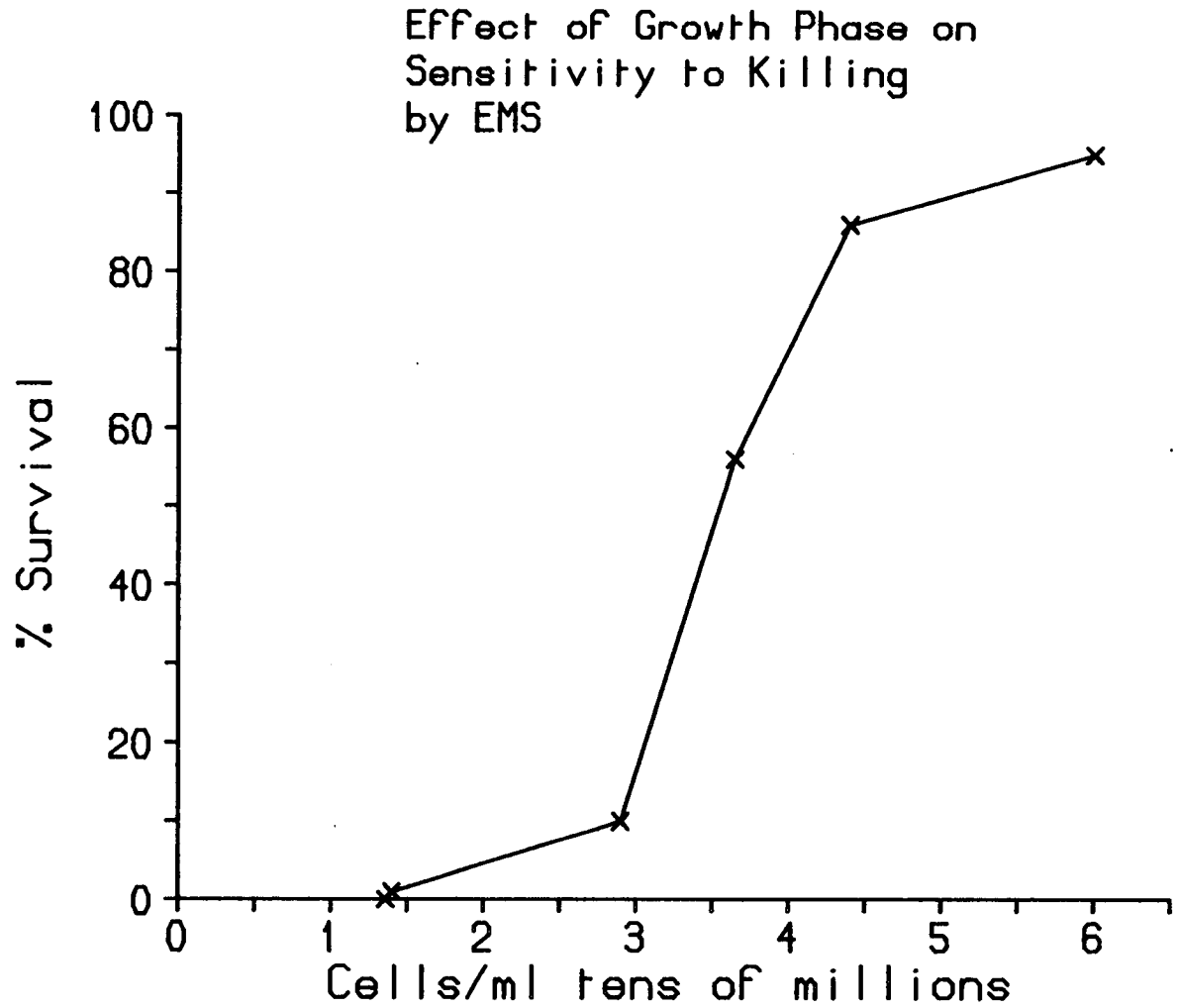
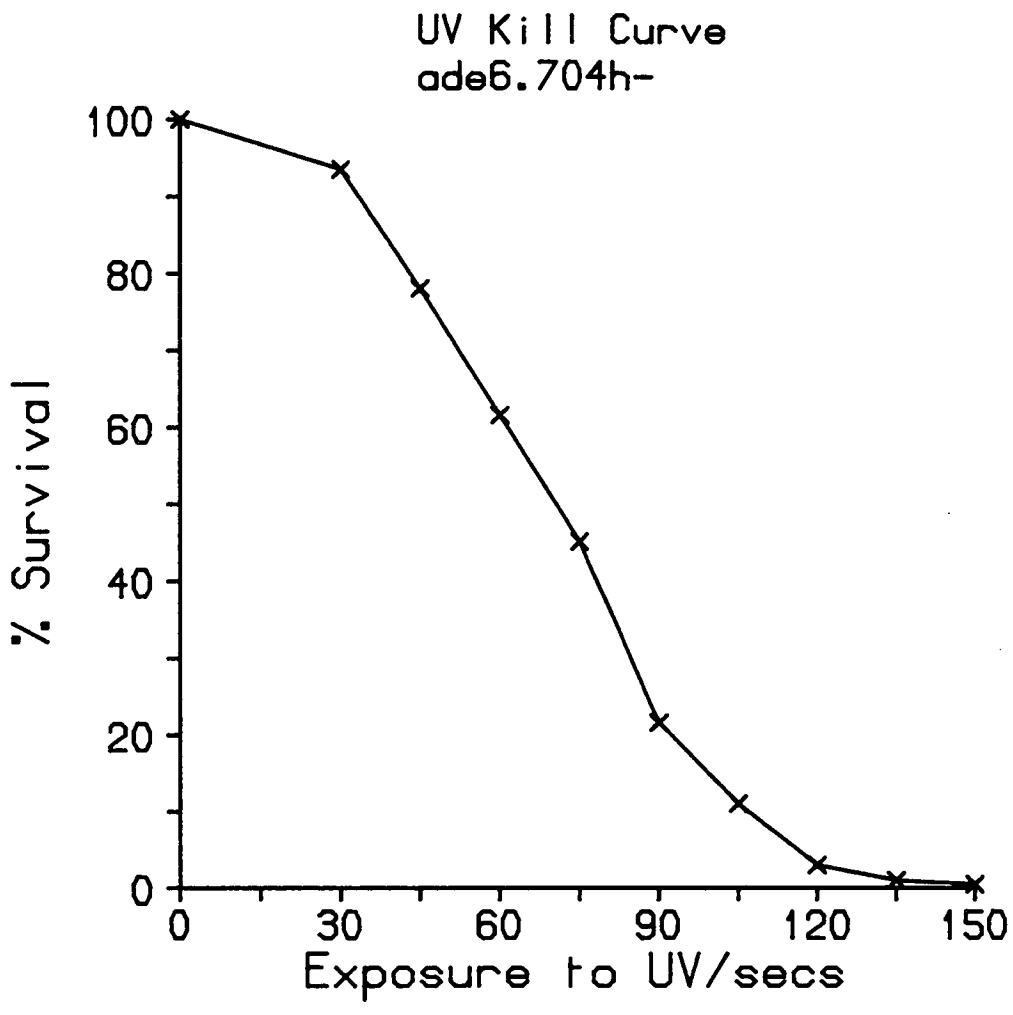

Effect of Growth Phase on
Sensitivity to Killing
by EMS
% Survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Cells/ml tens of millions

Figure 3.4


UV Kill Curve
ade6.704h-
100
80
60
40
20
0
% Survival
0
30
60
90
120
150
Exposure to UV/secs

treatment (3.5% EMS v/v in phosphate buffer for 60 mins at 28°C, see Methods). Samples of treated and untreated cells were plated for survival and culture viability. The results are shown in figure 3.3. It can be seen that a similar effect of "cell age" on the lethal effects of EMS was observed to that reported by Parry *et al.* (1976) in *S. cerevisiae*. Previous work on replicating instabilites in *S. pombe* has routinely used stationary phase cultures of cells for mutagenic treatment (Nasim and Auerbach, 1967), the treatment giving roughly 50% survival. Consequently in this study cultures for EMS mutagenesis were harvested at 3-4 X 10^7 cells/ml and treatment carried out as described above. The culture viability and survival was determined for each mutagenic experiment by plating samples of untreated and treated cells.

3.3 UV KILL CURVES

Evidence from both *S. cerevisiae* (Parry *et al*, 1976) and *S. pombe* (Fabre, 1970) shows that exponentially growing cells are more UV resistant that stationary phase cultures. However as with EMS, UV mutagenesis has routinely been carried out on stationary phase cultures of *S. pombe* (Nasim and Auerbach, 1967; Nasim, 1974). A culture of *ade6.704h-* was grown to stationary phase, harvested and exposed to UV (see Methods) for varying periods of exposure. The results are shown in figure 3.4. An exposure of 75 seconds gave roughly 50% killing, after this cultures of *S. pombe* were routinely exposed to 75 seconds of UV-light in mutagenesis experiments. As with EMS mutagenesis after each experiment, treated and untreated samples were plated to determine survival and the original viability of the culture treated.

3.4 MUTATION FREQUENCIES

The induced mutation frequencies in this study were in agreement with those reported by other workers on *S. pombe*, some typical results are presented in table 3.1 along with previously published data for comparison.

TABLE 3.1

Typical Mutation Frequencies in S. pombe

Source of data	Strain	Mutagen	% Survival	% Mosaics amongst mutants	Mutation frequency per 10^4 survivors
Nasim and Auerbach, 1967	ade7.407h$^-$	EMS	60-80	71	82.0
	ade7.407h$^-$	UV	60-80	30	77.0
Loprieno, 1966	972h$^-$	EMS	0.44	52	7.9
	ade6/7h$^-$	EMS	34.9	43	44.9
This study	ade6.704h$^-$	UV	44	82	24.0
	972h$^-$	EMS	45	77	1.8
	ade7.50h$^-$	EMS	51	66	20.0
	972h$^-$	EMS	43	64	2.4

CHAPTER 4
PLATING CONTROL EXPERIMENTS

4.1 INTRODUCTION

As discussed in the introduction, previously published data have given strong evidence for the existence of replicating instabilities in *S. pombe*. Especially strong is the evidence provided by single cell isolation (Nasim and James, 1971) where mosaic colonies were shown to arise from single cells isolated by micromanipulation.

However most of the work done in the past on replicating instabilities in *S. pombe* has used the technique of replating primary mosaic colonies, generated by mutagenesis, and scoring the number of secondary mosaics in the progeny as being diagnostic of the presence of replicating instabilities. Criticism of past work has centred on the fact that this technique is inherently flawed. If a colony which contains a mixed culture of cells is resuspended and replated and, by chance, two cells of different genotypes land sufficiently close together on the plate to have the appearance of being only one colony when fully grown, then such a colony may be wrongly classified as being the result of a replicating instability. Where a mosaic colony is known to have arisen as the result of such an event it is by definition a spurious or false mosaic. The problem with replating primary, EMS generated, mosaics is that presumably both spurious mosaics, as defined above, and mosaics arising from genuine replicating instabilities will occur. Unless the frequency with which spurious mosaics occur has been measured for a given experiment then it would be possible to dismiss all secondary mosaics as being the result of spurious mosaicism. This explanation would, in *S. pombe* at least, be the simplest possible for the observation that in second and subsequent plating generations all the mosaics observed have the same genetic constitution as the primary mosaic from which they are derived (Nasim and Grant, 1973). It would, in any case, be impossible to justify proceeding with a molecular analysis of any individual clone on this basis.

As a result of such criticism it has been common practice to publish data relating to the levels of spurious mosaicism observed whenever there has been a paper relating to replicating instabilities. The technique used to measure the

levels of spurious mosaicism varies between papers but has the common feature of being based in some way on the mixing and plating of stable, non-mutagenised, cultures of genetically distinct strains.

Detailed examination of data published, using the plating technique as diagnostic of the presence of replicating instabilities, revealed a number of discrepancies with regard to both the frequency of secondary mosaics arising from primary mosaics and the level of spurious mosaicism due to clumping. The estimated levels of both the above varied by up to five-fold between papers. For example in 1972 James *et al.* published data giving the level of secondary mosaics from EMS primary mosaics as 0.89%. The level of spurious mosaicism presumably caused in the manner described above, was determined by two methods. A mixture of cells from red and white colonies were replated, without mutagenic treatment, the level of spurious mosaicism was estimated from this to be 0.12%. Synthetic mosaics were generated by deliberately placing cells from red and white colonies at the same point on a plate, these synthetic mosaics were then picked and replated. The estimated level of spurious mosaicism arrived at by this method was 0.21%. However in 1971 Nasim and James (1971) found that the average level of tertiary mosaics obtained after micromanipulation of 8 secondary mosaics was 0.17%. Two of the 8 secondary mosaics were also sampled using the plating technique and the mean value for the frequency of tertiary mosaics was 0.17% the same as the results obtained by micromanipulation. In this paper the level of spurious mosaicism using the plating technique was estimated to be 0.04% when measured by replating synthetic mosaics as described above. In a third paper Nasim and Grant (1973) state:

> "These primary mosaics were replated after proper sonication and five different lines were chosen in which the plated progeny had a higher frequency of secondary and tertiary mosaics."

For one of these five progeny, E25, the pedigree of which was provided as illustration of the level of mosaicism observed in three successive plating generations was 0.14%, 0.25% and 0.19%.

The table below summarizes the levels of genuine and spurious mosaicism reported in these 3 examples.

AUTHOR	YEAR	%2RY mosaics	%spurious mosaics
James et al	1972	0.89	0.21/0.12
Nasim&James	1971	0.17	0.04
Nasim&Grant	1973	0.14,0.25,0.19	

These discrepancies are not discussed by the authors despite the fact that in two cases the reported level of secondary mosaics appears to be no higher than the level of background due to spurious mosaicism in the other example.

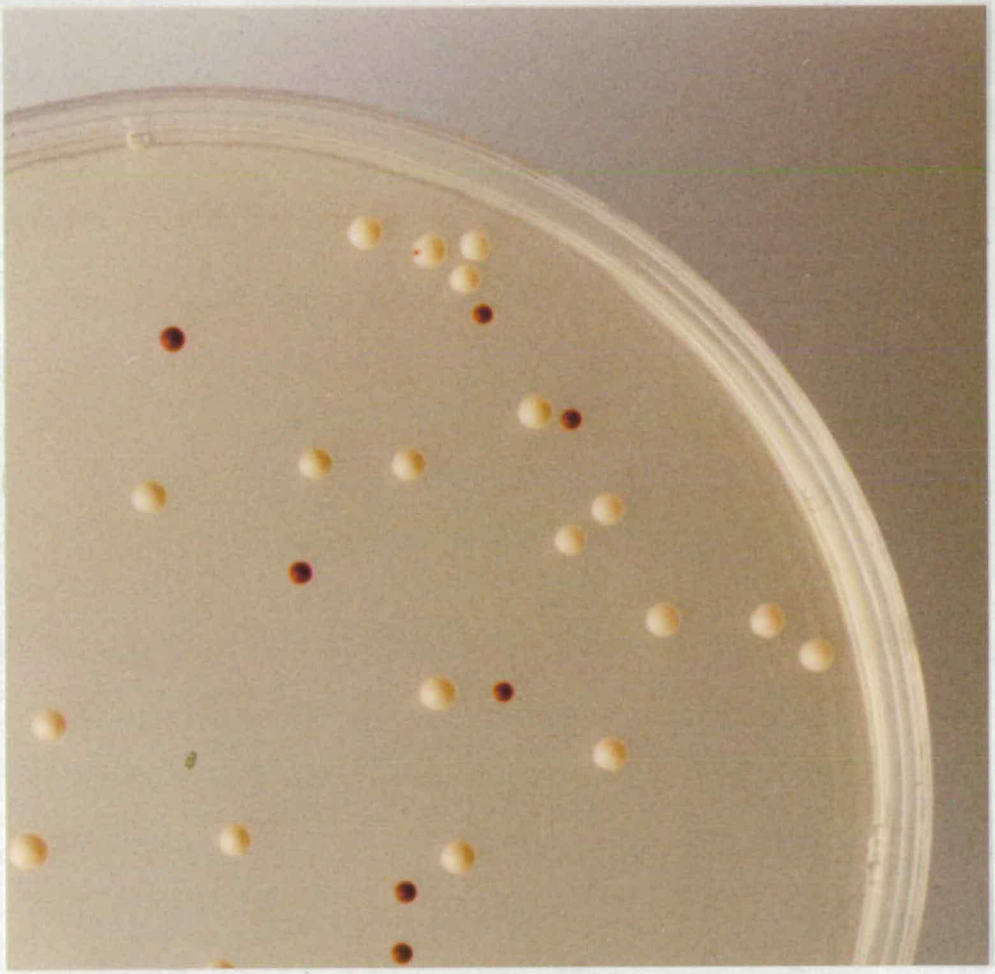
The examples above and the difficulties encountered in arriving at an estimate of false mosaics highlight the discrepancies in the data published by one group. However, examination of data published by other groups in the same field reveals similar differences in the estimates in the levels of both genuine and spurious mosaicism both between and within groups. For example Dubinin *et al.* (1972) estimated that under the experimental conditions used the level of spurious mosaicism observed was 1%, but Guglieminetti *et al.* (1967) working with mutations from 972*h*- to *ade6/7* observed 0 spurious mosaics when a mixed culture of 972 (white) and *ade6/7* (red) cells were plated at roughly 100 colonies per plate. As most of the work done in this field, especially relating to the problem of spurious mosacism has been carried out by Nasim *et al.* , discussion in this chapter of the problems encountered in using the replating technique for identifying replicating instabilities focusses on the work published by this group.

Because of these and other similar examples it was felt necessary to re-examine carefully the conditions used for plating cells from primary mosaics so that the background level of spurious secondary mosaics could be minimised. Unless this was done and false mosaics eliminated the expense and labour involved in the planned molecular analysis of mutants generated by replicating instabilities, identified by replating primary mosaics, could not be justified. It seemed that there were probably three main factors which could influence the level of spurious mosaicism:

1. The efficiency with which clumps of cells could be broken up;
2. The density at which cells were plated;
3. The ratio of red and white cells in a colony.

A series of experiments was designed to test the effects of each of the factors above on the levels of spurious secondary mosaicism.

Colour plate showing a typical false mosaic



4.2 RESULTS AND DISCUSSION

4.2.1 DISPERSING CLUMPS OF CELLS BY SONICATION

Sonication was carried out in phosphate buffer as described in the Methods. Care was taken to ensure that only one variable was altered at any one time by using the same type of vessel, the same volume of liquid, inserting the probe to the same depth and using the same duration of sonication each time. The standard curve only goes up to power setting 5 as it was not found possible to maintain a steady current at settings greater than this value. As well as testing the effects of altering the power setting at which sonication was carried out, different volumes of cell suspension were plated out to see if this affected the efficiency of cell separation on the plate.

The efficiency of sonication in separating clumps of cells was measured by scoring the number of colonies, or plating units, on the plates for each power level. The average number of colonies for each set of data was then converted into a percentage increase of plating units compared to the unsonicated control for that group. The results are depicted graphically in figure 4.1 (the raw data for the graph can be found in Appendix B).

On the basis of these results it was decided that the most efficient separation of cell clumps was achieved by using power setting 4. It was also decided that all plating experiments should be carried out using a sample volume of 0.2ml. The results show no significant difference between the different plating volumes, however it was considered to be another potential source of variation and it was eliminated as a source of systematic error by using a standard volume.

For one group in the experiment a visual inspection of the sonicated cells was carried out to estimate the efficiency of cell separation, and the results were as shown in table 4.1.

It is apparent from this that sonication does greatly reduce the proportion of clumped cells in a suspension. Many of the cells which were seen as pairs could be seen to be two daughter cells undergoing the final stages of cell division; this explains the fact that the number of cells in this category falls much less rapidly than the proportion of cells existing in clumps with increasing sonication. From the point of view of reducing incidence of

Figure 4.1
The Effect of Sonication:
Apparent increase in plating units

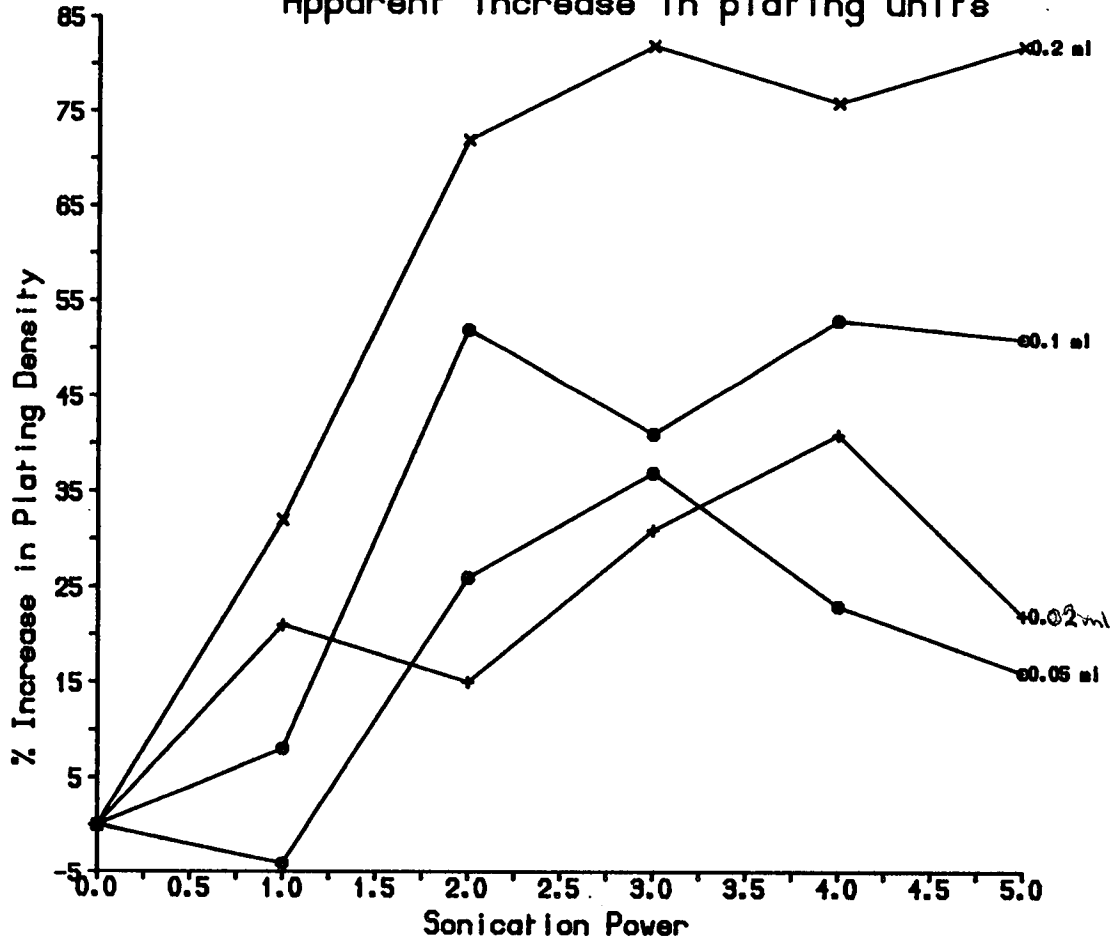


TABLE 4.1

The efficiency with which sonication separates clumps of cells, visual inspection of sonicated suspensions

Power	% single cells	% cells in pairs	% cells in clumps	sample size
0	12	40	48	259
2	69	21	10	237
4	87	12	1	204

spurious mosaicism the most significant figure in the above table is probably the 1% of cells existing as clumps even at the most effective level of sonication as will be discussed later.

4.2.2 DIFFERENT BUFFERS AND CELL CLUMPING IN *S. POMBE*

Sonication was shown in the previous section to be effective in breaking up clumps of cells in *S. pombe* suspensions, however it was also seen that even at the level of sonication which gave the greatest increase in apparent plating units not all the clumps were fully dispersed. It was therefore decided to test the effect of using different buffer systems on cell clumping.

S. pombe, unlike some other yeasts, is not known to flocculate, however some cultures do show the tendency to clump (Mitchison, 1970). The buffers used to try to reduce the level of clumping in the *S. pombe* suspensions included M/15 phosphate buffer (Nasim, 1967) and 40mM citrate buffer (Nasim, 1985) both of which are routinely used in *S. pombe* work.

It has been shown (Jansen and Mendlik, 1951) that removing bi- and poly-valent cations prevented flocculation from occuring in normally flocculent yeasts, for this reason the effect on cell clumping of phosphate buffer + 10mM EDTA was tested.

Jansen and Mendlik have also shown (Jansen and Mendlik, 1953) that pure sodium salts of alkyl sulphates, such as SDS, are adsorbed onto yeast cell surfaces causing a decrease in flocculance amongst flocculating yeasts. Thom and Raper report in A Manual for Aspergilli (Thom and Raper, 1945) that 0.01-0.001% SDS was successfully used for separating spores of *Aspergillus*. Consquently phosphate buffer + 0.001% SDS was tested for its effects on cell clumping.

0.001% Tween 80 was used as a wetting agent in experiments on *A. nidulans* by de Bertoldi and Griselli (1980) for separating conidia and so was also tested for its effect on cell clumping in *S. pombe* as 0.001% Tween 80 in phosphate buffer.

The effectiveness of the five buffer systems described above in reducing cell clumping was measured against a distilled water control. The experiment was carried out by harvesting a *S. pombe* culture grown in YEA , washing and

Figure 4.2a

Effect of Different Buffers on Cell Seperation

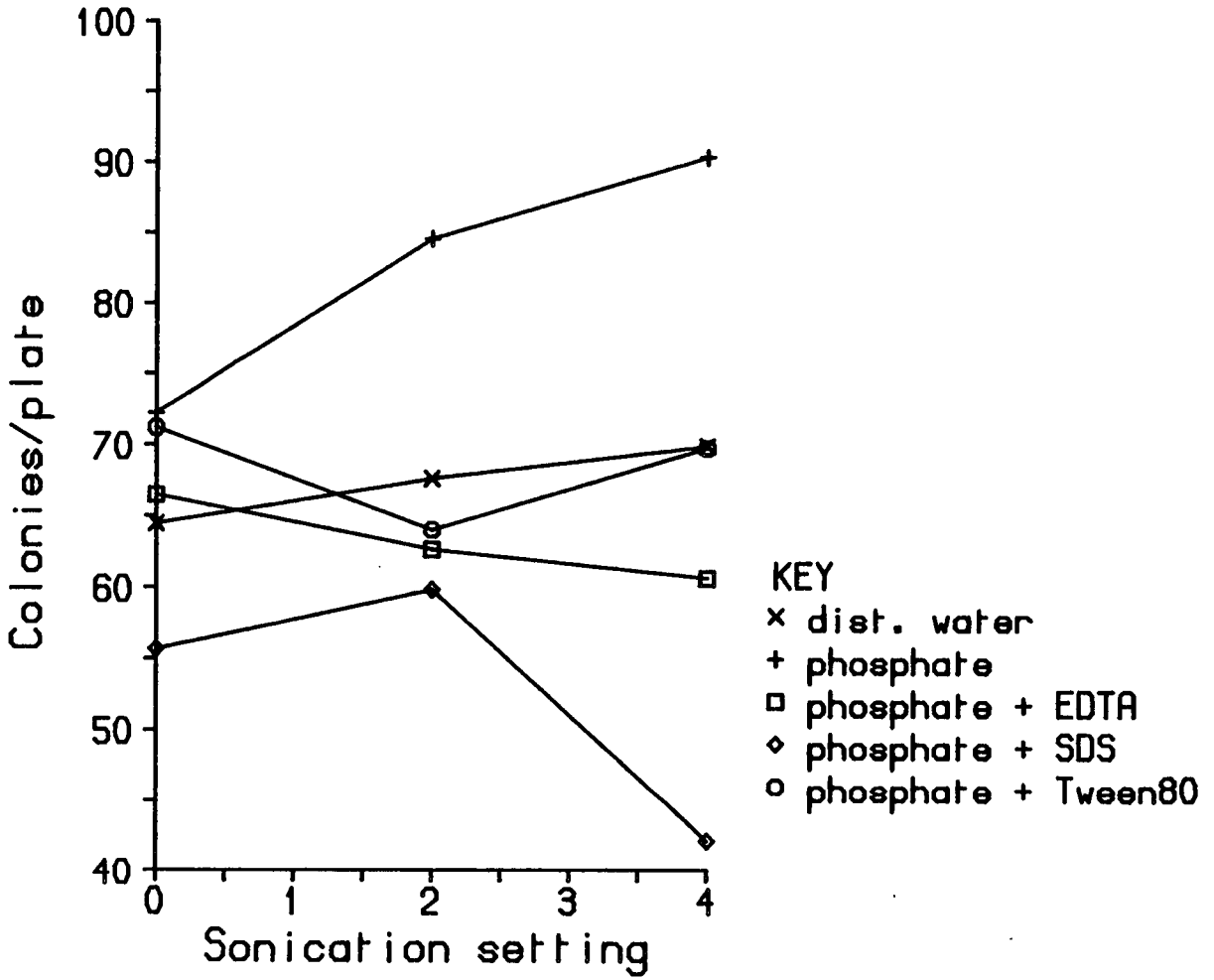
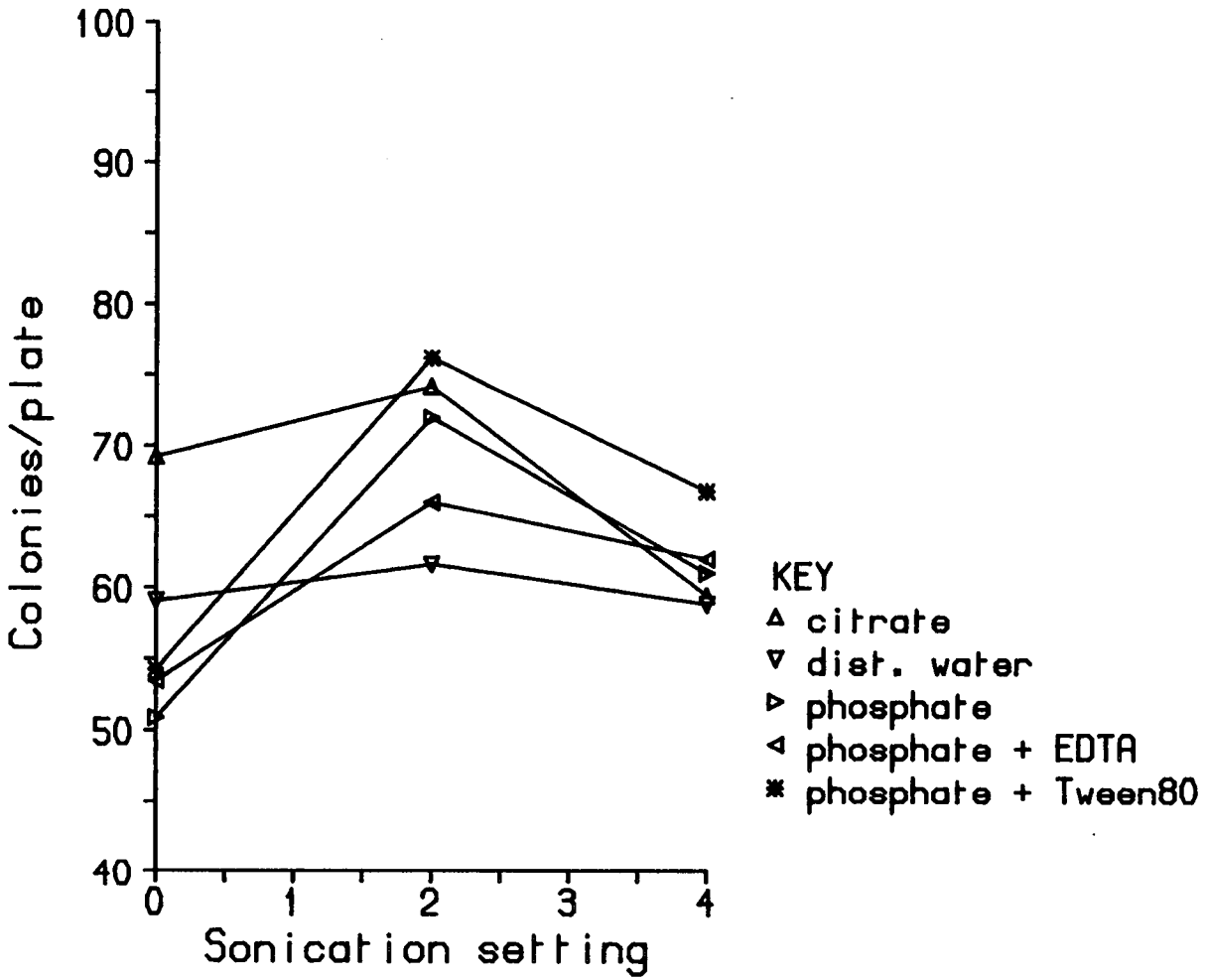


Figure 4.2b

Effect of Different Buffers on Cell Seperation



resuspending the cells to 1 X 10^7 cells/ml in the appropriate buffer. Sonication was carried out as described in the Methods at power settings 2 and 4. The treated suspensions along with an untreated control were diluted and plated. After several days' incubation at 30°C the plates were scored for the number of colonies on a plate. The average value of the replicates was worked out and the apparent number of plating units calculated. The results are depicted graphically in figures 4.2a and 4.2b (raw data in Appendix B).

None of the buffer systems tested appeared to give a significantly larger reduction in cell clumping than any of the others. The apparent increase in plating units was reproducibly marginally greater for phosphate buffer, but due to the large standard deviation of all the values this is not statistically significant. Phosphate buffer was finally selected as the buffer of choice for all experiments involving sonication, dilution and plating as it was routinely used for making cell suspensions for both EMS and UV mutagenesis.

4.2.3 PLATING DENSITY AND SPURIOUS MOSAICISM

Another factor contributing to the level of spurious secondary mosaicism is the density at which a cell suspension is replated. *S. pombe* colonies have to be grown to a size of 2-3mm in diameter in order to allow both the appearance of the red pigment, characteristic of *ade6* and *ade7* mutants and for the development of the mosaic colonies generated by a replicating instability. It is consequently possible that two cells with different phenotypes (ie red and white) may land sufficiently close to each other on a plate to give a mature colony which appears to be a mosaic.

To overcome the problem of spurious mosaicism it has been common practice in past work to replate primary, EMS generated, mosaics at plating densities as low as 20 colonies per plate (James *et al*, 1972). To take this approach a stage further and to be able to predict with confidence the greatest number of expected spurious secondary mosaics for a given plating density a series of experiments was carried out to give a standard curve for % spurious mosaicism against cell plating density. To achieve this standard curve, phenotypically different cells were mixed together and plated out, without mutagenic treatment. Any apparent mosaic colonies seen to arise on these plates had to be spurious or false mosaics, assuming that the chance of a replicating instability arising spontaneously is vanishingly small. This would

appear to be a fairly safe assumption in the light of results published by Friis *et al.* (1971) which show that the spontaneous mutation rate for the five loci (*ade 1, 3, 4, 5* and *9*) controlling the adenine biosynthetic pathway before *ade6* is about 2 X 10^{-6} per mitosis.

In controlling the number of possible variables it was decided to plate cells at a known, constant ratio of red to white colonies. The ratio chosen was 1:1 as this gives the greatest probability that if two cells land sufficiently close together to grow as one apparent colony they would be of different phenotypes (ie red and white). This would give the worst possible case for spurious mosaicism, and if this could be successfully reduced to a very low level then secondary mosaics arising from primary, EMS, mosaics plated under the same conditions could be identified with confidence as being genuine replicating instabilities. In order to control the ratios of red and white cells in the cell suspension the cultures used were grown separately in liquid culture and the density of the culture determined before harvesting.

The experiments were carried out by harvesting the cells, washing and resuspending in phosphate to the required cell density. The cell supensions were then mixed and sonicated, as described in the Methods before serial dilution and plating. After 3 days' incubation at 30°C the plates were then scored for the numbers of red, white and mosaic colonies on each plate. The average density of colonies per plate was calculated within replicates. The percentage of spurious mosaics was calculated from the number of apparent mosaic colonies observed in each set of replicates. The experiments were carried out in such a way that each replicate contained about the same number of colonies in total. This was approximately 1,700 colonies per replicate in the case of the *972h-/ ade6.704h-* (or *ade7.50h-*) curve and 1,200 colonies per replicate for the *ade6.704h-* (or *ade7.50h-*)*/ ade1.40h-* curve. Although this meant that different numbers of plates were used at different cell densities, it means that each point on the standard curve should have equal weight.

Two standard curves were constructed, one for *972h-* (phenotypically white) with *ade6.704h-* or *ade7.50h-* (both phenotypically red), the other was *ade1.40h-* (phenotypically white) with *ade6.704h-* or *ade7.50h-*. This would allow both of the mutation systems that give rise to red/white mosaic colonies on mutagensis to be studied. The two systems are the *972h-* (white) to *ade6.704h-* or *ade7.50h-* (red) , see Guglieminetti *et al*, 1967, Loprieno *et al*,

Figure 4.3

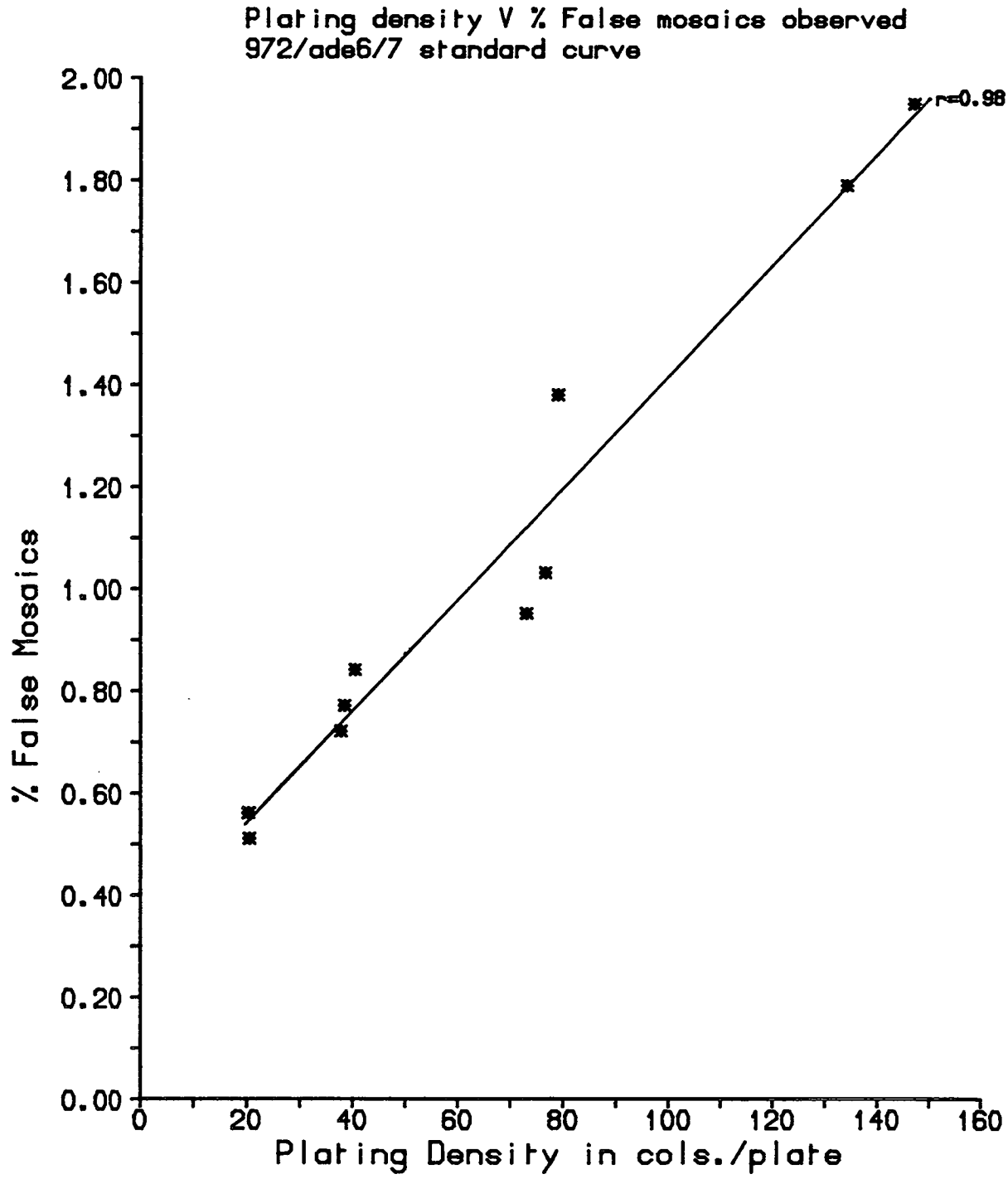
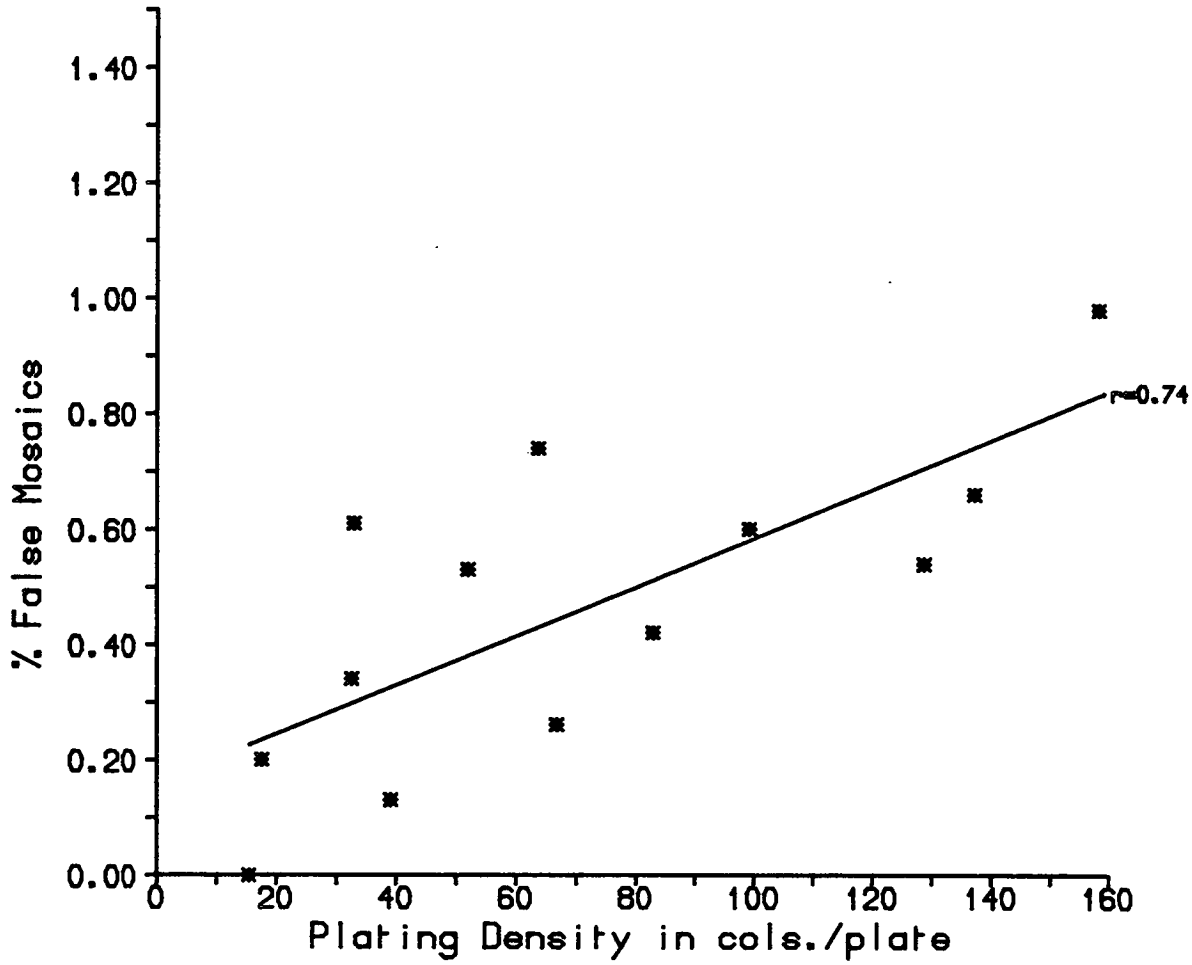

Plating density V % False mosaics observed
972/ade6/7 standard curve
r=0.98
% False Mosaics
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
20
40
60
80
100
120
140
160
Plating Density in cols./plate

Figure 4.4

Plating density V % False mosaics observed
ade6/7/ade1.40 standard curve



1968; and the *ade6.704h-* or *ade7.50h-* (red) to *ade6/7 ade1/3/4/5* double mutants (white), see Nasim, 1967, Nasim and Grant, 1973.

It was necessary to construct two standard curves because the strains used have different growth rates on YEA. Wild-type strains of *S. pombe* grow much faster than *ade6* and *ade7* mutants, strains carrying mutations at one of the preceding loci (*ade 1, 3, 4* and *5*) and double mutants (eg. *ade 1/ade 6*) have an intermediate growth rate. These differences in growth rate could mean that levels of spurious mosaics could be different for the two systems. The results of these experiments are shown in in figures 4.3 and 4.4 (raw data in Appendix B).

The data obtained can best be described over the range of plating densities used by a linear model. Regression lines were fitted to the data and found to have correlation coefficients, r, of 0.98 and 0.74 for the *972/ade6/7* and the *ade6/7/ade1* curves respectively. These high values for the correlation coefficient strongly suggest that the points vary in a linear fashion. To find out if the lines are statistically significant an analysis of variance was performed for each set of data (using standard regression analysis). An analysis of variance performed on the data for the *972/ade6/7* line showed that the regression line drawn is significant to well beyond the p=0.001 or the 0.1% level. In other words the probability of getting such data by chance is much less than 0.1%, this means that in statistical terms the line is highly significant. When the same analysis is carried out for the *ade6/7/ade1* regression line it was found that $p=0.005 > p > p=0.001$. In other words the regression line is signficant to the 0.5% level but not to the 0.1% level, this is still highly significant in statistical terms (values of p=0.05 or the 5% level are generally accepted as being statistically significant).

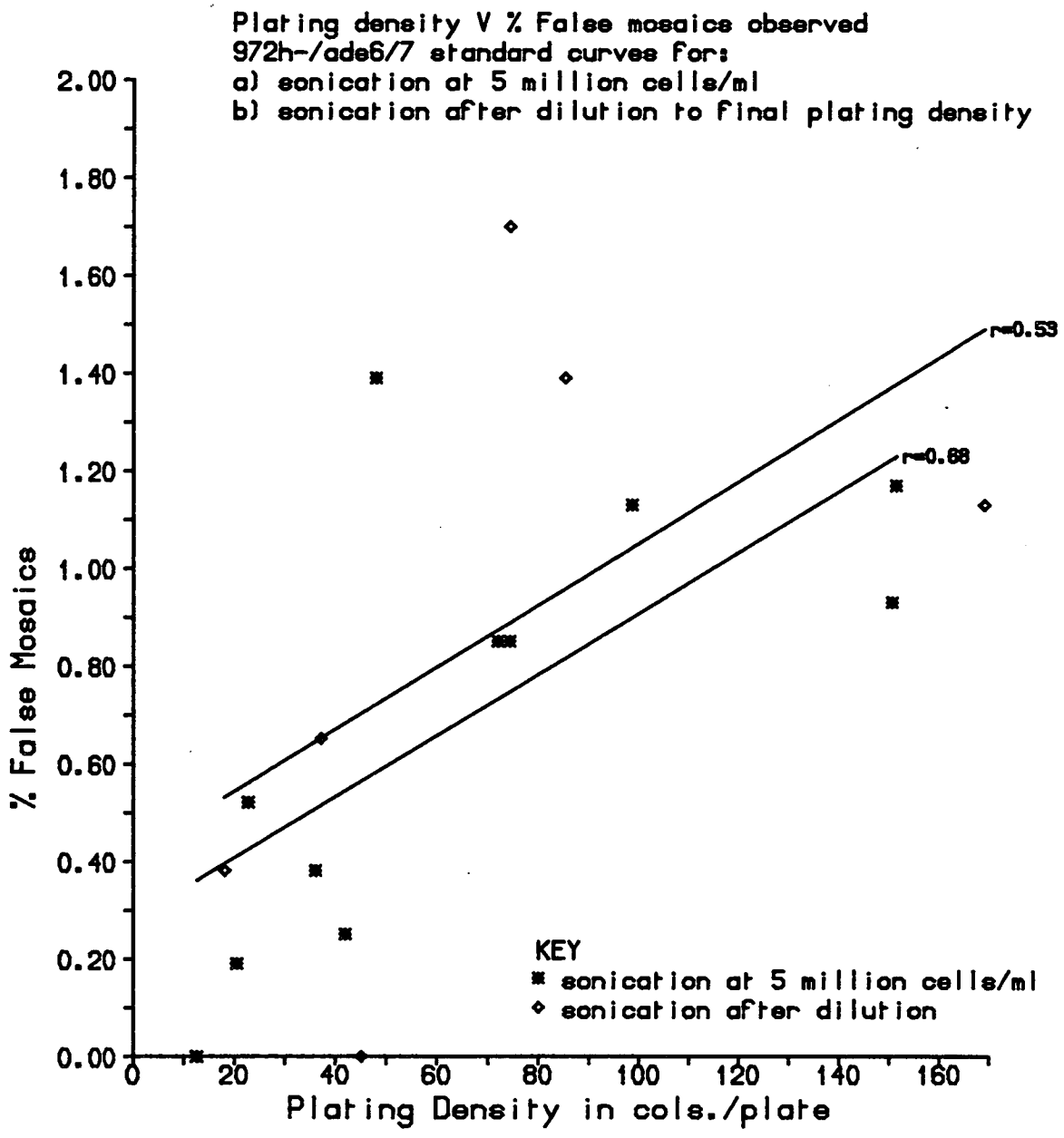
Although the two regression lines differed in slope they both contained features which raised grave doubts about the validity of replating primary mosaics as a method for identifying replicating instabilities. The first point was that the level of spurious mosaicism was much greater than had been expected, for example at 30 colonies per plate one can expect on the basis of these results to get an average of 0.65% spurious mosaics simply by plating a 1:1 mixture of *972h-* and *ade6.704h-* (or *ade7.50h-*) cells. Similarly at 30 colonies per plate the level of spurious mosaicism observed for a 1:1 mixture of *ade1.40h-* and *ade6.704h-* (or *ade7.50h-*) cells would be, on average, 0.29%.

Also the slope of the lines was greater than was expected which meant that the observed level of spurious mosaicism rose rapidly with the plating density used.

In conclusion these results make two points, one of which may be of fundamental importance to the argument as to whether replating primary mosaics is an acceptable method for identifying replicating instabilities. The first observation is that the level of spurious mosaicism varies linearly with the plating density used. The second and more crucial observation is that the level of spurious mosaicism is much higher at very low plating densities than might be expected on the basis of earlier work. The experimental set up used in generating the data for the standard curves was re-examined with a view to identifying and eliminating this second feature. As shown earlier in the chapter the effects of sonication and of varying the buffers used for making the cell suspensions had already been investigated and optimal conditions for the reduction of cell clumping decided on for each. When the standard curves were constructed the cells had been mixed at a density of 1 X 10^7 cells/ml (chiefly because this is the cell density used for mutagenesis). It was thought that perhaps this could be the cause of the high level of spurious mosaicism. To see if this was in fact the case two further experiments were carried out, this time just one of the two original systems was used, the *972h-* and *ade6.704h-* (or *ade7.50h-*) system. In one experiment the cell density at which the *972h-* and ade 6/7 *h-* cells were mixed was reduced from 1 X 10^7 cells/ml to 5 X 10^6 cells/ml. The rest of the experiment was carried out exactly as described before. In the second experiment the cell samples were mixed at 1 X 10^7 cells/ml but were sonicated after dilution to the cell density used for plating out. In this experiment the cell density on sonication therefore varied depending upon the final plating density desired (for example if it was desired to plate cells at an average density of 50 colonies per plate then the cells would be serially diluted to 250 cells/ml before sonication). In any case the cell density was always below 1000 cells/ ml on sonication. The results were measured and analysed in the manner described above and can be seen in figure 4.5 (raw data in Appendix B).

Regression lines were again fitted to the data. The cells mixed and sonicated at 5 X 10^6 cells/ml gives a correlation coefficient, r, of 0.68 the line being significant to the 5% level on analysis of variance. This means that there is a strong correlation of the data points with linearity and that the regression

Figure 4.5


Plating density V % False mosaics observed
972h-/ade6/7 standard curves for:
a) sonication at 5 million cells/ml
b) sonication after dilution to final plating density
% False Mosaics
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
20
40
60
80
100
120
140
160
Plating Density in cols./plate
r=0.53
r=0.68
KEY
sonication at 5 million cells/ml
sonication after dilution

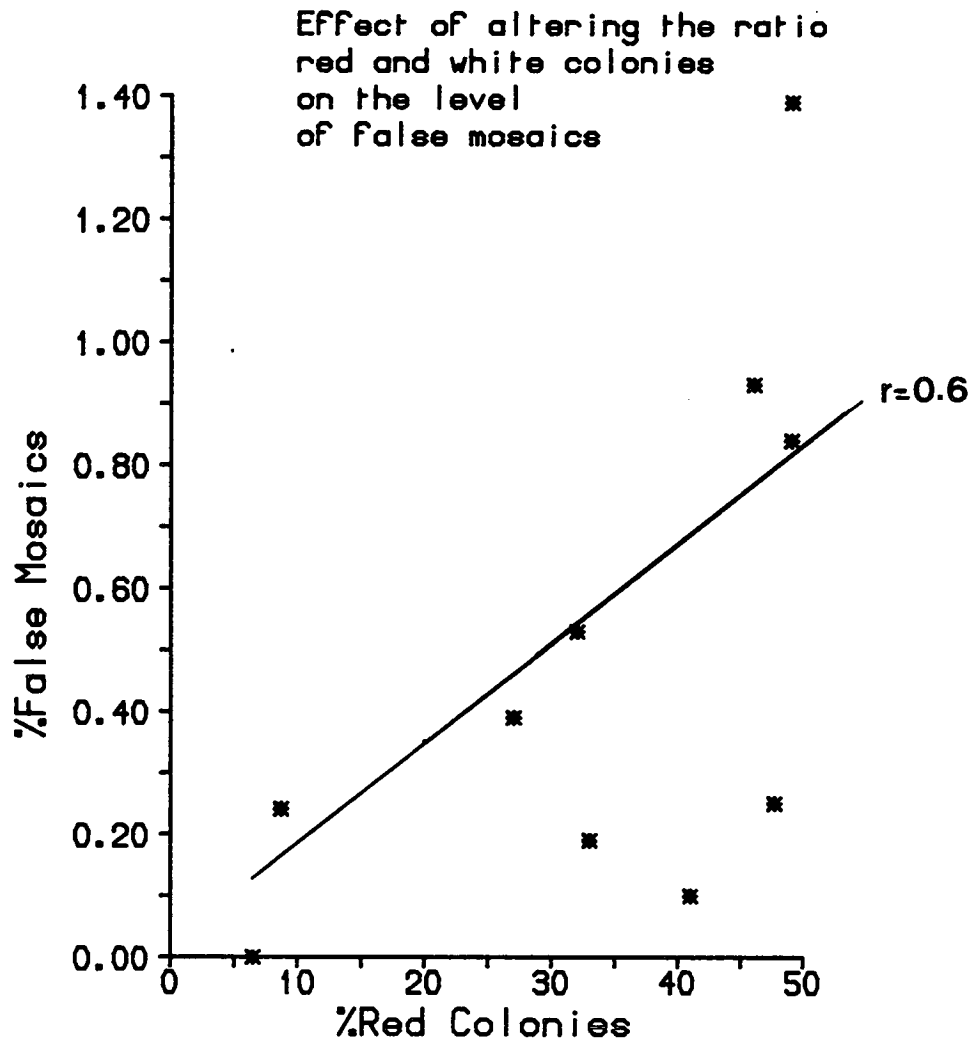
analysis is statistically significant. For the cells sonicated after dilution the points fit a linear model but not with a high degree of certainty that the model is appropriate, the correlation coefficient being 0.53 (a correlation coefficient of 1 indicates perfect correlation with linearity , whereas a correlation coefficient of 0 indicates no correlation with linearity). The analysis of variance of this line showed no statistical significance ($p>0.1$ or the 10% level). This does not make the line invalid but more likely points out the fact that there are too few data points in this experiment (only 6).

The result of the first experiment indicates that altering the density at which the cells are mixed reduces the level of spurious mosaicism; however analysis carried out to find out if the 5×10^6 cells/ml and the 1×10^7 cells/ml standard curves are different showed no statistically significant differences. Leaving aside the question of statistical significance for the second experiment the results seem to indicate that it apparently makes no difference to the level of spurious mosaicism observed whether the cells are sonicated before or after serial dilution. Indeed if all the data obtained for cell suspensions originally mixed at 1×10^7 cells/ml are added together then a regression line with a correlation coefficient of 0.85 can be drawn through the points. It would appear from the results of these two experiments that the density at which cells are suspended on sonication has little bearing on the level of spurious mosaicism observed. In terms of the objective of reducing the level of spurious mosaicism to a point where one would expect on average no spurious mosaics at all for a given plating regime, it appeared that this approach had not been successful. It was becoming obvious at this stage that, in my hands at least, the technique of replating primary EMS-generated mosaics was unlikely to be able to fulfil the objectives stated at the start of this chapter.

4.2.4 ALTERING THE GENOTYPE RATIOS AND SPURIOUS MOSAICISM

Having established that the level of spurious mosaicism on a plate was linearly related to the cell plating density over the range of plating densities used, it was decided to see if the ratio of red and white colonies on a plate had a further effect on the level of spurious mosaicism. This was done by holding the plating density constant at 30-40 colonies per plate and altering the ratio of red to white cells in the mixture. The experiment was carried out by mixing cell suspensions at 1×10^7 cells/ml in the manner described above. The results are depicted graphically in figure 4.6 (raw data in Appendix B).

Figure 4.6


Effect of altering the ratio
red and white colonies
on the level
of false mosaics
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
%False Mosaics
0
10
20
30
40
50
%Red Colonies
r=0.6

As expected the level of spurious mosaicism varied linearly with the ratio of red to white colonies on the plate. Regression analysis of the data gave a correlation coefficient of 0.6, the line being significant to the 5% level.

From the results of the last two sections it is clear that it is possible to construct a set of standard curves which would describe every ratio of cell types and plating density. Using such curves it would be possible to predict the of level spurious mosaicism expected under all conditions. In terms of the stated objectives of this piece of work, however, this would not be an acceptable solution to the problem of identifying with confidence mosaic colonies which are genuinely the result of a replicating instability. At best with such an approach using, for the sake of argument, plating conditions under which the level of spurious secondary mosaicism was estimated to be 0.2% but the observed level of secondary mosaicism 0.4% one could say that on average one in two of the mosaics observed should be the product of a genuine replicating instability.

In case some primary, EMS generated, mosaics did show levels of secondary mosaicism well above the background level of spurious mosaicism predicted by the standard curves about 20 primary mosaics were plated out under the same conditions as were described for the generation of the plating density controls. A yeast colony when resuspended in 5ml of buffer has on average a cell density of 3-5 X 10^6 cells/ml. Cultures of *972h-*, *ade6.704h-* and *ade7.50h-* were treated with EMS as described in the Methods, primary mosaics resulting from the mutagenic treatment were resuspended in 5ml of phosphate buffer, sonicated, serially diluted and plated out. After several days' growth at 30°C the plates were scored for red, white and mosaic colonies. The results of the experiment are shown in figures 4.7 and 4.8 (raw data in Appendix B), and the appropriate regression lines for the plating density controls are drawn in for comparison.

In many cases the levels of secondary mosaicism observed were well below the levels predicted by the regression lines, this probably reflects the fact that in many cases the ratio of red to white cells in the primary mosaics was far from being 1:1.

In conclusion it appears that replating primary EMS-generated colonies is not a satisfactory method for identifying replicating instabilities. This

Figure 4.7

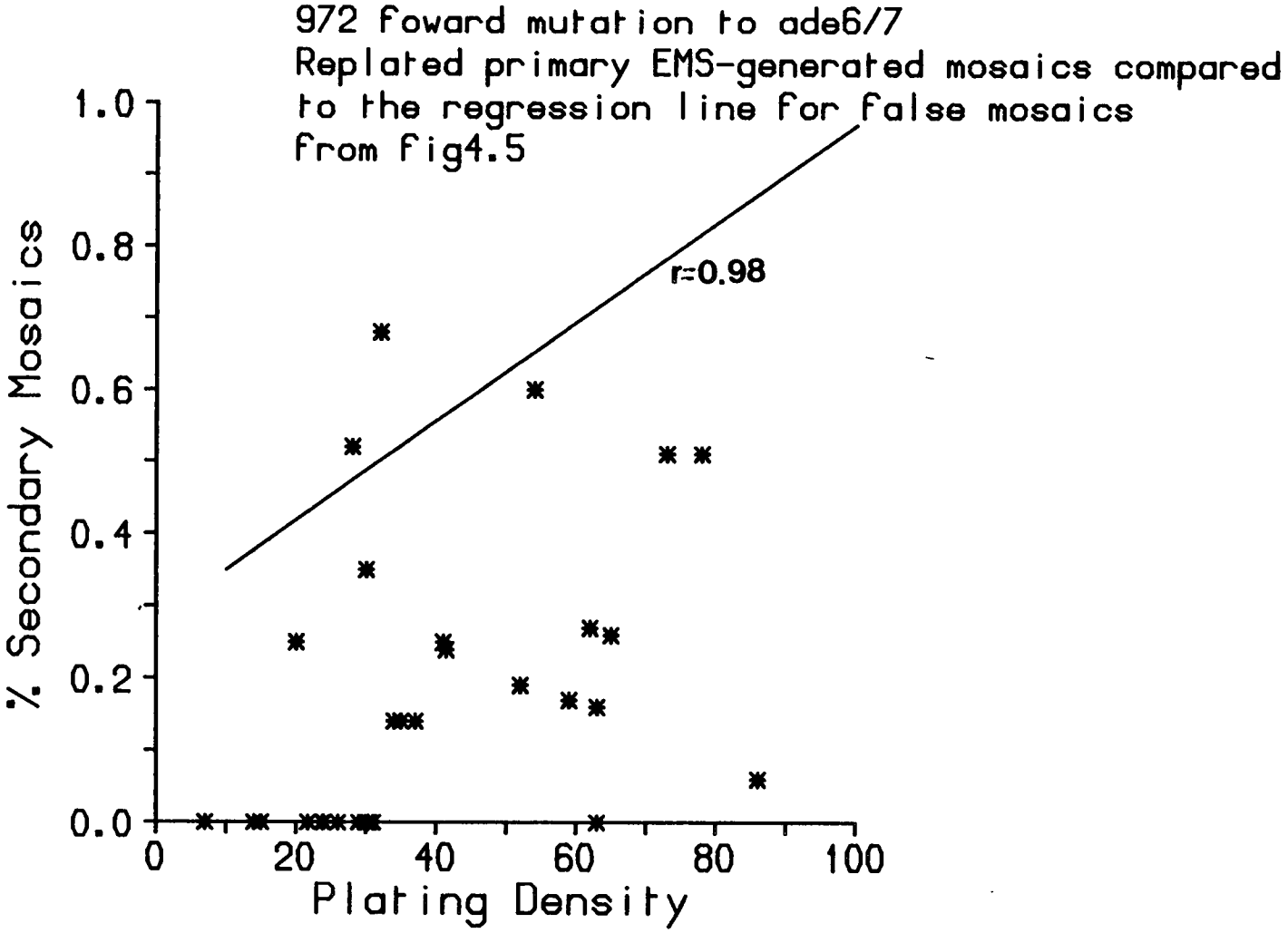
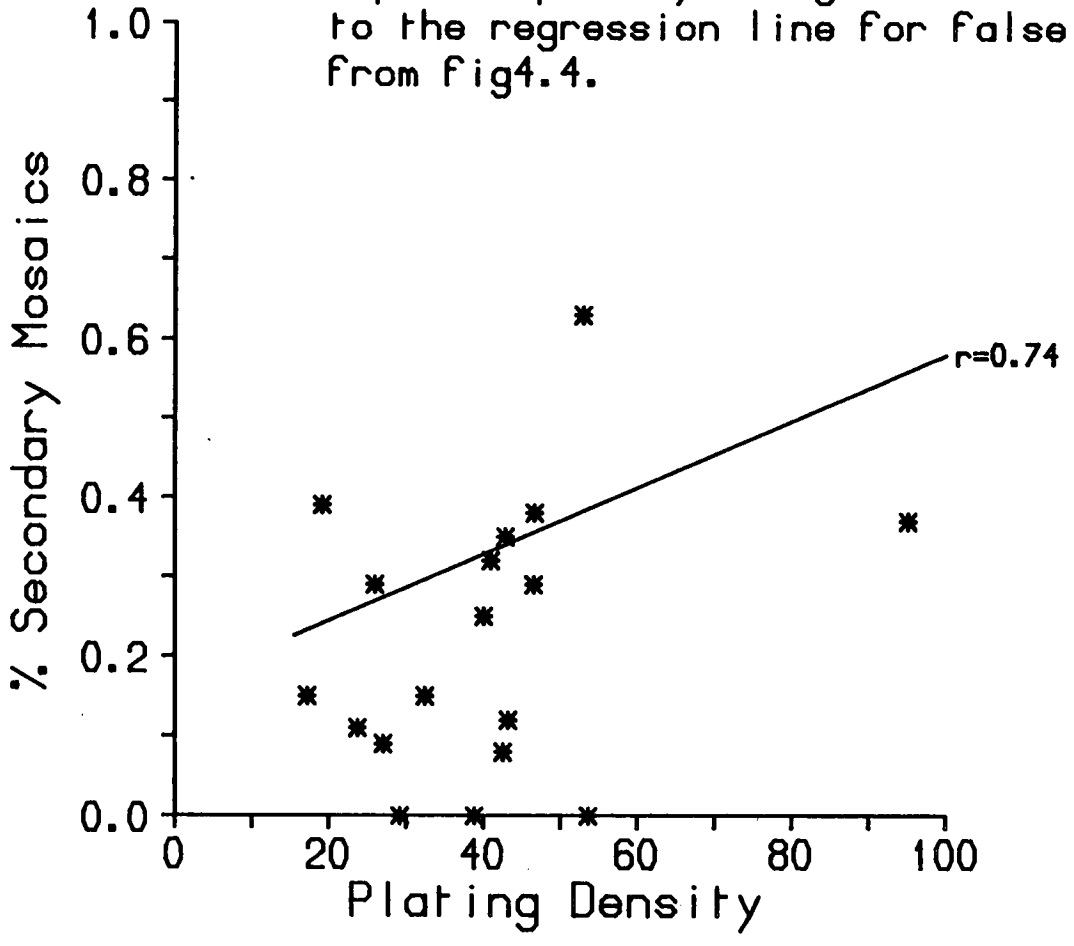

972 foward mutation to ade6/7
Replated primary EMS-generated mosaics compared
to the regression line for false mosaics
from fig4.5
r=0.98
% Secondary Mosaics
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Plating Density

Figure 4.8

Foward mutation from ade6/7 to ade6/7 adex,
Replated primary EMS-generated mosaics compared
to the regression line for false mosaics
from fig4.4.



conclusion is in conflict with those previously published on the subject, where it has been repeatedly claimed to be a sufficient test to diagnose replicating instabilities (see Nasim 1967). The difference is unlikely to be due to the quality of the data presented here, which are on the whole highly statistically significant. It could however be the result of having used different techniques to those in the original papers. There were two reasons why the original techniques were not followed, the first being, as already discussed, the desire to be able to control the ratios of red and white cells in a cell suspension. The second reason is that it would have been very difficult to repeat earlier work exactly as, in many of the papers, full descriptions of the methods used were not given.

The inability to make direct comparisons does not invalidate either this or earlier work, as long as the controls used in each case can be shown to be adequate. A major criticism of the present work could be that the control system used was based on mixing and plating separately grown liquid cultures, whereas mosaics arise as a sectored colony on a plate. It appears from the results in section 4.2.1 that the most probable cause of the high levels of spurious mosaicism observed at very low plating densities is the failure of sonication to disperse all the cell clumps in a suspension. In this case, it is arguable that the technique used here should ,if anything, underestimate the levels of spurious mosaicism as the two cultures are mixed just before plating. It was found impossible to design control experiments which were unflawed. For example the replating of synthetic mosaics has often been used as a control for spurious mosaicism and yet this does not allow any experimental control over the ratios of the genotypes in the colonies. If the replating of synthetic mosaics is an adequate control for the level of spurious mosaicism inherent in a given experimental system then surely it should not result in an estimate for spurious mosaicism which varies five-fold between experiments. However, in 1971 using this technique the level of spurious mosaicism in an experiment was estimated to be 0.04% (Nasim and James,1971), yet in 1972 the same group published an estimated level of spurious mosaicism of 0.21% using the same method (James *et al*, 1972). It is not possible to know if the experimental technique was exactly the same in the two cases as in one case no details are given of the plating densities used and in the other the ratio of the two cell types is not stated. Another practical difficulty is the fact that in dealing with a rare event a difference of only a few mosaics makes a large

TABLE 4.2

Comparison of the frequency of secondary mosaics from EMS and synthetic primary mosaics

Source of Data	Type of mosaic colony	Average plating density colonies/plate	Total colonies examined	Number of secondary mosaics	% Secondary mosaics
Nasim and James, 1971	EMS-generated synthetic	30-40	22,490 42,163	55 16	0.24 0.04
James et al, 1972	EMS-generated synthetic	NOT REPORTED	40,070 30,901	394 64	0.89 0.21
This study (ade 7.00h$^-$ -> ade 7.50 adex)	EMS-generated synthetic	39	25,594 10,684	62 17	0.24 0.16
This study (972 h$^-$ -> ade 6/7)	EMS-generated synthetic	35	18,092 3,040	37 17	0.56 0.56

difference in the observed percentage of mosaicism. In other words the system is inherently subject to random fluctuation in the observed levels of mosaicism.

In general it is not fruitful to draw comparisons directly between previously published data and the data presented here, for the reasons discussed above; however there is one case where this is possible. As the plating of synthetic mosaics was so frequently used as the control for the replating of primary, EMS generated, mosaics, it was decided that this approach should also be tried in this work. The synthetic mosaics were generated by plating a mixed culture of red and white cells, without mutagenic treatment. The spurious mosaics arising on these plates were picked, resuspended and sonicated as described above, before serial dilution and plating. The results are summarised in table 4.2, along with previously published data as a comparison.

In the work published to date it can be seen that in each case there is a significant difference between the estimated level of spurious mosaicism and the level of secondary mosaics obtained by replating primary mosaic colonies, however there is considerable fluctuation between the two papers. In the results of this work there is no significant difference between the levels of spurious mosaicism and the numbers of secondary mosaic colonies seen on replating primary mosaic colonies; the fluctuation between the two groups of results is lower and also is in rough agreement with those published by James *et al.* in 1972 for the level of spurious mosaicism. This result underlines the conclusion that the data presented here were valid.

Nonetheless Nasim published two pieces of extra data which do lend considerable support to the conclusion that replating is an adequate technique with which to identify replicating instabilities. In 1967 Nasim published work relating to the levels of secondary mosaicism observed when the primary mosaics were derived using different mutagens. It was found that although EMS, MNNG and UV generated primary mosaics which gave rise to secondary mosaics at a frequency of about 0.8%, those generated using NA and HA gave rise to 0% and 0.005% secondary mosaics respectively. Using the argument that a significant proportion of secondary mosaics are really spurious mosaics caused by cell clumping it is hard to explain these results without having to suppose that one of the groups of mutagens causes long-term effects on the cell surface. However neither the plating density used nor the ratios of

colonies observed is reported. When Loprieno carried out a similar forward mutation experiment using NA (Loprieno et al, 1968), however, it was found that mosaic colonies were observed for up to 4 plating generations after treatment. In Loprieno's work the levels of secondary and subsequent mosaicism ranged between 0.003% and 2.08%, however, there was no mention of controls in this paper. In the 1967 paper Nasim also publishes the results of mixing together and replating cells from a mosaic and a white colony where the two whites had different genotypes ~~are reported~~ The experimental system is depicted diagramatically in figure 1.6. It was found that in 60 out of 64 secondary colonies the mosaics had the same genotype as the primary mosaic used, and none had the genotype depicted as being the predicted result of cell clumping. It is not stated, however, whether or not the mixed cell suspension was sonicated before plating.

This clever approach is still subject to the criticism that one still cannot distinguish with certainty between a mosaic colony which is the result of a genuine replicating instability and a spurious mosaic arising through cell clumping, as it would not be at all surprising if most of the cells existing in clumps should be clumped with cells from the colony in which they had originally grown. This argument is particularly pertinent to these results where it is unknown whether sonication was used to disperse clumps of cells or not.

Despite there being flaws in both sets of work, the conclusion that replating primary mosaics generated by mutagenesis does not represent a sufficient test to diagnose the existence of replicating instabilities seems valid both with respect to the objectives stated in this chapter and, possibly, in general. The stated objective of this chapter was to reduce the level of spurious mosaicism associated with replating mosaic colonies to a level where it would be possible to justify the molecular analysis of an individual mosaic colony, however, this has not been found to be experimentally possible. The conclusion has to be reached whichever set of data is analysed that to select a colony for further analysis would be to play Russian Roulette with statistics.

4.3 SUMMARY

In sum, analysis of the factors influencing the level of spurious mosaicism inherent in the plating technique was undertaken, with the objective of finding conditions under which spurious mosaicism could be reduced to a minimum. If this was achieved then replating would have a very high probability of yielding genuine replicating instabilities for molecular analysis.

The results show that sonication is highly effective in reducing clumping of cells, but that the buffer used seems to have no effect on cell clumping. It is also shown that a linear relationship exists over the plating densities used between the density at which colonies are plated and the level of spurious mosaicism observed. A linear relationship is also found between the ratio of red to white colonies on a plate and spurious mosaicism. Comparison of replated primary EMS generated mosaics with the standard curves generated show there is little difference between the levels of mosaicism seen in the two cases. It was concluded that the plating technique did not identify genuine secondary mosaics with a sufficiently high probability to warrant the molecular analysis of secondary mosaics arising from replated primary EMS-generated mosaics.

CHAPTER 5
SINGLE CELL ISOLATION FROM MOSAIC COLONIES

AND TESTING CULTURES FOR DIPLOIDY

5.1 INTRODUCTION

The conclusion reached in the previous chapter was that replicating instabilities could not be selected reliably enough using the replating technique to warrant the molecular analysis of secondary mosaic colonies. The chief drawback to using plating as a selection system is that it is impossible to prove that a colony has arisen from a single, isolated cell. The only way to overcome this problem is by the micromanipulation of single cells to discrete locations on an agar slab. Indeed as a result of similar criticisms of the plating technique Nasim and James (1971) carried out single cell isolation from EMS-generated mosaic colonies using micromanipulation. This represents the strongest evidence to date for the existence of replicating instabilities in *S. pombe*. In this paper experiments were described in which cells from secondary mosaics were streaked along the edge of an agar block and individual cells then placed at well spaced locations on the block. Nasim and James found that 10 out of 5,816 cells isolated from 8 secondary mosaics gave rise to mosaic colonies, a mean frequency of 1.7×10^{-3} (0.17%) tertiary mosaics. This value is 10^3 greater than the spontaneous mutation rate for the five loci preceding *ade6* in the biosynthetic pathway (as measured by Friis et al, 1971), so these results cannot be attributed to spontaneous mutations. One of the most interesting features of replicating instabilities is that the mutation generated when a replicating instability is resolved is reported to be at the same locus in each successive generation (Nasim and Grant, 1973). Unfortunately this report is based on replicating instabilities selected using the plating technique, consequently it is disappointing that Nasim and James (1971) apparently did not determine the loci at which the mutations had arisen in the secondary and tertiary mosaic colonies when using the single cell isolation technique. On the basis of the work carried out by Nasim and Grant (1973) it was decided to use single cell isolation by micromanipulation to identify genuine replicating instabilities by following them for at least one further plating generation and checking the genetic nature of the stable mutants produced.

EMS and UV-generated mosaics were chosen for micromanipulation since these two mutagens have been reported (Nasim 1967, 1974) as being among the most effective in generating replicating instabilities. Three types of mosaic colonies were initially selected for micromanipulation:

1. Primary EMS-generated mosaics
2. Secondary EMS-generated mosaics
3. Primary UV-generated mosaics

Cultures of *S. pombe* grown to stationary phase in YEA contain a mixture of 78% G2 and 22% binucleate cells (Abbondandolo and Bonatti,1970) which leads to the prediction that many if not all mutations arising directly after mutagenic treatment should occur as mosaic rather than complete mutants. This prediction has indeed been fulfilled in the work of others (Abbondandolo and Bonatti, 1970; Abbondandolo and Loprieno, 1967; Nasim and Clarke, 1965) and in this work (see chapter 3). In addition, EMS acts primarily at the replication fork (Kilbey, 1984) and would therefore be expected to also give rise to mosaic colonies after mutagenesis. None of these mosaics are expected to be replicating instabilities. The latter might therefore represent a small proportion of the mosaics observed. However when estimates are made of the proportion of primary mosaics which are replicating instabilities (ie give rise to secondary mosaic colonies), the figure of 80% is frequently quoted. This is based on replating experiments carried out by Nasim (1967). As has already been shown (see chapter 4) replating primary mosaic colonies is an unsatisfactory method for identifying genuine replicating instabilities. In the experiments to be described here primary mosaics were selected for micromanipulation despite the probable low frequency of replicating instabilities. In case the proportion of primary mosaics which were in fact replicating instabilities was too low, primary mosaic colonies were also replated at very low densities after sonication and the resultant mosaic colonies were then analysed by micromanipulation. Neither approach, it will be appreciated, is ideal.

5.2 RESULTS AND DISCUSSION

5.2.1 MICROMANIPULATION OF PRIMARY MOSAIC COLONIES

In designing the experimental protocol a choice had to be made between micromanipulating many cells from one or two mosaic colonies or analysing a few cells from many mosaic colonies. Neither approach is ideal, if the rate of

induction of replicating instabilities amongst mosaic colonies arising from mutagenic treatment is less than the 80% which has been quoted then inspecting just a few primary mosaic colonies might not reveal any genuine replicating instabilities because the mosaic arose for other reasons. However if the level of unstable cells in a mosaic colony is low, possibly because their rate of resolution is high, then by inspecting just a few cells from many colonies it would not be surprising if many genuine replicating instabilities were missed. Inevitably therefore the method of analysis used has to be a compromise between these two extremes. It was decided to analyse about 200 viable cells from half a dozen colonies in each of the three categories above.

Should any of the primary mosaics analysed give rise to secondary mosaics it would be worth expending more effort to see if these then gave rise to tertiary mosaics following further micromanipulation. The other criterion usually adopted for a genuine replicating instability is that it should be locus specific, that is that the forward mutation should occur at the same locus in each successive generation. Consequently it would be necessary to test by complementation some of the white colonies which arise after single cell isolation from a mosaic colony at each generation to ensure that the mutation was locus specific.

The micromanipulation of mosaics from the three categories described above was carried out as described in the Methods and the results are summarized in tables 5.1, 5.2 and 5.3. The pedigree of those secondary mosaics selected for micromanipulation is given in table 5.4.

It can be seen from the results in tables 5.1, 5.2 and 5.3 that it was possible to isolate secondary mosaic colonies in all 3 categories tested. In the 3 cases where these secondary mosaics were analysed by micromanipulation tertiary mosaics were found to arise; in these cases all the white colonies had the same genotype (as determined by complementation analysis) in both colony generations and so fit the definition of a genuine replicating instability. The three replicating instabilities isolated were unstable at three different adenine loci so they could not be due to spontaneous mutation of a highly unstable locus which is mutating independently of the mutagenic treatment [cf Esposito's work on spontaneous mutants in the adenine biosynthetic pathway of *S. cerevisiae* where 43% of all the mutants arose at *ade6*, the equivalent to *ade3* in *S. pombe* (Esposito, 1967)]. Assuming that each of the five genes

TABLE 5.1

Results of single cell isolation from primary EMS-generated mosaics

Strain		1st colony generation of single cell isolates					2nd colony generation of single cell isolates				
		Red	White	Mosaic	Total	Locus of forward[1] mutation	Red	White	Mosaic	Total	Locus of forward[1] mutation
ade6.704 h^-	1	27	166	0	193	-					
	2	90	66	0	156	-					
	3	59	137	0	196	-					
	4	92	127	1	220	ade4 (5/5)	330	3	2	335	ade4 (3/3)
	5	114	105	0	219	-					
	6	178	32	1	211	ade3 (4/4)	215	18	3	236	ade3 (3/3)
ade7.50 h^-	1	58	284	0	342	-					
	2	35	164	0	199	-					
	3	130	58	0	188	-					
	4	122	105	1	228	ade3 (5/5)				N/D[2]	
Totals				3(0.13%)	2152				5(1.06%)	471	

Notes

[1] These figures show the number of independent tests of the locus of the forward mutation in the white colonies arising in the same colony generation as the mosaics to which they refer

[2] N/D = not determined.

TABLE 5.2

Results of single cell isolation from secondary mosaic colonies
(derived by replating primary-EMS generated mosaics)

Strain	1st colony generation of single cell isolates					2nd colony generation of single cell isolates[1,3]				
	Red	White	Mosaic	Total	Locus of forward mutation	Red	White	Mosaic	Total	Locus of forward mutation
ade6.704h$^-$ 1	310	12	4	326	ade1 (7/7)	515	16	1	532	ade1 (4/4)
2	49	185	0	234		173	12	1	186	ade1 (3/3)
3	34	222	0	256						
4a[2]	81	114	0	195						
4b[2]	72	119	0	191						
5	61	152	0	213						
			4(0.29%)	1415				2(0.28%)	718	

Notes

[1] See note 1, Table 5.1

[2] 4a and 4b are two separate mosaics which arose from the replating of a primary mosaic

[3] The two mosaics were taken from the four mosaic colonies which arose from single cell isolation from ade6.704h$^-$.

TABLE 5.3

Results of single cell isolation from primary UV-generated mosaics

Strain	1st colony generation of single cell isolates					2nd colony generation of single cell isolates				
	Red	White	Mosaic	Total	locus of forward mutation[1]	Red	White	Mosaic	Total	locus of forward mutation[1]
ade6.704 h^-										
1	132	41	0	173	-					
2	51	165	0	216	-					
3	29	255	0	284	-					
4	181	64	3	248	ade1 (4/4)				N/D[2]	
5	225	21	0	246	-					
6	97	68	1	166	N/D[2]				N/D[2]	
7	173	36	0	209	-					
Totals			4(0.2%)	1542						

Notes

[1] See Table 5.1 note 1

[2] See Table 5.1 note 2

TABLE 5.4

Results of Replating Primary EMS-generated mosaics.
Secondary mosaics used in Table 5.2.

Strain	Colony	Average plating density	Standard error	Number of secondary mosaics	Total colonies examined
ade6.704 h^-	1	13.1	±3.9	1	328
	2	26.1	±4.4	1	431
	3	15.8	±3.3	1	284
	4	16.9	±4.6	2	489
	5	15.4	±3.7	3	463

preceding *ade6* in *S. pombe* has an equal spontaneous mutation rate then the probability of getting identical spontaneous mutation for two generations in three different lines by chance is $(0.2)^6$ or 1 in 6.4×10^5, a vanishingly small figure.

It is difficult to draw any firm conclusions about the frequency of secondary mosaicism based on a relatively small sample size like this. However, the results confirm the conclusion that replating primary mosaic colonies derived from mutagenic treatment is of little assistance in specifically identifying genuine replicating instabilities. When the number of replicating instabilities determined by micromanipulation of primary and secondary EMS-generated mosaic colonies is compared it can be seen that 3/11 primary mosaic colonies gave rise to secondary mosaic colonies and 1/6 secondary mosaic colonies (derived by replating primary mosaics) gave rise to tertiary mosaic colonies. If it were possible to select replicating instabilities by replating at very low plating densities one would expect the number of replicating instabilities identified from secondary mosaic colonies to be greater than the number identified from primary mosaic colonies.

If the totals of the number of viable cells and mosaic colonies in the 1st generation derived by micromanipulation are added together for all 3 categories then it is found that 11 secondary mosaics were observed from 5,109 single cells (0.22%), a figure in agreement with the figure of 10 in 5,816 cells (0.17%) published by Nasim and James (1971). It is questionable however if such a figure has any real meaning. A more useful conclusion to be drawn from these results is that as only 6 out of 24 primary mosaic colonies examined gave rise to secondary mosaics the proportion of all EMS and UV-generated mutants which occur as replicating instabilities has probably been over-estimated in the past, instead of the 80% usually quoted a more realistic figure might be 25%.

In conclusion the data presented here establish that it is possible to isolate genuine replicating instabilities by the micromanipulation of single cells from mutagenically derived mosaics. In this work the analysis has been taken a stage further than that presented by Nasim and James (1971) as for three replicating instabilities locus specificity was proved over two generations of single cell isolation. It can be said with confidence that these three cases, at least, fit all the criteria laid down for a genuine replicating instability.

One possible objection to the technique of isolating single cells by micromanipulation is that it rests on the assumption that all the cells in a colony are haploid. This may not always be the case as it is known that all haploid cultures of *S. pombe* contain at least some diploid cells. In at least one case a replicating instability has been shown to be the result of diploidy in the culture from which it was mutagenically derived (Kurennaya and Devin, 1985). Having established that these three replicating instabilities were genuine then it was necessary to check if they were diploids before molecular analysis could be considered.

5.2.2 TESTING THE UNSTABLE STRAINS FOR DIPLOIDY

Amongst the explanations for the occurrence of replicating instabilities in *S. pombe* put foward in the past were the suggestions that they might be the result of either diploids or aneuploids occurring amongst the haploid population of cells. *S. pombe* strains although normally haploid always contain a few diploid cells presumed to arise by endomitosis (Leupold, 1955); the frequency with which this event occurs has never been determined (Leupold, personal communication) but has always been thought to be low as such diploid cells are unstable. Such a diploid would of course be homozygous with respect to its mating type (ie *h+/h+* or *h-/h-*) and unable to sporulate spontaneously. Homozygous diploids of this type are called heterothallic diploids; in contrast, a *h+/h-* diploid is capable of sporulating without being crossed to another strain and so is called a homothallic diploid. Although an unstable diploid or aneuploid carrying a recessive mutation at one of the adenine loci would of course be a simple explanation for replicating instabilities it has always been considered to be an unlikely explanation as it would be necessary to postulate also that alkylating agents and UV cause a large increase in the the level of diploids or aneuploids in the population to account for the high frequency at which replicating instabilities are presumed to occur.

Until recently no attempt has been made to examine the effect of aneuploidy on the viability of *S. pombe* cells. However in 1985 Niwa and Yanagida published a definitive piece of work in which they showed that only disomics aneuploid for chromosome III are viable in *S. pombe*. As only *Ade5* and *Ade6* out of the genes involved in the adenine biosynthetic pathway are located on this chromosome it is obvious that aneuploidy cannot be the explanation for replicating instabilities, in *S. pombe* at least.

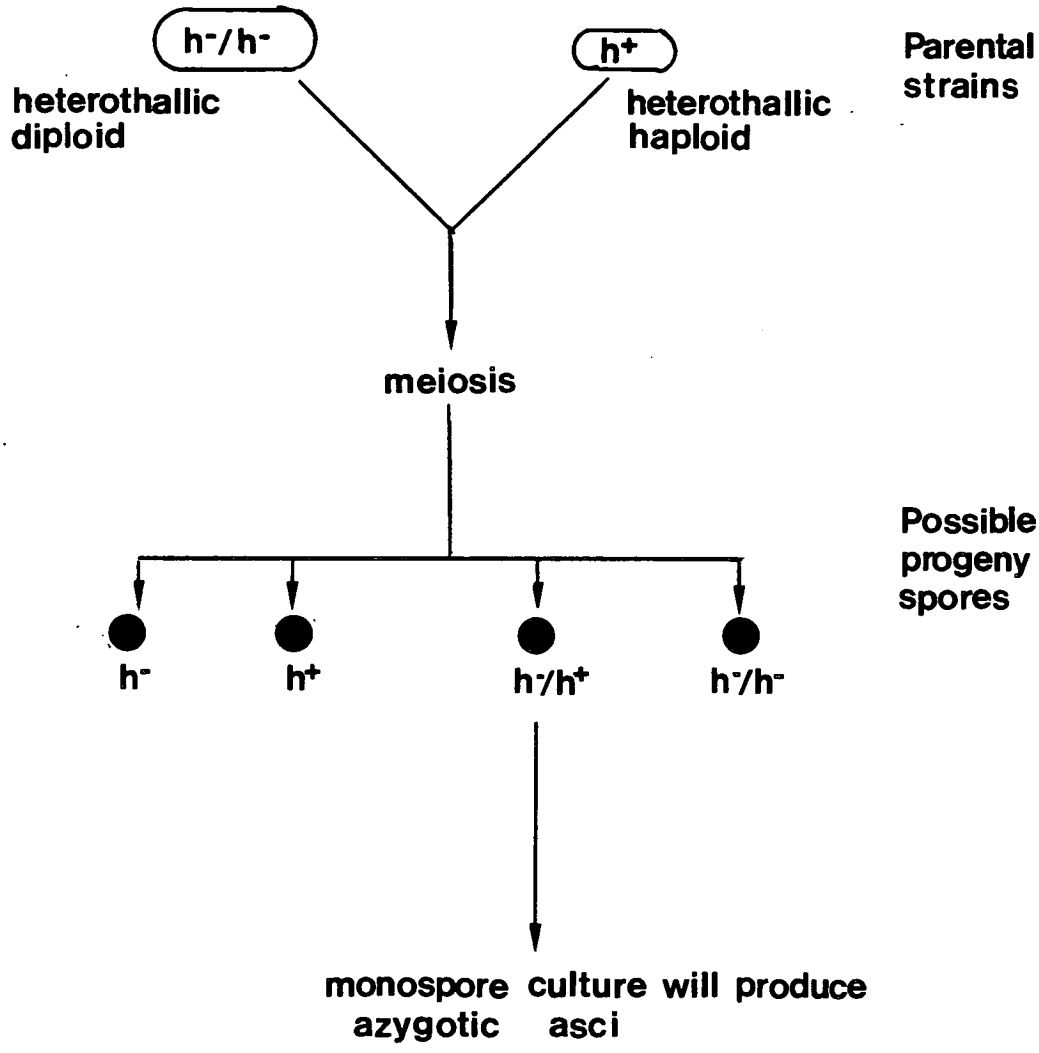
At the same time a paper was published by Kurennaya and Devin (1985) in which they showed that a UV-generated replicating instability on which they were working was a diploid carrying a recessive *ade3* mutation. In this paper the authors also report on their unsuccessful attempts to isolate genuine replicating instabilities from mosaic colonies resulting from the UV mutagenesis of a freshly isolated haploid *ade7.407h-* strain. On the basis of these results the authors concluded that all replicating instabilities in *S. pombe* are due to the presence of unstable heterozygotic diploids generated during mutagenic treatment. In reaching this conclusion the authors appear to have ignored the sizeable difference in the level of secondary mosaicism as reported by them (18.2%) and that reported by Nasim (*circa* 0.8%) for the replating of primary mosaic colonies.

The criteria used by Kurennaya and Devin (1985) for the identification of diploidy in *S. pombe* were low spore viability in conjunction with the production of azygotic asci from some of the spores after the putative diploid had been crossed to a normal haploid strain. The reason that low spore viability is considered indicative of diploidy in one of the parental strains can be found in the work of Niwa and Yanagida (1985) on triploid meiosis and aneuploidy in *S. pombe*. They found that less than 30% of the spores produced by triploid meiosis were viable as compared to 80-90% viability for spores from a normal diploid meiosis. The low spore viability is due to the fact that the only aneuploidy tolerated by *S. pombe* is disomy for chromosome III. When triploid meiosis occurs most of the spores which are viable segregate as haploids or diploids.

The second criterion for recognising a heterothallic diploid is the ability of some of the spores resulting from a cross between such a diploid and a haploid cell to form azygotic asci. Azygotic asci are those which arise in a colony, plated on to sporulation medium, without having been first crossed to another *S. pombe* strain of opposite mating-type. In a homothallic diploid, such as those resulting from the fusion of two cells of opposite mating type, sporulation occurs spontaneously at the end of vegetative growth. In contrast heterothallic diploids (ie those with the genotypes *h+/h+* or *h-/h-*) are unable to sporulate (Gutz et al, 1974). The replicating instability on which Kurennaya and Devin (1985) were working was thought to be a heterothallic diploid arising from endomitosis in an *ade7.407h-* strain (and hence was an *h-/h-* diploid). To prove the existence of such a diploid it is necessary to put the culture through

Figure 5.1

Triploid meiosis in S. pombe



a triploid meiosis with a normal *h+* haploid (see figure 5.1), under these conditions some of the resultant spores will be *h+/h-* diploids. When plated onto MEA or SPA these will be able to sporulate spontaneously, in other words they will now be able to form azygotic asci.

Kurennaya and Devin (1985) found that 17 spores from 120 asci in a cross of their replicating instability to a haploid *h+* strain gave rise to azygotic asci whereas they found 0 azygotic asci in spores from 116 asci analysed from control crosses of known haploids. In this paper Kurennaya and Devin do not mention the level of azygotic asci observed in colonies arising from these spores. In my opinion a low level of azygotic asci (<<1%) may be expected in a high proportion of monospore cultures due to spontaneous mutations from *h+* to *h90*. The rate of spontaneous mutations from *h+* to *h90* has been measured as being in the region of 3×10^{-4} per mitosis (Egel *et al.*, 1980), consquently in a normal *h+* colony of 2.5×10^{7} cells roughly 7,500 *h90* cells ($3 \times 10^{-4} \times 2.5 \times 10^{7}$) are expected. When such a culture is plated onto MEA these *h90* cells will form diploids and sporulate, so in most *h+* cultures plated onto MEA one might expect to see a very low level of azygotic asci. For this reason the criterion used in this work to recognise heterothallic diploids is slightly different from that used by Kurennaya and Devin, being a low level of spore viability in conjunction with a high level of azygotic asci.

The colonies selected for testing for diploidy were two red colonies, selected at random from each of the two colony generations for the *ade3* and *ade4* replicating instabilities plus one or more of the tertiary mosaics arising from these two replicating instabilities. The replicating instability which gave rise to *ade1* mutants was isolated later than the other two and so it was possible to directly test the original mosaic colony. This made it unnecessary to test any of the colonies arising from this original mosaic. Control experiments using freshly isolated haploids of *ade6.704* (*h+* X *h-*) and *ade7.50* (*h+* X *h-*) were carried out at the same time.

The experiment was carried out as described in the Methods; scoring the spores from crosses for azygotic ascus was done by visual inspection under a microscope. Where present the proportion of azygotic asci was estimated and the spore classed as being in one of the three following categories:

1. Containing >>10% azygotic asci
2. Containing 1-10% azygotic asci
3. Containing <<1% azygotic asci

It was not possible to measure accurately the number of azygotic asci as there were a large number of colonies to be scored and a limited time to do it in (even under refrigeration asci walls break down after about a week). A diploid spore would be predicted to fall into category 1 (ie >>10% azygotic asci) as it would be expected to give rise to a culture containing both diploid (*h+/h-*) and haploid *h+* or *h-* cells Such a culture when plated onto MEA would give rise to a large number of asci. Spores falling into the third category can be predicted to be those *h+* colonies where mutations giving rise to the self-sporulating *h90* strain have occurred. One might expect the majority of spores to fall into this class. Even by visual inspection of cell suspensions, therefore, it is possible to distinguish between these two events quite easily.

The results of this experiment are shown in table 5.5. A binomial statistical analysis of the data was carried out and table 5.6 contains the variance and the standard error for each of the results. A quick test to see if two results are significantly different is to see if they both fall within the range delinated by twice the standard error; if they do then the results are probably not significantly different.

Using this test it can be seen that there is no significant difference between the results obtained for the two control samples (*ade6.704h+* X*h-* and *ade7.50h+* X*h-*) so the results can be added together and compared to the results obtained for the stable red and mosaic colonies tested. Using twice the standard error as a method of comparison it was found that none of the colonies tested showed a significantly increased frequency of spores which fell into category 1 as compared to the control. On the basis of this result the conclusion can be drawn that there is no evidence for a raised level of diploidy amongst colonies arising from the genuine replicating instabilities isolated here as compared to the controls.

Kurennaya and Devin (1985) conclude on the basis of their results that the

TABLE 5.5

Spore viability and determination of the levels of azygotic asci arising from the spores of test and control crosses

Cross number	Cross	Type of colony[1]	% viable spores	Number of spores giving rise to azygotic asci			Total spores examined
				levels of asci seen			
				>>10%	1-10%	<<1%	
1	ade6.704(h^+ x h^-) control		82	6	3	29	126
2	ade7.50(h^+ x h^-) control		85	6	2	29	104
3	ade6.704h^+ x ade6/ade4h^- replicating instability[2]	1st Gen[3]/stable red	31	0	0	5	14
4	" x "	1st Gen[3]/stable red	88	0	0	3	28
5	" x "	2nd Gen[3]/stable red	81	0	0	2	34
6	" x "	2nd Gen[3]/stable red	100	1	0	4	22
7	" x "	2nd Gen[3]/mosaic	91	0	0	19	62
8	" x "	2nd Gen[3]/mosaic	67	0	0	5	28
9	" x ade6/ade3 replicating instability[2]	1st Gen[3]/stable red	46	3	0	0	10
10	" x "	1st Gen[3]/stable red	95	0	1	9	41
11	" x "	2nd Gen[3]/stable red	89	0	2	7	23
12	" x "	2nd Gen[3]/stable red	82	0	0	5	22
13	" x "	2nd Gen[3]/mosaic	68	0	0	5	21
14	" x ade6/ade1 replicating instability[2]	1st Gen[3]/mosaic	89	0	2	24	85

Notes

[1] Type of colony refers to the phenotype of the single cell isolate colony used.

[2] Genotype of unstable lines isolated in Tables 5.1 and 5.2

[3] 1st Gen = 1st colony generation of single cell isolates
2nd Gen = 2nd colony generation of single cell isolates.

TABLE 5.6

Analysis of binomial data presented in Table 5.5.

Cross Number (see Table 5.5)	Level of azygotic asci					
	>>10%		1-10%		<<1%	
	p	se	p	se	p	se
1	0.048	±0.019	0.024	±0.013	0.230	±0.038
2	0.068	±0.023	0.019	±0.013	0.279	±0.044
1+2	0.052	±0.015	0.022	±0.010	0.252	±0.027
3					0.357	±0.128
4					0.107	±0.058
5					0.059	±0.043
6	0.046	±0.044			0.182	±0.082
7					0.307	±0.059
8					0.179	±0.072
9	0.300	±0.145				
10			0.024	±0.024	0.220	±0.074
11			0.087	±0.059	0.304	±0.096
12					0.227	±0.187
13					0.238	±0.093
14			0.024	±0.016	0.282	±0.049

Analysis used:

$p = X/N$

$q = 1-p$

$se = \sqrt{pq/N}$

where X = number of spores in that category
N = total number of spores

most likely explanation for replicating instabilities was diploidy, but the results here are clearly at variance with this conclusion. The data relating to the control experiments also differs from that reported by them. Kurennaya and Devin report in their discussion that they observed 0 spores giving rise to azygotic asci in 116 tetrads analysed from test crosses, in contrast the results presented here show that 12 of 230 spores gave rise to azygotic asci at a level indicative of the spore having been a heterozygotic diploid (ie >>10%). If all haploid cultures of *S. pombe* contain some diploid cells (see Gutz *et al.*, 1974 and Egel et al, 1980) it might be expected that diploid spores should occasionally arise in any cross between two haploid cultures of *S. pombe*. Indeed if the data presented by Kurennaya and Devin (1985) is examined it can be seen that in at least one of the control test crosses (*ade7.704h-* X *975h+*) 5 spores out of 21 tetrads yielded azygotic asci. In the data presented here 8 of the diploid spores came from just two asci which suggests that in these cases two diploids fused to undergo tetraploid meiosis to give rise to 4 viable diploid spores.

The methods used in this experiment were based on those described by Kurennaya and Devin (1985). No mention was made in their paper of the possibility of azygotic asci arising as a result of *h+* to *h90* mutations, consequently I thought at first that azygotic asci would only arise from *h+/h-* diploid spores. Although, as discussed, it is relatively easy to separate these two events on the basis of the levels of azygotic asci seen it would still be preferable to have an unequivocable test for diploid spores. After consideration the solution to this problem turned out to be very simple. A diploid spore will give rise to a colony containing both *h+* and *h-* cells and so should give a positive iodine reaction with both *972h-* and *975h+* lawns when replica plated onto MEA (see Methods). In contrast a spore which is *h+* and contains some *h90* mutants will give a positive iodine reaction only when plated onto a *972h-* lawn; even if a few *h90* cells exist in a *h+* colony the low proportion of ascospores (<<1%) generated by these cells on a *975h+* lawn would never be seen in such a low resolution technique as this.

To see if this idea worked in practice a small scale control was carried out with two crosses (*ade6.704h-* X *h+* and *ade7.50h-* X *h+*), and the resultant spores were analysed as before (by visual inspection) and then the colonies were plated onto *972h-* and *975h+* lawns. By visual inspection only 1 of the spores analysed was found to have >>10% azygotic asci, 3 gave rise 1-10%

TABLE 5.7

Comparison of mating type and levels of azygotic asci seen in spores test crosses

Cross	Spores giving >> 10% azygotic asci		Spores giving 1-10% azygotic asci		Spores giving <1% azygotic asci		Total spores examined
	number	mating type	number	mating type	number	mating type	
ade6.704 (h^+ x h^-)	0	-	3	$3h^+$	14	$14h^+$	45
ade7.50 (h^+ x h^-)	1	h^+/h^-	0	-	15	$15h^+$	51

asci and 29 gave rise to <<1% asci. After treatment with iodine vapour the spore in the >>10% category gave a positive reaction with both the *972h-* and the *975h+* lawns and so must have been a *h+/h-* diploid, all the other spores gave a positive reaction with the *972h-* lawn only and so must have been as predicted *h+* spores. The results are shown in table 5.7. Unfortunately it was not possible to re-test the stable reds and mosaic colonies from the three replicating instabilities but in the future this would be the method of choice for identifying heterozygotic spores (ie *h+/h-* spores). Not only does it give unequivocal results but it is much quicker and easier to perform hence allowing larger scale experiments to be planned.

5.3 SUMMARY

In conclusion the results reported in this chapter show that in the three classes of mosaic colony investigated it was possible to isolate genuine replicating instabilities. The replicating instabilities isolated fulfilled both the criteria used to identify genuine replicating instabilities; that is, firstly they gave rise to multiple mosaic colonies over more than 1 colony generation and secondly, the mutations involved were locus specific.

Contrary to the findings of Kurennaya and Devin (1985) there was no evidence to support the idea that any of the three replicating instabilities isolated were diploids carrying a recessive adenine mutation. It is true that the evidence presented here does not show conclusively that the replicating instabilities isolated were not diploids but the data does strongly point to this being the case. A simpler and quicker method for identifying heterozygotic diploid spores of *S. pombe* was worked out and shown to be effective.

CHAPTER 6
MOLECULAR ANALYSIS OF THE *ADE1* INSTABILITY

6.1 INTRODUCTION

In the last chapter, 3 genuine replicating instabilities were isolated by micromanipulation of single cells from mutagen-derived mosaic colonies. Unlike the replicating instability isolated by Kurennaya and Devin (1985) there was no evidence to suggest that these replicating instabilities were heteroallelic diploids. The three replicating instabilities gave rise to different, locus specific mutants, with frequency at which the mosaic colonies arose at these loci being in the order of 2×10^{-3}, which is about 10^3 greater than the spontaneous mutation rate at these loci (Friis *et al*, 1971). It seems highly likely that the replicating instabilities obtained were therefore a consequence of the mutagenic treatment.

On the basis of these results it seemed reasonable to begin molecular analysis of the replicating instabilities obtained. The mechanism by which replicating instabilities are generated must have a basis in the lesions generated by mutagenic treatment. The majority of lesions observed after both UV and EMS mutagenesis are point mutations (Sega, 1984; Lawerence, 1982; Auerbach and Kilbey, 1971). This has been shown in *N. crassa* (Kilbey *et al*, 1971; Malling and de Serres, 1968), *S. cerevisiae* (Lawerence and Christensen, 1979,1978; Prakash, 1974) and *S. pombe* (Abbondandolo and Loprieno, 1967; Gutz *et al*, 1974). However evidence does exist to show that both mutagens are capable of promoting other types of lesion including non-revertible mutations presumed to be extensive intragenic deletions (Kilbey *et al*, 1971; Malling and de Serres, 1968). UV has been shown to cause both segmental interchanges (McClintock, 1945) and gross chromosomal rearrangements (Perkins and Radford in Fincham, Day and Radford, 1979) although it is not normally considered to cause structural changes in chromosomes. EMS has likewise been shown to give rise to structural changes such as translocations (Yost *et al*, 1967), deletions (Bishop and Lee, 1969), inversions (Syzlinka, 1963, 1963a), single stranded breaks (Sharma and Grover, 1969) and to promote gene conversion in stationary phase cultures of *S. cerevisiae* (Davies and Parry, 1976).

6.1.1 DUPLICATED SEGMENTS OF DNA AND INSTABILITIES

It has been suggested that unstable phenotypes might be the result of a duplication of the gene in question (Kohli, personal communication). Rambosek and Kinsey (1984) showed that the genetic instability of an *am* mutant of *N. crassa*, generated by nitrous acid mutagenesis, was due to a 33 bp duplication covering the splice site between intron 1 and exon 2. In the revertant the duplication is precisely excised.

Data from *S. cerevisiae* also show that duplicated regions of DNA behave in a mitotically unstable manner. This is thought to be largely due to gene conversion between duplicated regions (Jackson and Fink, 1981). Work done in Leupold's group on tRNA gene families in *S. pombe* also shows that conversion within gene families can give rise to both mitotic (Minet *et al*, 1980) and meiotic (Heyer *et al*, 1986) instability. It was shown by Ratz (1985) that in *S. pombe* a *SupX-UGA* active suppressor located <0.3 cM from *Sup10* on chromosome I showed an unusually high spontaneous mitotic reversion rate of between 10^{-3} and 10^{-4} (approaching the levels of instability seen in a replicating instability). The genetic instability of the *SupX* gene was shown to be a consequence of its proximity to the *Sup10* gene, the majority of revertants being caused by gene conversion.

A recent report by Hayles (1986) describes the unstable behaviour of a spontaneously arising duplication in *S. pombe*. Hayles found that a class of temperature sensitive mutants of *cdc2* had a spontaneous reversion rate of 4 X 10^{-6}. These revertants were themselves unstable, spontaneously mutating to *cdc*$^-$. Southern analysis of the revertants showed that they all contained an extra copy of the *cdc2* gene. The size of the duplication involved has not yet been precisely determined , but it is believed to be in excess of 100kb. The duplication of the *cdc2* region isolated by Hayles was found as a result of the selective pressure exerted by growing the *cdc* mutants at the restrictive temperature. Obviously, then, mutations alleviating the restricted phenotype such as the duplication in question are positively selected for in this case. Such selective pressure is unlikely to be operating on the *ade1* locus in the present study as the pathway is blocked at a second, later step as well. Consequently, if duplication is the explanation for the replicating instability observed, it would not be expected to give the cell in question any selective advantage.

In summary, there is good evidence to suggest that duplications of varying sizes can play a role in genetic instability and and the first example given, the duplication explaining the behaviour of an unstable *Neurospora am* mutant fits all the criteria used to define a replicating instability.

6.1.2 THE EXPERIMENTAL APPROACH USED

Whether or not the mutational event causing the instability is a point mutation or a structural change in the chromosome, the first step in the molecular analysis is the same, that is, a restriction analysis of the locus in question, and its flanking regions, to detect any gross structural changes. If the replicating instability resulted in structural changes in the chromosome, then Southern hybridisation using a suitable probe might reveal changes in restriction fragment sites between mutant and non-mutant alleles. This technique is limited by the size of the DNA fragments which can be separated by agarose gel electrophoresis (see Maniatis *et al*, 1982). It is possible to separate fragments over a range from 20kb to about 0.4kb by choosing the appropriate concentration of agarose. Changes in the DNA resulting in fragments larger or smaller than this will be missed using gel electrophoresis and Southern blotting. Point mutations in the chromosome will only be revealed after sequencing, unless a restriction site is destroyed or created. However, elimination of structural changes had to precede any sequencing programme. Ideally, molecular analysis of replicating instabilities at all adenine loci should be carried out. The conclusions reached here would carry more weight if it had been possible to carry out the molecular analysis of all three replicating instabilities isolated in this study. However probes were only available for the *ade1* locus making molecular analysis possible only in this case.

6.1.2.1 THE MUTANTS STUDIED

Six colonies which were descendants of the original *ade1* mosaic were used for molecular analysis. These were quaternary red, white and mosaic colonies isolated by single cell micromanipulation from two of the tertiary mosaics derived from the original *ade6.704/ade1* mosaic. 200ml stationary phase cultures of cells (grown in YEA + 75µg/ml adenine sulphate) were used for the preparation of total genomic DNA (see Methods).

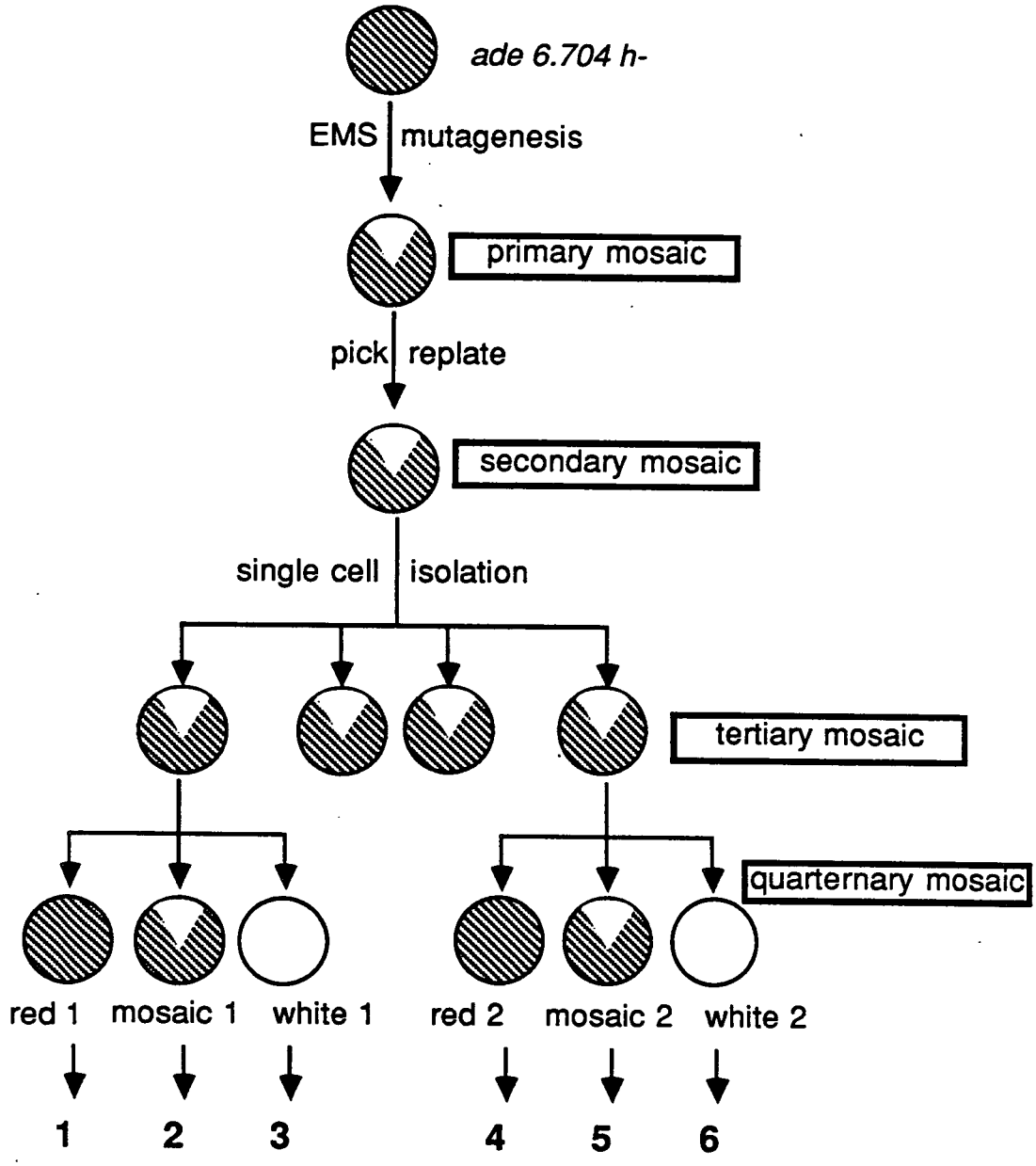


Figure 6.5. Pedigree of *Ade* mutants used for preparation of genomic DNA used in the Southern analysis.

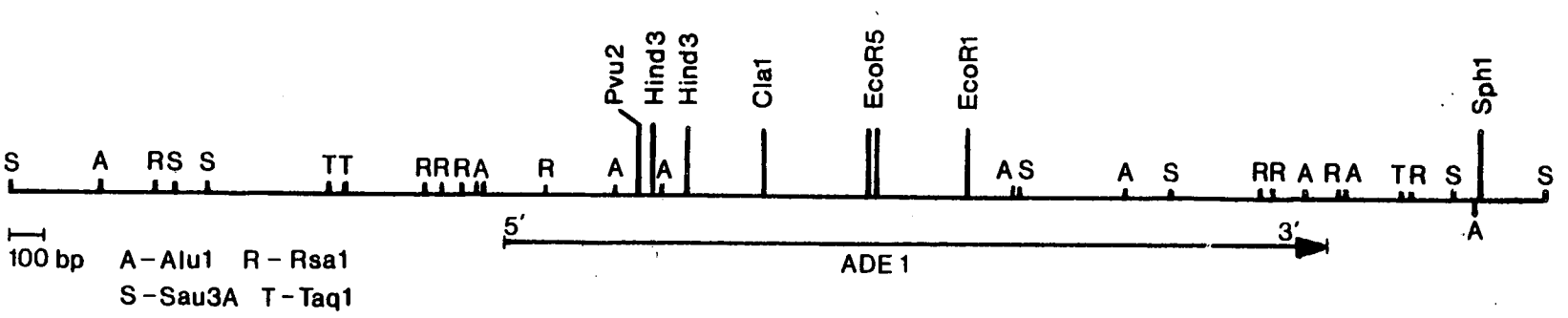
The cultures used were:

1. Red colony from secondary mosaic 1
2. Mosaic colony from secondary mosaic 1
3. White colony from secondary mosaic 1
4. Red colony from secondary mosaic 2
5. Mosaic colony from secondary mosaic 2
6. White colony from secondary mosaic 2

The pedigree of these six colonies is depicted in figure 6.5. At the same time, a loop of cells from each flask was streaked on YEA to estimate the phenotypic composition of each culture. The two red and two white cultures were found to have no colonies of the opposite phenotype. The mosaic colonies were found to contain less than 5% white colonies, in each case. White colonies from the two mosaic cultures were tested by complementation analysis and, in each case, 3/3 colonies were found to be *ade1*, so the mosaic colonies chosen for DNA isolation arose as a result of the replicating instability and not spontaneous mutations. Two facts are thus known about DNA preps. 1–6: firstly, they were all derived from the same original cell, two plating generations previously; and secondly, the red colonies were phenotypically wild-type with respect to the *ade1* gene.

6.1.2.2 THE PROBE

The *Ade1* gene was originally cloned by Peter Schuchert (Berne, Switzerland, unpublished data) from a library, constructed by inserting Sau3A partial digest fragments into the BclI site of the plamid pWH5. The construct, pPS6, contains the *Ade1* gene in a 4.5kb insert (see figure 6.6). Peter Schuchert also carried out some preliminary restriction mapping of the region of chromosome II in which the *Ade1* gene is located. The plasmid pPS6 was then sent to Edinburgh where the *Ade1* gene and its flanking regions have been sequenced by Rod McKenzie. A restriction map of the region is given in figure 6.7. The 5′ end of the *ade1* gene is situated 375 bases 5′ to the PvuII site and the coding sequence extends to about 1kb 3′ to the EcoRI site. The coding sequence is uninterrupted by introns and is some 2.4kb in length. The probe used was the 2.5kb HindIII fragment of pPS6 (see figure 6.8).



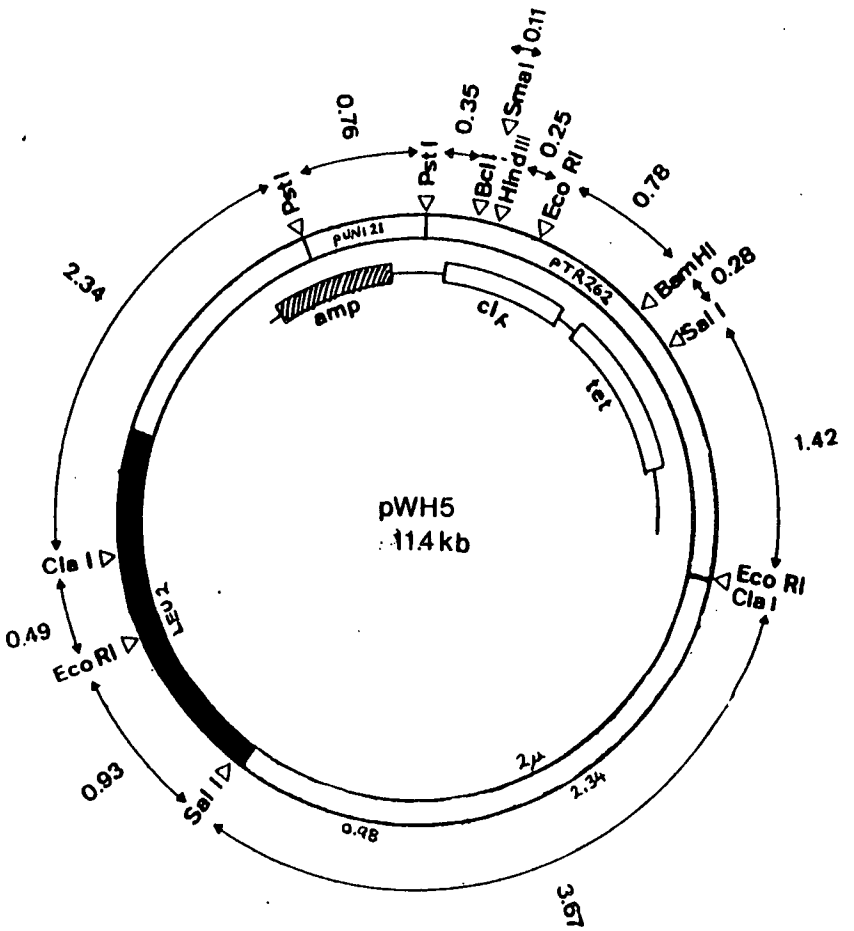


Figure 6.6

Showing the construction of pPS6, which is the Sau 3A fragment of S. pombe DNA, above, cloned into the Bcl I site of pWH5.

OPPOSITE FIGURE 6.7

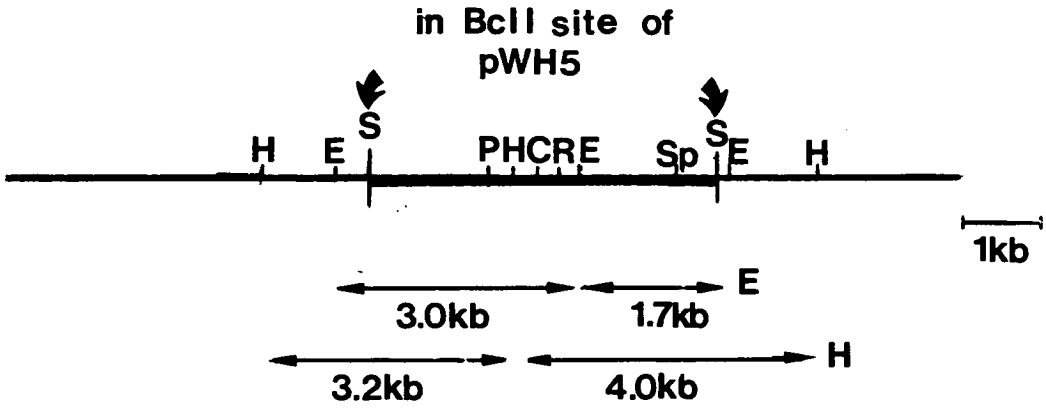
RESTRICTION SITES AROUND THE *ADE1* LOCUS ON CHROMOSOME II

Information from P. Schuchert, Berne.

Symbol	Enzyme	Hybridisation to *972* DNA
H	HindIII	3.2/4.0 kb
E	EcoRI	1.7/3.0 kb
S	Sau3A	-
P	PvuII	9.6/2.9 kb
C	ClaI	5.1/2.1 kb
R	EcoRV	9.0 kb
Sp	SphI	6.7/9.0 kb

No sites for the following enzymes in the insert:
PstI, XhoI, BamHI, SalI, XbaI
SmaI, HpaI, StuI, PvuI.

Figure 6.7


in BclI site of
pWH5
S
S
H
E
PHCRE
Sp
E
H
1kb
E
3.0kb
1.7kb
H
3.2kb
4.0kb

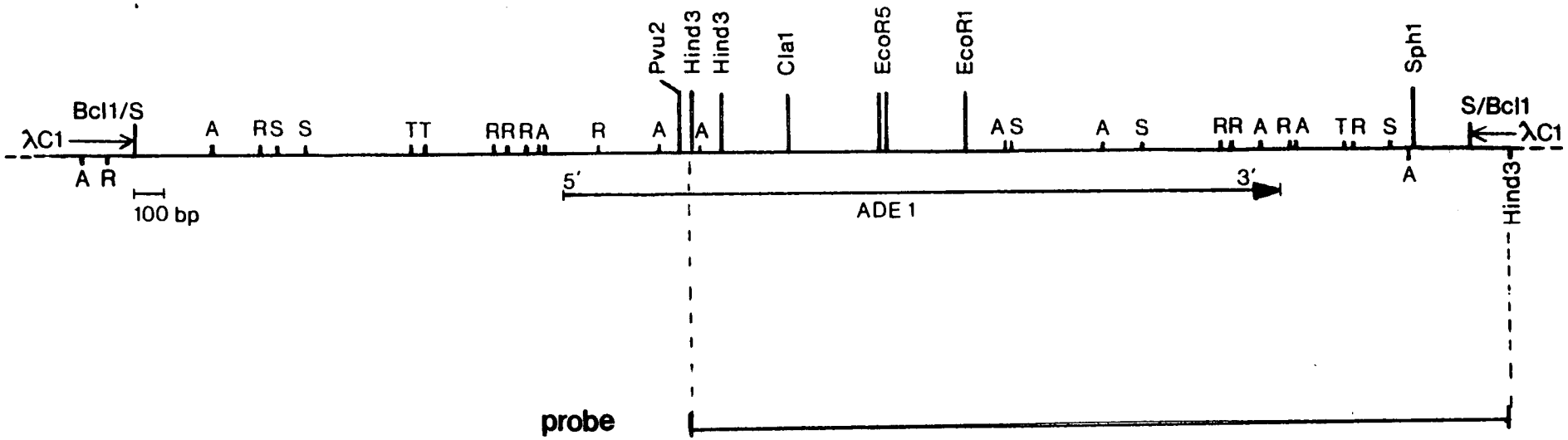


Figure 6.8

Showing the Hind III fragment of pPS6 used in Southern hybridisation experiments.

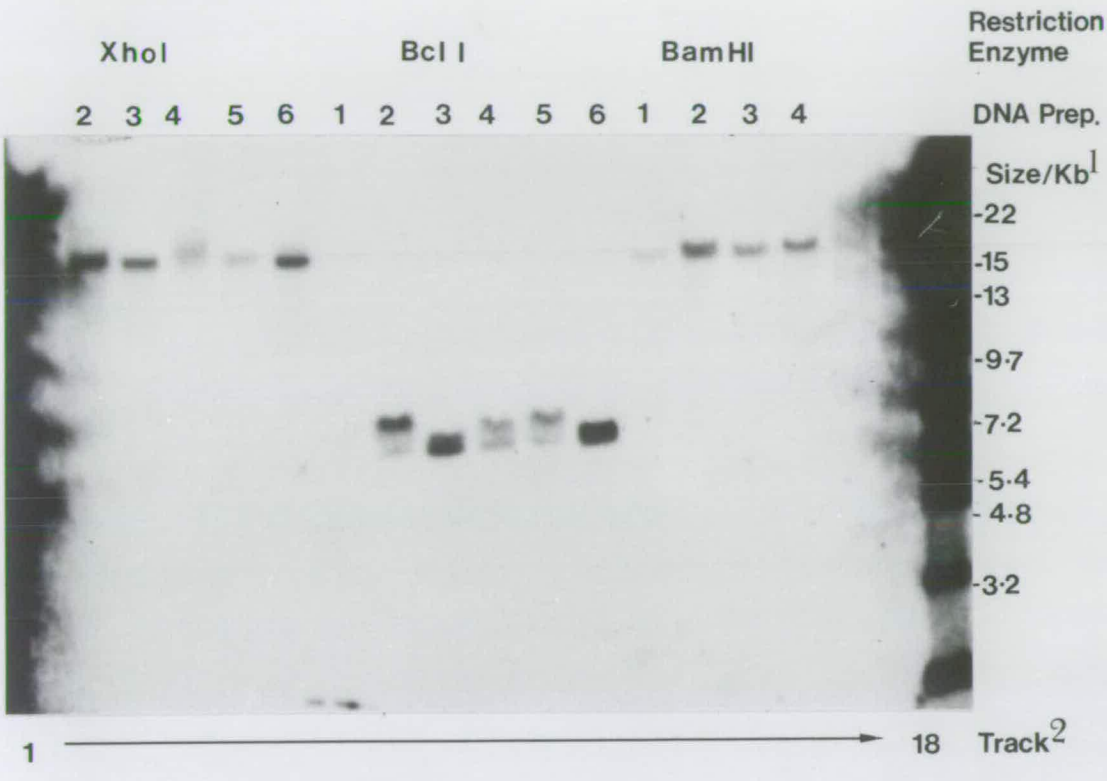
6.2 RESULTS

Two experiments were performed using the six different total genomic DNA samples described above. In each case single restriction enzyme digests were carried out firstly with cutters which left the gene intact and secondly with cutters which also cut within the region of the coding sequence covered by the probe. The restriction enzymes chosen which did not cut within the region of the probe were also infrequent cutters of DNA (on the basis of their cutting frequencies in Lambda and Adenovirus2). It was hoped that this approach would, in addition to revealing structural changes close to the region covered by the probe, also reveal changes further away from the *ade1* locus by producing large fragments of DNA (>10kb). The enzymes chosen for the first experiment were XhoI, BclI, PvuI, BamHI, ApaI and SalI. A combination of Lambda digests was designed to give a ladder of MW markers from 30-1kb (single digests of Lambda with SalI, EcoRI and BglII). The 3 Lambda digests were carried out separately and samples of each mixed together after the addition of "stop" buffer immediately prior to loading the gels. 5µg samples from each of the six DNA preps. were digested using each of the six enzymes (a total of 36 digests). The resulting samples were run on two 20 track 0.5% agarose gels as described in the Methods, with the MW ladders in the two outer most tracks.

The gels were blotted onto nitrocellulose paper, hybridised and autoradiographed as described in the Methods. A standard curve was prepared from the Lambda ladder and the MW of the bands hybridising to the probe estimated from their electrophoretic mobilities (on the autoradiograph). The autoradiographs of the two Southerns from the first experiment can be seen in figures 6.1 and 6.2. Tables 6.1 and 6.2 give the sizes and the mobilities of the bands. In figure 6.1 , track 8 (DNA prep 1,BclI) could not be seen so the positions of the bands in this track were visualised after lengthy exposure of the X-ray film resulting in over-exposure of the other tracks on this filter. Over-exposing the the filters on film revealed no further hybridising bands. Unfortunately tracks 1, 17 and 18 in figure 6.1 are obscured by the signal from the MW marker tracks.

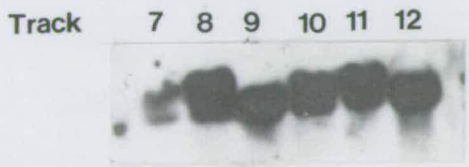
Of the six enzymes used, four gave significant restriction fragment size changes within the group of DNA samples (XhoI, BclI, ApaI and SalI). BamHI and PvuI did not appear to give significant changes in pattern within the group.

Figure 6.1



1 Marker positions determined from an underexposure of blot

2 Tracks 1, 17 & 18 obscured by markers



Overexposure, showing track 7, missing above

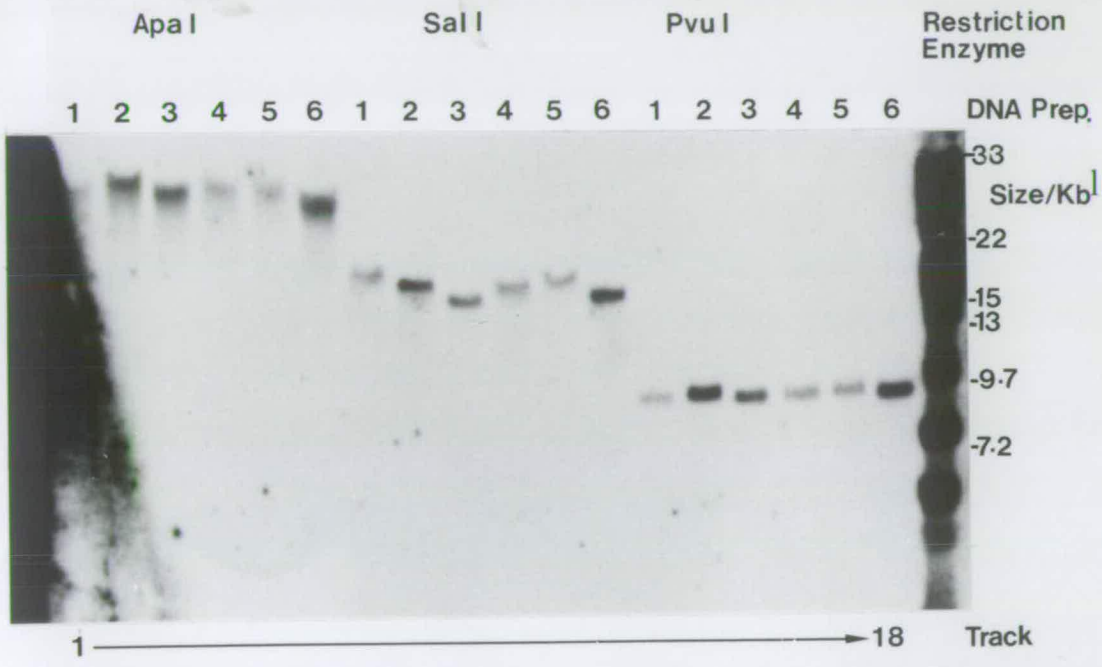
TABLE 6.1

Data from measuring electrophoretic mobilities of bands hybridizing to Ade1 probe seen in Figure 6.1

Track	DNA prep	Restriction Enzyme used	Electrophoretic mobility (cm)	Band size (kb)	Restriction[1] fragment size change (bp)
1	1	XhoI	-	-	-
2	2		0.50	21.8	+900
3	3		0.55	21.6	+700
4	4		0.40	24.4	+3,500
5	5		0.50	21.8	+900
6	6		0.60	20.9	0
7	1	BclI	4.0/4.4	7.2/6.6	+300/0
8	2		3.9/4.35	7.4/6.6	+500/0
9	3		4.2/4.35	6.9/6.6	0/0
10	4		4.0/4.35	7.2/6.6	+300/0
11	5		3.9/4.30	7.4/6.7	+500/100
12	6		4.15/4.3	7.0/6.6	+100/0
13	1	BamHI	0.8	18.6	0
14	2		0.8	18.6	0
15	3		0.8	18.6	0
16	4		0.7	20.0	+200
17	5		-	-	-
18	6		-	-	-

[1] Compared to smallest fragment in group (by enzyme used).

Figure 6.2



l See note l Fig. 6.1

TABLE 6.2

Data from measuring electrophoretic mobilities of bands hybridizing to Ade1 probe seen in Figure 6.2

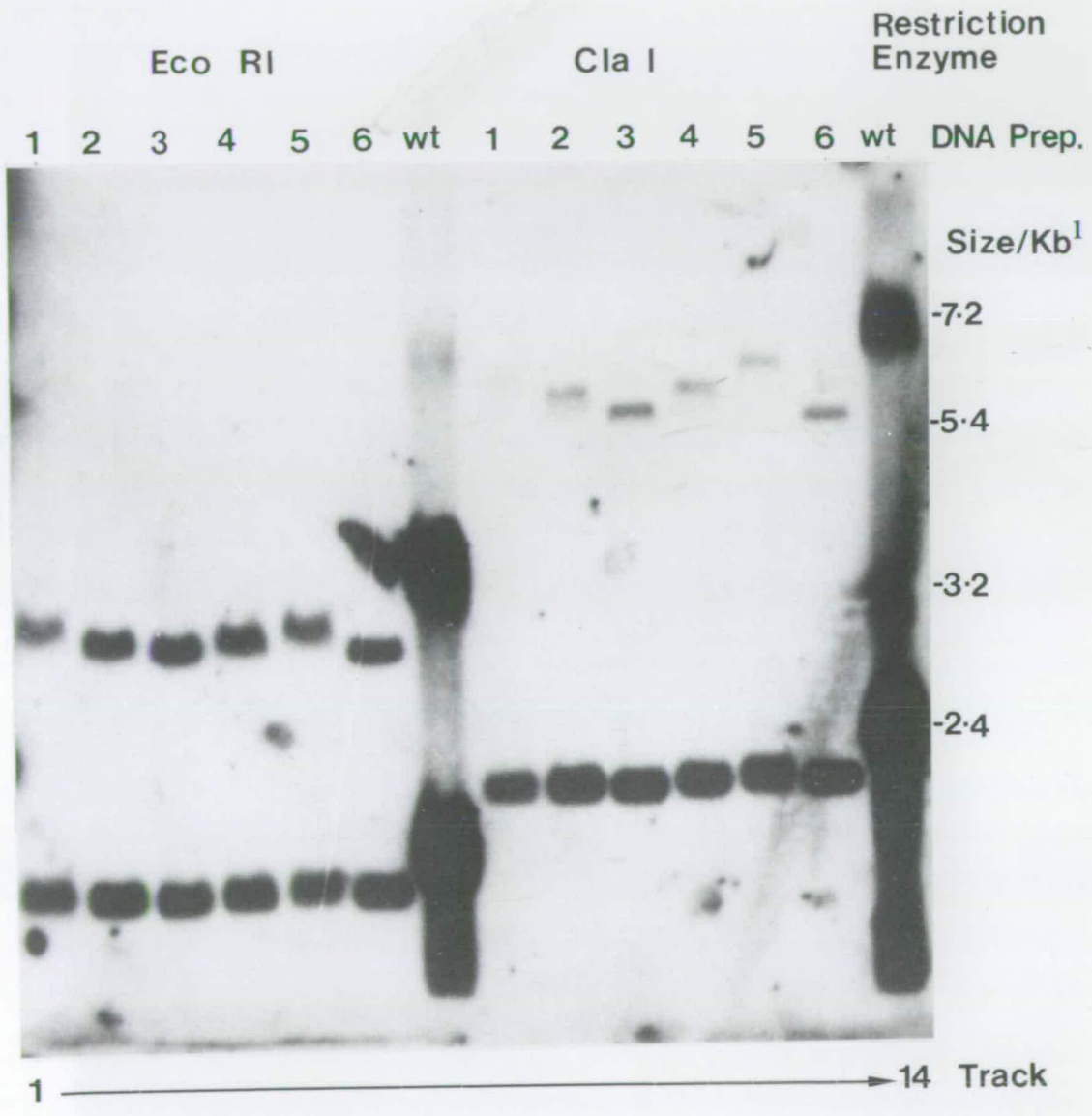
Track	DNA prep	Restriction Enzyme used	Electrophoretic mobility (cm)	Band size (kb)	Restriction[1] fragment size change (bp)
1	1	ApaI	0.1	30.9	+3,400
2	2		0.0	32.9	+4,800
3	3		0.3	28.8	+1,300
4	4		0.0	32.3	+4,800
5	5		0.1	20.9	+3,400
6	6		0.4	27.5	0
7	1	SalI	1.9	15.8	+1,800
8	2		2.1	15.1	+1,100
9	3		2.4	14.0	0
10	4		2.1	15.1	+1,100
11	5		2.0	15.5	+1,500
12	6		2.3	14.3	+300
13	1	PvuI	4.2	9.5	+200
14	2		4.2	9.5	+200
15	3		4.3	9.3	0
16	4		4.2	9.5	+200
17	5		4.2	9.5	+200
18	6		4.3	9.3	0

[1] See note 1 Table 6.1.

For each of the enzymes showing significant differences, the pattern of fragment sizes seen is the same: namely, DNA preps 1 and 5 are approximately the same size. Similarly prep 2 = 4 and prep 3 = 6. The order of the bands on the gel gives the following pattern for the size of the fragment to which the probe hybridised 1=5>2=4>3=6. The consistency of the pattern with the different enzymes makes it unlikely that the results are artefactual. DNA preps 3 and 6 were both obtained from stable *ade1 ade6* double mutants (see figure 6.5) and these bands appear to be consistently smaller than the other four samples. A striking feature of these results is that, instead of DNA samples from the red and mosaic colonies having a similar restriction pattern to samples of the same phenotype, the two reds are more like one of the two mosaics (red 1 = mosaic 5 and red 4 = mosaic 2). However the most striking feature of the gels is the appearance of two distinct bands in each of the tracks representing DNA digested with BclI (fig 6.1, tracks 8-13). This would suggest either that the mutagenesis has generated a BclI site within the region covered by the probe, independent of the observed instability; or, that there are two sequences hybridising to the *ade1* probe.

The results of the second experiment are given in figures 6.3 and 6.4, with tables 6.3 and 6.4 containing the sizes and mobilities of the bands observed. In this case single enzyme restriction digests using EcoRI and ClaI were carried out for all six DNA preps and also samples of *972h-* DNA. At the same time, the digests using BclI and PvuI were repeated, and *972h-* digests were added as controls for both enzymes. The digests were carried out as before using 5µg of DNA per digest and the same MW markers were used. Since the fragments were expected to be smaller, so the concentration of the agarose in the gels was increased to 0.7% to give a better linear separation of small fragments. The gels were blotted and autoradiographs taken from the filters as before. The mobilities of the hybridizing fragments were used to estimate their molecular weights from a standard curve, constructed from the mobilities of the MW markers. Overexposure of the X-ray film to the filters revealed no extra bands other than those seen in figures 6.3 and 6.4. In figure 6.3 it can be seen that both EcoRI and ClaI gave only two hybridizing bands in all six DNA preps from the *ade1* replicating instability, and, the case of *972h-* DNA, restriction with BclI gave only one hybridizing band this time (figure 6.4). On the basis of these results there is no evidence to suggest that a duplication of the coding sequence of the *ade1* gene exists. Presumably the two hybridizing

Figure 6.3



[1] See note | Fig. 6.1

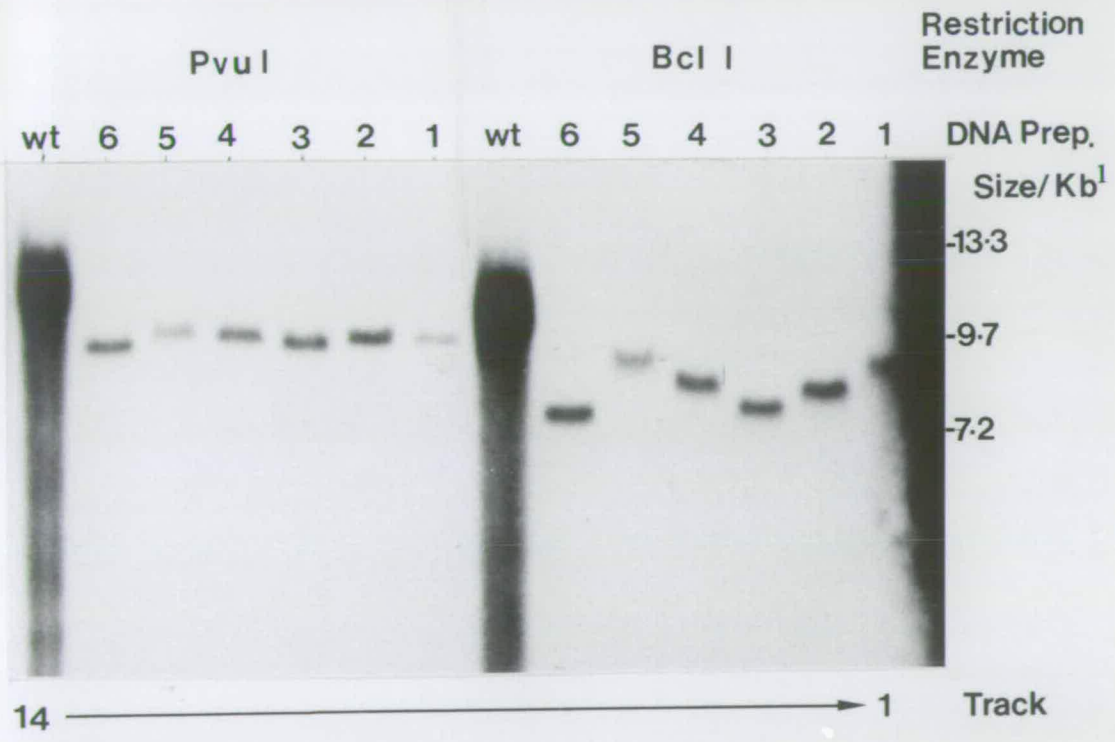
TABLE 6.3

Data from measuring the electrophoretic mobilities of bands hybridizing to Ade1 probe seen in Figure 6.3

Track	DNA prep	Restriction Enzyme used	Electrophoretic mobility (cm)	Band size (kb)	Restriction[1] fragment size change (bp)
1	1	EcoRI	10.35/14.30	2.92/2.10	+100/10
2	2		10.60/14.40	2.85/2.09	+30/0
3	3		10.70/14.40	2.82/2.09	0/0
4	4		10.60/14.30	2.85/2.10	+30/10
5	5		10.40/14.25	2.92/2.11	+100/20
6	6		10.70/14.30	2.82/2.10	+0/10
7	972h⁻		9.90/14.10	3.13/2.13	+310/40
8	1	ClaI	6.70/13.80	6.17/2.17	+770/20
9	2		7.00/13.85	5.75/2.16	+350/10
10	3		7.20/13.90	5.52/2.15	+120/0
11	4		6.90/13.80	5.89/2.17	+490/10
12	5		6.60/13.80	6.27/2.17	+870/10
13	6		7.30/13.80	5.40/2.17	+0/10
14	972h⁻		6.10/13.40	7.00/2.24	+1,600/90

[1] See note 1 Table 6.1.

Figure 6.4



1 See note 1 Fig. 6·1

TABLE 6.4

Data from measuring the electrophoretic mobilities of bands hybridizing to Ade1 probe seen in Figure 6.4

Track	DNA prep	Restriction Enzyme used	Electrophoretic mobility (cm)	Band size (kb)	Restriction[1] fragment size change (bp)
1	1	BclI	4.70	8.81	+1,400
2	2		5.00	8.13	+720
3	3		5.30	7.59	+180
4	4		5.00	8.13	+720
5	5		4.60	9.02	+1,610
6	6		5.40	7.41	0
7	972h$^-$		4.30	9.77	+2,360
8	1	PvuI	4.15	10.12	+350
9	2		4.20	10.00	+230
10	3		4.30	9.77	0
11	4		4.20	10.00	+230
12	5		4.15	10.12	+350
13	6		4.30	9.77	0
14	972h$^-$		3.70	11.40	+1,630

[1] See note 1 Table 6.1.

bands seen in the first BclI digests were artefacts.

In all cases the DNA preps from the *ade1* replicating instability produced smaller fragments than the *972h-* DNA. However, the *972h-* DNA was rather heavily contaminated with ethidium bromide and was also overloaded on the gel. Both these factors would be expected to affect the electrophoretic mobility of the *972h-* samples, therefore it is impossible to say whether or not the differences are meaningful. Once again, in all the bands in which there are differences in restriction fragment size (BclI, the larger of the EcoRI and ClaI bands) the pattern seen is the same, and is also the same as seen in the first two gels, that is 1=5>2=4>3=6. Again, in all cases, the mutant phenotype is associated with the smallest bands. From the EcoRI digest, it is possible to ascertain that the 5' end of the gene appears to be associated with the observed changes in restriction fragment size. The ClaI digests also show variation in only one of the two bands. Although the orientation of the ClaI sites around the gene are not known, it would seem reasonable to propose that the band showing heterogenity is also 5' to the ClaI site in the gene.

Comparison of the size estimates for the fragments generated by the enzymes BclI and PvuI showed that both the estimated size of the fragment and the differences between the fragments were greater for the gels depicted in figure 6.1 and figure 6.2 than those in figure 6.4. This means that the data presented here can be regarded as qualitative only. Nonetheless a number of observations from these results appear to be sufficiently consistent so that any hypothesis purporting to explain the molecular basis of this replicating instability would have to account for them. These are:

1. The generation of the *ade1 /ade6* double mutant is associated with restriction site heterogenity around the *ade1* locus. On the basis of the restriction pattern changes seen for digests using EcoRI the heterogenity appears to be in the 5' region of, or 5' to, the gene.
2. Not all enzymes tested showed significant heterogenity in band sizes but in those which did the pattern of changes is the same for each enzyme, suggesting that this pattern is significant.
3. Where heterogenity exists, there are at least three sizes of band.
4. The larger the hybridizing fragment, the larger the size differences appear to be.
5. There is no evidence to suggest that restriction sites have been lost during the generation of the mutant *ade1* allele.
6. There is no evidence to suggest that there is a duplication of the coding sequence of the *ade1* gene.

6.3 DISCUSSION

These results are of course preliminary and, before firm conclusions can be drawn, it will be necessary to develop more data from a range of instabilities. It is possible, however, to re-examine hypotheses about the molecular nature of the unstable state in the light of these results. Already it is apparent that some explanations appear improbable, as they cannot easily fit the observations made, whilst other categories of explanation look more promising.

6.3.1 DUPLICATIONS AND THE UNSTABLE STATE

If a duplication of the gene had occurred, cutting within the coding sequence would have generated at least three fragments, each of which should hybridise to the probe. This clearly has not happened. This major class of explanation for the unstable state seems, as a consequence, to be unlikely.

However, although there is no evidence to support the proposal that the *ade1* locus is duplicated, the observations made here do not rule out duplications entirely. As discussed above, the technique used is limited by the range of sizes of DNA within which structural changes could be observed. Large duplications in excess of about 30kb and very small duplications would not have been observed. A large duplication containing the *ade1* locus might well give a tendency to chromosomal rearrangment and hence mitotic instability. If such a large duplication were to exist however, rearrangments might occur anywhere within the duplicated region, and this would be difficult to reconcile with the apparent specificity of the changes producing the stable mutant observed here. Likewise, a small duplication in the coding sequence of the *ade1* gene could formally explain the replicating instability, if the duplicated sequence were precisely excised, restoring gene activity, in a similar manner to that found by Rambosek and Kinsey (1984) in the *am* gene of *Neurospora*. This possibility can not be ruled out on the grounds of the data presented here, but it appears unlikely, as it would be necessary to also postulate coincident multiple deletion events in the 5′ region of the *ade1* gene, in order to account for the differences in fragment sizes.

A duplication of sequence 5′ to the coding region, perhaps involved in the control of transcription could account for the observations made above, as this

would have probably have remained undetected in this system, since the probe did not extend to the 5' end of the coding sequence. If such a duplication existed it might have generated the observed unstable phenotype by promoting genetic recombination at the *ade1* locus. Non-tandem duplications in *S. cerevisiae* have been shown to promote intrachromosomal gene conversion with a mean frequency of 2×10^{-4} per mitosis (Jackson and Fink, 1981). Gene conversion at a high frequency (10^{-2} to 10^{-3}) has also been observed in yeast plasmids containing two heteroallelic mutant copies of the *S. cerevisiae HIS3* gene, where the copies were inserted as inverted repeats (Embretson and Livingston, 1984). It might seem reasonable, therefore, to propose that the mutagenic treatment had generated an imperfect duplication of sequences 5' to the coding region of the *ade1* , gene which are involved in the transcriptional control of the gene. Such a duplication could give rise to mitotic instability, either by causing unequal crossing over between sister chromatids, or by promoting intrachromosomal gene conversion, resulting in the loss of an active control function. A mechanism of this kind might lead to the deletion of some of the restriction sites in the region. However, since this was not observed this perhaps seems rather improbable, although not impossible. The obvious way to investigate this would be to extend the sequencing programme into the 5' region by "chromosome walking" and to compare the restriction sites in upstream regions.

6.3.2 DELETIONS AND THE UNSTABLE STATE

The *ade1* stable white mutants both give the smallest hybridising bands with any of the restriction enymes used. Thus, they appear to have lost DNA sequence within the 5' region of the gene. However, a simple deletion of material generating the mutant is not consistent with the size changes obtained with different enzymes. A deletion event would be expected to give rise to the same size change in the restriction fragment regardless of which enzyme was used to digest the DNA. Since, as noted in the preceding section, not all the enzymes tested appeared to give significant heterogenity, and since the observed size changes varied between enzymes, a deletion event would seem unlikely. Even if one were to postulate that the deletion covered one of the restiction sites in the wild-type locus, thereby uncovering a different, originally more distant site, for that enzyme, this could only explain a difference in restriction fragment size changes between two of the enzymes used. In order to explain the differences seen in restriction fragment size

changes between all the enzymes tested, it would be necessary to postulate that the deletion had removed all the original restriction enzyme sites bringing a new set of sites near to the coding sequence.

The quasi-palindromic model suggested by Ripley to explain the generation of spontaneous frame-shift mutants (Ripley, 1982) cannot easily accomodate the observations listed above. This model postulates that spontaneous frame-shift mutants could be generated by the insertion or deletion of short sequences of bases where areas of mismatch occur within stretches of secondary structure of DNA, the so called quasi-palindromic sequences (Ripley, 1982; Todd and Glickman, 1982). This model can not be easily fitted to the data for two reasons; firstly, the size changes observed between restriction fragments of the mutant and normal alleles are too large to be explained by this model; secondly, even if the model were adapted to allow for the insertion or deletion of relatively long sequences of DNA, it would be necessary also to postulate multiple events to account for the fact that there is no evidence to suggest that restriction sites have been added or lost during the formation of the mutant locus.

6.3.3 MOBILE ELEMENTS AND THE UNSTABLE STATE

The observations above could be related to the movement of a mobile genetic element in the *S. pombe* genome, although transposable elements have not so far been reported in *S. pombe* . It is not, however, unreasonable to suppose that these may exist, considering the wide distribution of such elements in both prokaryotic and eukaryotic systems (see Shapiro, 1983). In the present case an inserted element could be envisaged as producing the observed instability in one of two ways: either, the mutagenic treatment may have produced a "hot-spot" for transposition 5′ to the *ade1* locus; or, as a consequence of mutagenesis, a mobile element has inserted 5′ to the *ade1* gene and the observed instability is a result of its excision. Although both these suggestions pose mechanistic problems which will be discussed later, in one way this idea fits the observations better than any of the others discussed so far, since it can explain all the variations observed in restriction fragment sizes. As mentioned previously, one of the most striking peculiarities of the observations made was that some enzymes appeared to show significant heterogenity in band sizes between mutant and non-mutant alleles of *ade1* whilst others did not. This feature could be explained by the insertion or

excision of an element to produce the observed instability. If the production of mutation at *ade1* was due to the insertion of a mobile element, and that element carried a restriction site for an enzyme, it might appear that a deletion event had occured. The size of the apparent "deletion" would depend on the relative positions of the original site in the 5' chromosomal DNA and the site of insertion of the element carrying the new restriction site. The situations in which no apparent heterogenity exists between mutant and non-mutant alleles could be accounted for if the mobile element had no site for that particular enzyme. The apparent lack of size change in this case would be a function of the size of the mobile element, relative to the size of the hybridising fragment and the degree of resolution achieved by the Southern blots.

The concept of a "hot-spot" or preferential site for transposition is not without precedent, since some mobile elements have a high specificity for target site selection; for example IS4 is observed at only 3 sites in the chromosome of all *E. coli* K12 strains tested (Iida *et al*, 1983). Other mobile elements show regional specificity, such as IS1 (Iida *et al*, 1983), whilst other such as bacteriophage Mu appear to insert in the host chromosome at random sites (Toussaint and Resibois, 1983). In this context, it is interesting to note that the Ty elements of *S. cerevisiae* seem to show a preference for the 5' regions of genes (Roeder and Fink, 1980,1983) and that in *Drosophila melanogaster* two out of three preferential sites for P element insertion in the *RpII215* locus are in the 5' non-coding region of the gene (Serales *et al*, 1986). This may perhaps served to strengthen the suggestion that a mobile element is involved in the *ade1* replicating instability, as it was observed that the changes in the locus giving rise to the mutant phenotype were at the 5' end of the gene.

The "hot-spot" could equally well be envisaged as a region of the chromosome especially prone to transposition as a specific site . An example of regional specificity can be found in *S. cerevisiae* where a "hot-spot" for Ty transposition has been found between the 5' ends of two tRNA genes $tRNA_3^{Leu}$ and $tRNA_3^{Glu}$ on the left arm of chromosome III (Warmington *et al*, 1986). The concept of a susceptible region perhaps ties in better with the observations than the idea of a specific site, as it is possible to envisage that the inactivation of the gene may depend on how near to the coding sequence the element inserts. This would explain the observation that the mutant *ade1* locus is always associated with the smallest restriction fragments. A major problem

with this model is that the rate of transposition into the region would have to be of the order of 10^{-3} to account for the observed frequency of instability. This is far in excess of the 10^{-5} to 10^{-9} quoted for IS elements (Iida *et al*, 1983) and 10^{-8} for Ty elements (Roeder and Fink, 1983). There are examples, however, where the rate of transposition of an element can be considerably higher, for example the "high-hopper" mutant of Tn10 transposes at between 10^{-3} and 10^{-4} (Kleckner, 1983). The element Tn5, which codes for kanamycin resistance, shows considerably elevated rates of transposition immediately after entry into a cell (Biek and Roth, 1980). Probably the best known example of elevated rates of transposition is seen in the phenomenon of hybrid dysgenesis in *Drosophila* (reviewed by Bregliano and Kidwell, 1983). During dysgenesis reversions of P element induced white locus mutations occur at a frequency of greater than 10^{-3} (O'Hare and Rubin, 1983).

The difficulty in explaining an increased frequency of transposition might be overcome by the proposal that the mobile element was inserted into the locus as an immediate consequence of the mutagenic treatment. In this case, the observed instability of the locus would result not only from excision of the element, but also from homology-dependent recombination with other elements. Homology-dependent recombination between Ty elements in *S. cerevisiae* is well documented (Scherer and Davis, 1980; Roeder and Fink, 1980; Breilmann *et al*, 1985) and is 100-fold more frequent than Ty transposition (Roeder and Fink, 1983). However, this suggestion has the added complication that, with the notable exception of the induction of Lambda prophage, mobile elements are not believed to transpose as a result of enviromental stress (Edlin *et al*, 1986) or mutagenic treatment (Eeken and Sobels, 1983, 1986).

It would be possible to test for the presence of a mobile element in several ways. One would be to carry out extensive restriction mapping of the region 5' to the *ade1* locus of both mutant and normal alleles. If an area in the mutant alleles could be shown to have gained or lost several restriction sites, it would be a good candidate for an insertion sequence. This region could then be cloned and probed to genomic digests from different *S. pombe* strains, and changes in the numbers and sites of hybridising bands would be indicative of a mobile element. Another approach would be to sequence the 5' region of both mutant and wild-type alleles and compare the sequences for signs of DNA insertion. As restriction fragment heterogenity was observed for EcoRI digests,

the putative insert must be not more than 2kb 5′ to the coding sequence of the gene, thus making sequencing of mutant and non-mutant alleles feasible.

6.3.4 GENE CONVERSION AND THE UNSTABLE STATE

Another possible explanation for the observations made above is the generation of an endonuclease sensitive site in the 5′ region of the ade locus by the mutagenic treatment. Unrepaired double stranded breaks in DNA are lethal to *S. cerevisiae* even if present at the level of just one or two per genome, since such lesions are dominant (reviewed in Kunz and Haynes, 1981). Wild-type *S. cerevisiae* cells can repair double stranded breaks very efficiently, using a pathway which is recombinogenic in nature. A model for the mechanism by which this recombinational repair occurs has been put foward (Orr-Weaver and Szostak, 1983; Szostak *et al*, 1983). Briefly, this model suggests that double stranded breaks become enlarged into double stranded gaps by exonuclease digestion, and these gaps are then repaired by gene conversion. Whether the details of the model are correct or not, it is certain that double stranded breaks in DNA and single stranded sections of DNA are highly recombinogenic. The suggestion that these results could be explained by the generation of an endonuclease sensitive site during mutagenesis is not entirely without precedent. Site specific induction of gene conversion has been observed in the *ade6* mutant M26 of *S. pombe* (Gutz, 1971; Goldman, 1974; Goldman and Smallets, 1979). This particular mutant has a very strong marker effect on meiotic gene conversion in heterozygous diploids with conversion of the M26 allele to wild-type occuring 12 X more frequently than conversion in the opposite direction (Gutz, 1971). The results have been interpreted in terms of site-specific breakage at the M26 lesion leading to strand degradation and subsequent gene conversion. It was concluded (Goldman and Smallets, 1979) that the results were consistent with the *ade6* M26 mutant having a preferential site for endonucleolytic cleavage.

In order to sustain the idea that the *ade1* replicating instability could be explained by site-specific gene conversion, several assumptions are necessary. Firstly, for the mechanism by which the replicating instability generates mutations at the *ade1* locus to be intergenic gene conversion, it would be necessary for there to be some sequence homology between the *ade1* locus and another locus or loci in the *S. pombe* genome. The region of homology necessary to promote gene conversion need not be extensive, for example the

homology between the serine tRNA genes mentioned above is about 200 base-pairs (Thuriaux, 1985). It has also been proposed that gene conversion can traverse extensive regions of inhomology in a model presented by Golin *et al.* (1986). It would also be necessary to postulate that the induced gene conversion was intergenic rather than intragenic as the unstable behaviour resides in mitotically dividing haploid populations of cells. Intergenic mitotic gene conversion of this sort has indeed been observed in *S. pombe* between the dispersed, but related, serine tRNA genes (Kohli *et al*, 1984; Szankasi *et al*, 1986). The frequency with which gene conversion takes place between these genes is however very low, at between 10^{-6} and 10^{-7} per mitosis (Szankasi *et al*, 1986) with is 10^{3} less than the observed frequency of secondary mosaics at the *ade1* locus. It is possible, therefore, to envisage two ways in which sufficient homology to promote intergenic gene conversion at the *ade1* locus might exist. The first being if 5' promoter sequences to *ade1* had homology with the promoter sequences of other genes in the adenine biosynthetic pathway, such sequences might exist to allow for the co-ordinated expression of the adenine genes. Also, it is possible that regions of repetitous DNA are located 5' to the *ade1* locus which display homology with other repetitous sequences elsewhere in the genome. As the probe covered only the coding sequence and some sequence 3' to the *ade1* gene, sequence homology in the 5' region would not have been detected with this probe.

There are some major flaws in this hypothesis -the first being that intergenic conversion, unlike intragenic conversion, does not appear to cause sequence changes in the regions surrounding the area of homology (Heyer *et al*, 1986). Secondly the frequency with which intergenic conversion is believed to occur is far too low to account for the observed frequency of instability of about 10^{-3} at the *ade1* locus. There is of course one notable exception to both these rules and that is in the switching of mating-type in both *S. pombe* and *S. cerevisiae* (Beach, 1983; Klar *et al*, 1980; Klar and Strathern, 1984). In *S. pombe* the switching of mating-type occurs by intergenic conversion between the tightly linked expression and storage loci for mating-type information. This switching is initiated by site-specific strand cleavage at the *smt* locus, the production of the break and its healing are thought to be cell cycle specific and separated by a whole cell cycle (Beach, 1983). Thus, it may be that unrepaired double strand breaks are highly recombinogenic due to their lethal nature to cells. In such a situation, a site which was hypersensitive to

endonuclease cleavage could promote the formation of heteroduplex DNA with a considerable degree of "illegitimate" pairings and so lead to gene conversion.

It would be possible to test the idea that the observed instability was caused by mitotic recombination using genetical methods. Such an approach has been attempted before (Nasim, 1974) but no signficant difference was found between the levels of secondary mosaicism observed between the three UV-sensitive mutants tested and the controls. I believe that there are two reasons why significant differences might have been missed: firstly, the experiments were done using the replating technique to score the frequency of secondary mosaics, and in view of the comments made about this technique in chapter 4, it is perhaps not surprising that it was found difficult to interpret the results obtained and that they seemed to conflict with those of Dubinin *et al.* (1972). Also, the UV-sensitive mutants selected would perhaps not be expected to affect the sort of recombinational mechanism suggested here, the mutants in question being *rad1.1* ,*rad5.518* and *rad10.198*. The strain *rad1.1* was selected because it drastically reduces the frequency of UV-induced mutation, however no clear cut "*rec-*" phenotype has been observed, and *rad1.1* displays wild-type levels of spontaneous mitotic and meiotic recombination (Grossenbacher-Grunder and Thuriaux, 1981). The *rad5.158* and *10.198* strains are strong mutators (Phipps *et al*, 1985) and there is no reason *a priori* why they should affect the induction of site-specific recombination.

There are two tests which could usefully be applied to see if, in fact, recombination is involved in the observed instability. The first would be to expose the unstable *ade6* strain already isolated to a second dose of UV or perhaps even better to X-rays (which are not thought to produce replicating instabilities, see Nasim, 1967) and to see if the treatment increased the instability at the *ade1* locus. It would be easy to check that the *ade1* mutants observed were the same as those observed previously by Southern blotting restriction digests of the DNA and comparing them to the digests obtained above. The rationale behind this experiment is that mitotic recombination in yeast is induced by exposure to UV and X-rays (see Kunz and Haynes, 1981), consequently, an increased frequency of mutation would be expected at all loci. However, an increase of mutations at the *ade1* locus above and beyond the observed frequency at other loci might be interpreted as being indicative of a site in the locus with a predilection to mitotic recombination. The second approach would be to induce replicating instabilities in the UV-sensitive strain

rad2.44 a mutant which shows a 10-fold increase in the level of spontaneous mitotic recombination (Grossenbacher-Grunder, 1985). The increase is attributed to an increase in the size of a recombination-proficient subpopulation of cells in cultures of mitotically dividing *S. pombe* (Grossenbacher-Grunder, 1985). If replicating instabilities could be induced in this strain, then one might expect to observe an increased freqency of instability if replicating instabilities were indeed due to recombination in mitotically dividing cells.

6.4 SUMMARY

In conclusion, restriction digests of DNA samples of mutant and non-mutant *ade1*, loci generated by a replicating instability, showed significant heterogenity in fragment size for most of the enzymes tested. This represents the first physical evidence as to the molecular nature of a replicating instability and excludes trivial causes for the observed instability such as diploidy. In addition no evidence was found to support the proposal that there was a duplication of the *ade1* coding sequence. A number of hypotheses as to the nature of the unstable state were put foward and methods by which they could be tested discussed. As the results were only qualitative it is not possible to attach more weight to any one of the suggestions than the others.

CHAPTER 7
CONCLUSIONS

On the basis of the data presented here it is possible to draw a number of conclusions concerning the possible nature of replicating instabilities in *Schizosaccharomyces pombe*.

Single cell isolation from mutagenically derived mosaics over two plating generations shows that replicating instabilities do indeed exist in *S. pombe*. The average frequency of 2×10^{-3} with which secondary mosaics were observed in this study was in agreement with the figure of 1.7×10^{-3} reported by Nasim and James (1971) based on micromanipulation. The observed frequency was, however, four-fold less than the 0.8% commonly quoted as a result of experiments carried out by replating primary mosaics (Nasim, 1967; James *et al.*, 1972). This, along with the data on plating control experiments presented in chapter 4 seems to indicate that replating is not a techinque which enables the distiction of genuine from false mosaics with confidence. Thus it appears that replicating instabilities are harder to isolate than past literature suggests.

Genuine replicating instabilities in *S. pombe* do indeed continually generate locus-specific mutants in agreement with the obervations made in *Drosophila* (Auerbach, 1947). Replicating instabilities can occur independantly of diploidy and in the three cases investigated here, dipoidy was excluded by an analysis of azygotic asci.

The limited molecular analysis carried out suggested that in the case of one unstable strain, not only were the mutations generated repeatedly from the replicating instability, locus specific, but that the mutant derivatives appeared to be identical in two different samples. The molecular results were preliminary only, so it is impossible to say with certainty what the nature of the molecular events was. However, the generation of the mutant and non mutant derivatives appear to involve significant changes in the 5' region of the gene. In addition the Southern analysis revealed no evidence to suggest that the instability involved a duplication of the *ade1* coding sequence.

Future work clearly should be directed towards the molecular analysis of more independantly isolated instances of genuine replicating instabilities.

APPENDIX A
STOCK SOLUTIONS AND MEDIA

MEDIA

(Gutz *et al.* 1974)
These recipes are for a litre of dist. water

YEAST EXTRACT LIQUID (YEL)
30g Glucose
5g Yeast Extract

MINIMAL LIQUID (ML)
(Sherman *et al.* 1974)
40g Glucose
6.7g Difco Yeast Nitrogen Base w/o Amino Acids

YEAST EXTRACT AGAR (YEA)
As for YEL + 20g/l Agar

MINIMAL AGAR (MA)
As for ML + 20g/l Agar

SPORULATION AGAR (SPA)
10g Glucose
1g Potassium dihydrogen orthophosphate
10µg Biotin (from a 10µg/ml stock in 50% EtOH)
1mg Calcium Pantothenate (10mg/ml stock)
10mg Nicotinic Acid (10mg/ml stock)
10mg Meso-inositol (10mg/ml stock)
20g Agar

MALT EXTRACT AGAR (MEA)
30g Malt Extract
20g Agar

PREPARTION
Liquid media were dispensed into aliquots before sterilization in the auto-clave at 15 lb/sq in for 20 minutes. Solid media were autoclaved at 15lb/sq in for 20 minutes before pouring into plates (85mm sterile plastic from Beveridge) or sterile capped tubes for slopes.

STOCK SOLUTIONS AND BUFFERS

Maniatis *et al.* 1982 except where otherwise stated.

Adenine sulphate
Stock solution made up at 5mg/ml, in dist. water,

sterilize by autoclaving.

44mM Citrate buffer pH 5.6
(Njagi 1982)
3g citric acid
9g trisodium citrate
1l dist. water
Sterilise by autoclaving.

20mM Citrate/phosphate pH5.8
2.84g sodium phosphate (dibasic Na_2HPO_4)
dissolve in 900ml of dist. water, pH to 5.8 using conc. citric acid, make up to 1l with dist. water, sterilize by autoclaving.

50mM Citrate/phosphate, 1.2M Sorbital,
40mM EDTA, 50mM b-Mercaptoethanol pH5.8
7.1g sodium phosphate (Na_2HPO_4)
218.6g Sorbitol
16.5g EDTA (tetra-sodium salt)
dissolve in 900mls of dist. water, pH to 5.8 using conc. citric acid, make up to 1l with dist. water, sterilize by autoclaving.
Add 40µl b-Mercaptoethanol per 10ml before use.

50 X Denhardts solution
5g Ficoll-400 (Pharmacia)
5g PVP-40 (polyvinyl-pyrrolidone MW 40,000 Sigma)
5g BSA (Sigma, fraction V)
dissolve in dist. water to 500ml
filter through 0.45µm filters, dispense into aliquots, store at -20°C.

1M Dithiothreitol (DTT)
3.09g DTT
20ml 0.01M sodium acetate (pH 5.2)
Dissolve the DTT in the sodium actate, sterilize by filtration
Dispense into 1ml aliquots and store at -20°C.

DNAase
1mg/ml stock in 50% glycerol.
Dilute and use at 10^{-4} in nick translation buffer/ 50% glycerol,this can be kept at -20°C

0.5M EDTA (pH 8.0)
232.1g EDTA.$2H_2O$ (tetrasodium salt)
Dissolve in 800ml dist. water pH to 8.0 using conc. HCl, make up to 1l, dispense into aliquots and sterilize by autoclaving.

Ethidium bromide (10mg/ml)
Add 1g of ethidium bromide to 100ml of dist. water.

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.
Wrap container in foil and store at 4°C.

Gel loading buffer
6 X buffer
0.25% bromophenol blue
0.25% xylene cyanol
30% (v/v) glycerol in dist. water
Store at 4°C.

b-Mercaptoethanol
Usually obtained as a 14.4M solution. Store in a dark bottle at 4°C
Do not autoclave b-Mercaptoethanol or solutions containing it.

1M $MgCl_2$
Dissolve 203.3g of $MgCl_2.6H_2O$ in 800ml of dist. water, adjust the volume to 1l.
Dispense into aliquots and sterilize by autoclaving.

10 X Nick Translation buffer
5ml 1M Tris pH 7.2
1ml 1M $MgSO_4$
100µl DTT
500µg/ml BSA
Make up to 10ml in dist. water, filter sterilize, dispense into aliquots and store at -20°C
(Working conc. 50mM Tris, 10mM $MgSO_4$, 0.1mM DTT, 50µg/ml BSA.)

M/15 Phosphate buffer pH 6.8
(Njagi 1982)
5.75g dipotassium hydrogen orthophosphate (K_2HPO_4)
4.49g potassium dihydrogen orthophosphate (KH_2PO_4)
Make up to 1l in dist. water, sterilize by autoclaving.

10 X Restriction enzyme buffers
low salt buffer
100mM Tris.Cl (pH 7.5)
100mM $MgCl_2$
10mM DTT

medium salt buffer
500mM NaCl
100mM Tris.Cl (pH 7.5)
100mM $MgCl_2$
10mM DTT

high salt buffer
1M NaCl
500mM Tris.Cl (pH 7.5)

100mM $MgCl_2$
10mM DTT

The solutions are made up in dist. water, filter sterilized and stored at -20°C

3M sodium acetate pH 5.2
408.1g sodium acetate.$3H_2O$
Dissolve in 800ml of dist. water, adjust the pH to 5.2 using glacial acetic acid. Adjust the volume to 1l, dispense into aliquots, sterilize by autoclaving.

3M sodium chloride
175.3g NaCl
Dissolve in 800ml of dist. water, adjust volume to 1l, dispense into aliquots and autoclave to sterilize.

20% Sodium dodecyl sulphate (SDS)
200g SDS (electrophesis-grade)
Dissolve in 900ml of dist. water, adjust pH to 7.2 using conc. HCl, make up to 1l, dispense in to aliquots.

20 X SSC
175.3g NaCl
88.2g trisodium citrate
Dissolve in 800ml of dist. water, adjust pH to 7.0 with 10N NaOH, make up to 1l,sterilize by autoclaving.

1M Tris
121.1g Tris base
Dissolve in 800ml of dist. water, pH to required pH using conc HCl, make up to 1l, sterilize by autoclaving.

50 X Tris-acetate gel buffer
242g Tris base
57.1ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)
Make up to 1l, sterilize by autoclaving.

10 X TE
12.1g Tris base
4.1g EDTA (tetrasodium salt)
Dissolve in 800ml dist. water, pH to 7.6 using conc. HCl, adjust volume to 1l,autoclave to sterilize.
(Working concentration 10mM Tris, 1mM EDTA.)

APPENDIX B
RAW DATA FOR FIGURES IN CHAPTER 4

DATA FOR FIGURE 4.1.

Sonication power	Current (Amps)	Plating volume/ml	Average plating density	Standard error	% increase in plating units
0	0	0.05	22.3	±2.2	0
1	0.8	0.05	21.5	±0.6	-4
2	1.4	0.05	27.5	±7.5	26
3	2.0	0.05	30.5	±3.3	37
4	2.3	0.05	27.5	±4.2	23
5	2.8	0.05	26.0	±6.0	16
0	0	0.1	36.3	±7.0	0
1	0.8	0.1	39.3	±8.4	8
2	1.4	0.1	56.0	±12.3	52
3	2.0	0.1	51.3	±11.0	41
4	2.3	0.1	55.6	±7.4	53
5	2.8	0.1	54.7	±1.5	51
0	0	0.2	61.6	±3.2	0
1	0.8	0.2	81.3	±11.0	32
2	1.4	0.2	106.0	±6.2	72
3	2.0	0.2	112.0	±3.5	82
4	2.3	0.2	108.6	±13.2	76
5	2.8	0.2	117.7	±14.6	82
0	0	0.2	66.4	±9.4	0
1	0.8	0.2	80.4	±11.4	21
2	1.4	0.2	76.0	±15.0	15
3	2.0	0.2	87.6	±12.3	31
4	2.3	0.2	93.6	±15.5	41
5	2.8	0.2	81.0	±5.3	22

DATA FOR FIGURE 4.2

Buffer system	Sonication power	Average colonies/plate	Standard error
Distilled water	0	64.4	±5.1
	2	67.6	±4.9
	4	69.6	±8.4
Phosphate	0	72.2	±9.2
	2	84.6	±10.3
	4	90.4	±14.5
Phosphate + 10mM EDTA	0	66.4	±7.1
	2	62.2	±3.4
	4	60.6	±5.8
Phosphate + 0.01% SDS	0	55.6	±3.1
	2	59.8	±10.5
	4	42.2	±4.7
Phosphate + 0.01% Tween 80	0	71.2	±8.8
	2	64.0	±6.4
	4	69.8	±6.9
Citrate	0	69.2	±14.2
	2	74.2	±11.8
	4	59.4	±8.9
Distilled water	0	59.0	±5.5
	2	61.6	±3.6
	4	58.8	±8.6
Phosphate buffer	0	50.8	±9.0
	2	72.0	±11.7
	4	61.0	±9.1
Phosphate + 10mM EDTA	0	53.4	±5.5
	2	66.0	±11.1
	4	62.0	±6.8
Phosphate + 0.01% Tween 80	0	54.2	±4.7
	2	76.2	±11.3
	4	66.8	±7.5

DATA FOR FIGURE 4.3

Average plating density	Standard error	% Red colonies	% Mosaic colonies	Total Colonies examined
147.1	±14.0	50.0	1.95	2,206
73.0	±8.0	49.0	0.95	1,678
40.5	±7.9	49.0	0.84	1,660
20.3	±5.2	50.0	0.56	1,969
79.0	±10.5	56.0	1.38	1,597
37.8	±6.0	57.0	0.72	1,397
20.5	±5.0	56.0	0.51	1,168
134.2	±26.5	44.0	1.79	1,342
76.6	±12.1	47.4	1.03	1,456
38.5	±8.4	43.7	0.77	1,423

DATA FOR FIGURE 4.4

Average plating density	Standard error	% Red colonies	% Mosaic colonies	Total Colonies examined
137.1	±14.9	34.0	0.66	1,371
99.2	±10.6	34.0	0.60	1,448
66.7	±8.8	31.0	0.26	1,134
51.9	±8.6	32.0	0.53	1,506
32.9	±6.7	39.0	0.61	493
17.6	±4.4	39.7	0.20	528
128.6	±6.4	42.0	0.54	1,286
63.6	±10.2	37.0	0.74	1,209
32.5	±5.5	42.5	0.34	1,170
15.4	±3.9	40.6	0	1,029
158.2	±10.5	58.0	0.98	1,424
82.9	±9.3	60.0	0.42	1,657
39.1	±7.7	58.0	0.13	1,526

DATA FOR FIGURE 4.5

Sonication procedure	Average plating density	Standard error	% Red colonies	% Mosaic colonies	Total colonies examined
Sonication at 0.5×10^7 cells/ml	150.6	±14.0	43.7	0.93	1,506
	74.3	±8.0	48.2	0.85	1,412
	42.0	±4.6	47.7	0.25	1,594
	20.5	±4.9	50.2	0.19	1,620
	151.4	±19.0	54.3	1.17	1,363
	72.0	±12.0	52.7	0.85	1,298
	36.1	±6.3	55.5	0.38	1,299
	98.6	±9.5	46.0	1.13	887
	48.1	±7.1	49.0	1.39	721
	22.7	±5.2	47.0	0.52	771
	12.5	±3.9	43.0	0	660
Sonication after dilution to plating density used	74.4	±9.2	51.4	1.70	1,116
	37.2	±7.8	52.5	0.65	1,076
	18.1	±5.3	50.0	0.38	1,065
	169.1	±14.2	51.0	1.13	1,691
	85.3	±8.9	50.0	1.39	1,023
	45.1	±8.7	46.0	0	1,712

DATA FOR FIGURE 4.6

% Red colonies	% Mosaic colonies	Average plating density	Total colonies examined
8.7	0.24	56.0	840
47.7	0.25	42.0	1,594
49.0	0.84	40.0	1,660
49.0	1.39	48.1	721
46.0	0.93	45.1	1,712
6.5	0	47.0	1,409
33.0	0.19	42.0	1,049
41.0	0.10	40.0	990
27.0	0.39	51.0	770
32.0	0.53	52.0	1,506

DATA FOR FIGURE 4.7

Average plating density	% Red colonies	% Mosaic colonies	Total colonies examined
23.8	11.5	0	357
30.4	1.2	0	426
21.7	8.0	0	326
26.0	23.8	0	390
15.0	3.8	0	159
7.0	11.8	0	136
32.0	16.2	0.68	293
14.0	13.1	0	298
59.0	27.0	0.17	595
28.0	27.0	0.52	572
63.0	10.0	0.16	630
35.0	10.0	0.14	717
52.0	6.6	0.19	521
29.0	6.4	0	575
41.4	10.0	0.24	414
20.0	10.0	0.25	397
78.0	6.4	0.51	779
37.0	5.9	0.14	711
73.0	21.0	0.51	583
34.0	19.0	0.14	679
31.0	13.0	0	464
24.0	26.0	0	472
41.0	21.0	0.25	814
54.0	37.0	0.60	1,019
63.0	11.4	0	1,268
30.0	26.0	0.35	573
65.0	8.4	0.26	1,176
86.0	3.9	0.06	1,640
62.0	28.0	0.27	1,108

DATA FOR FIGURE 4.8

Average plating density	% Red colonies	% Mosaic colonies	Total colonies examined
42.5	82.0	0.08	1,276
52.9	43.0	0.63	1,588
53.6	96.0	0	1,554
46.5	33.0	0.29	1,396
17.2	19.0	0.15	652
40.0	24.0	0.25	1,601
46.6	33.0	0.38	1,864
38.8	7.0	0	1,434
40.9	27.0	0.32	1,556
43.2	48.0	0.12	1,641
25.9	47.0	0.29	1,636
42.8	37.0	0.35	1,710
95.1	19.0	0.37	3,234
32.4	35.0	0.15	1,296
27.0	42.0	0.09	1,076
23.7	38.0	0.11	952
29.2	45.5	0	992
19.1	15.0	0.39	736

APPENDIX C
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