Genet. Res., Camb. (1981), **38**, *pp.* 267–279
Printed in Great Britain

Mitochondrial mutagenesis in *Saccharomyces cerevisiae*: the origin of *mit*$^-$ mutants

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(*Received* 24 *May* 1979 *and in revised form* 15 *May* 1981)

SUMMARY

Yeast cells contain many copies of mitochondrial (*mit*) genomes. The question we tried to answer was how *mit*$^-$ mutations occurring in one genome as a result of mutagenic treatment might yield homoplasmic mutant cells. Three processes were considered. First, that these cells originate by segregation of mutant and standard alleles during cell division. Secondly, that they originate through intracellular selection, for which cell division is not required. Thirdly, that recombination involving the mutant and standard alleles is non-reciprocal and unidirectional *mit*$^+ \rightarrow$ *mit*$^-$ so that the mutant allele is spread into the entire population of mitochondrial genomes within a cell, thus making it homoplasmic *mit*$^-$. The results indicate that the first process, although efficiently producing homoplasmic cells from heteroplasmic zygotes (for review see Birky, 1978), seems to play only a minor, if any, role in producing homoplasmic mutant progenies from mutagenized cells. The most important is the second process, that is, intracellular selection occurring in cells which have one or a few genomes carrying *mit*$^-$ mutations, while the remaining genomes are irreversibly damaged. The third process, unidirectional *mit*$^+ \rightarrow$ *mit*$^-$ conversion, does not seem to play any part.

INTRODUCTION

Cytoplasmic genomes, that is genomes of mitochondria and chloroplasts, are present within cells in many copies. Therefore, studies on spontaneous and induced mutagenesis of these genomes involve, apart from the events occurring at the level of single copies of each genome, processes leading to the formation of clones homoplasmic for a given mutation. It was the latter aspect of mitochondrial mutagenesis which interested us in the present work.

So far two types of mitochondrial mutation other than the well known deletion mutants called mitochondrial petites or ρ^-, have been identified. First, mutations conferring drug resistance. When the drug is present in the medium the *drug*^{r} mutations confer on mitochondria (or a portion thereof) a selective advantage over

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their *drug*^{s} alleles. Indeed, there is direct evidence (Beale, Knowles & Tait, 1972, see also Queiros & Beale, 1974) that homoplasmic *drug*^{r} clones of Paramecium originate through the intracellular selection of mitochondria carrying mutant mitochondrial genomes. Indirect evidence (Birky, 1973; Dujon *et al.* 1976; Putrament *et al.* 1976; Putrament *et al.* 1975) shows that similar mechanisms are involved in the formation of *drug*^{r} mutant clones in yeast. Intracellular selection may also be involved in the origin of revertant clones of *mit*$^-$ mutants; the data to be presented here strongly suggest such a possibility.

The second type of mutation occurring in mitochondrial genomes and not leading to a complete loss of mitochondrial functions, is exemplified by *mit*$^-$ mutations (Slonimski & Tzagoloff, 1976). They occur in mitochondrial genes specifying the components of respiratory phosphorylation enzymes. In contrast to their respective *mit*$^+$ alleles, *mit*$^-$ mutations lead to inability of the cells to grow on non-fermentable substrates. Under standard experimental conditions the *mit*$^-$ mutations do not have selective advantage over their *mit*$^+$ alleles, so that homoplasmic *mit*$^-$ clones cannot originate through intracellular selection from heteroplasmic cells containing *mit*$^+$ and *mit*$^-$ alleles.

The question we tried to answer was, how such homoplasmic *mit*$^-$ clones can be formed:

(1) The *mit*$^-$ clones might be formed by segregation from cells in which a single mitochondrial genome carries the mutation, while the remaining genomes carry its wild-type allele. The situation would be similar to that observed in the diploid progeny of *drug*^{r} × *drug*^{s} crosses with an extremely biased input of mitochondrial genomes from one of the parental strains. Therefore for the *mit*$^-$ mutant clone to be established many cell divisions would be required (for review see Birky, 1978).

(2) Although the *mit*$^-$ mutants have no selective advantage over *mit*$^+$ alleles, they must have a selective advantage over the ρ^- mutants. We have already presented indirect evidence leading to this conclusion (Putrament *et al.* 1976). Direct evidence comes from the fact that ρ^- mutations are lethal for the cells carrying a nuclear mutation *op1* (Kovacova, Irmlerova & Kovac, 1968), while *mit*$^-$ are not (Kotylak & Slonimski, 1977). Therefore the *mit*$^-$ mutant clones might originate through intracellular selection from cells in which a single copy of mitochondrial genome carries the *mit*$^-$ mutation, while all other genomes are irreversibly damaged. If this is true then *mit*$^-$ mutant clones will be recovered from cells which did not divide during or after mutagenic treatment, and before being plated to identify the mutants. The frequency of the *mit*$^-$ mutants recovered may be expected to be correlated with the frequency of ρ^- mutations.

(3) The *mit*$^-$ mutant clones might originate without segregation from cells containing initially only a single mutant genome. The homoplasmic condition might be achieved by unidirectional non-reciprocal recombination *mit*$^+ \rightarrow$ *mit*$^-$. The *mit*$^-$ mutants thus arising will retain their property of unidirectional non-reciprocal recombination, so that an overwhelming majority or even the entire diploid progeny from isonuclear and isomitochondrial *mit*$^-$ × *mit*$^+$ crosses will be converted into *mit*$^-$.

Obviously these three pathways which might lead to the formation of homo-

plasmic *mit*$^-$ clones do not exclude each other, and in fact they may even be complementary. It is possible, however, that one of them plays a predominant role under the experimental conditions applied for isolation of *mit*$^-$ mutants (Kotylak & Slonimski, 1977; for review of other methods see Putrament *et al.* 1978).

The experimental approach we adopted consisted of:

(1) Comparison of the frequencies of *mit*$^-$ mutations under conditions either (*a*) allowing, or (*b*) preventing segregation of *mit*$^-$ and *mit*$^+$ alleles. When manganese was added it was necessary to estimate the number of divisions (if any) which the cells might undergo in the cation-containing medium, as well as induction of ρ^- mutations. The latter was the measure of irreversible damage to mitochondrial genomes.

(2) Testing in appropriate crosses whether the *mit*$^-$ mutants which originated without segregation of *mit*$^-$ and *mit*$^+$ alleles showed unidirectional *mit*$^+ \rightarrow$ *mit*$^-$ conversion.

MATERIAL AND METHODS

(i) *Strains*

The strains used are listed in Table 1. For screening *mit*$^-$ mutants strain 777-3A, carrying the nuclear mutation *op1* was used. The advantages of this mutation were discussed by Kotylak & Slonimski (1977). Strains AB1-4A/8 and AB1-11D are isonuclear, isomitochondrial with strain 777-3A (Kruszewska & Szczesniak, 1980).

(ii) *Media*

YEPglu2 – 1 % yeast extract, 1 % peptone, 2 % glucose; YEPglu10 – as above, but with 10 % glucose. YEPglyc – as above, but with 2 % glycerol instead of glucose. YEPdif – YEPglyc containing in addition 0·5 % glucose. For treatment with manganese YEPglu2 medium with pH adjusted to 6·0 was used. For selection of *ery*^{r} mutants YEPglyc medium was supplemented with phosphate buffer pH 6·2 (final concentration 0·05 M) and erythromycin (final concentration 0·5 %).

Minimal medium containing 0·67 % yeast nitrogen base medium without amino acids, but with 2 % or 5 % glucose, as indicated.

(iii) *Mutagenic treatment*

Strain 777-3A and its derivatives tend to form clumps. To remove them, the cells, grown to a late exponential phase, were centrifuged at low speed for 3–5 min. Single and budding cells which remained in the supernatant were used as inocula. They were added at a final density of $1–5 \times 10^5$/ml to YEPglu2 medium, pH 6·0 containing 4–10 mM-$MnSO_4$ (referred to as manganese medium). After 24 or 48 h incubation at 28 °C in a shaker, the cells were counted and plated. The *op1* strain was plated on YEPglu10. The *OP* strains were plated either on YEPglu2, or on YEPdif. In the former case the percentage of ρ^+ colonies (whole + sectored) was determined by tetrazolium salt staining. In the latter case ρ^+ and ρ^- colonies could be distinguished after 5–6 days incubation by their size, and by the slight buff colour of the ρ^+ colonies and sectors.

Estimation of cell survival was based on comparison between cell counts in a haemacytometer and the number of colonies on the plates.

We were not able to distinguish between viable and non-viable cells by staining with cotton blue.

Table 1. *Genotypes and origin of strains used*

Strain	Nuclear genotype	Mitochondrial genotype	Origin
777-3A	*a*, *ade1*, *op1*	ρ^+	P. P. Slonimski
AB1-11D	*α*, *ade1*	ρ^+	A. Kruszewska
AB1-4A/8	*a*, *his*	ρ^+	A. Kruszewska
AB1-4A/8	*a*, *his*	ρ^0	A. Putrament
DT15	*a*, *leu4*, *his*	ρ^0	G. Fink
rho$^-$ testers:			
A211	*a*, *his1*, *trp1*	ρ^- *oxi1*$^+$ *oxi2*$^+$	Kotylak & Slonimski 1977
DT4A1	*a*, *his1*, *trp1*	ρ^- *oxi1*$^+$	Kotylak & Slonimski 1977
RP156	*a*, *his1*, *trp1*	ρ^- *oxi2*$^+$	Kotylak & Slonimski 1977
RP131	*a*, *his1*, *trp1*	ρ^- *oxi3*$^+$	Kotylak & Slonimski 1977
RP617/A1B1	*a*, *his1*, *trp1*	ρ^- *box*$^+$	Kotylak & Slonimski 1977

Both ρ^0 strains were obtained from respective ρ^+ by incubation in YEPglu2 with 50 μg/ml ethidium bromide for about 100 cell generations. The ρ^- testers have lost most of their mitDNA, and retained the regions as designated. A *mit*$^-$ mutant is classified as e.g. *oxi3*$^-$ when in the diploid progeny of this mutant crossed with ρ^- *oxi3*$^+$ tester the *mit*$^+$ mitochondrial genotype is restored.

Abbreviations and gene symbols:

mitDNA – mitochondrial DNA

ρ^+, cells in which mitDNA molecules carry complete sets of mitochondrial genes, although some of them may be mutant. In such mitDNA molecules reside mitochondrial genomes (Birky, 1978).

ρ^- and ρ^0, mutants which have extensive deletions and complete loss of mitochondrial genomes, respectively. They have irreversibly lost the capacity of respiration and of carrying out mitochondrial protein synthesis.

ρ^+ *mit*$^-$, mitochondrial mutants which have lost the ability to respire, but are capable of carrying out mitochondrial protein synthesis (Slonimski & Tzagoloff, 1976).

ρ^- *mit*$^+$, ρ^- mutants carrying the wild-type allele of a particular *mit*$^-$ mutant (see Table 1).

oxi1$^-$, *oxi2*$^-$, *oxi3*$^-$, *mit*$^-$ mutants lacking cytochrome c oxidase activity.

box$^-$, *mit*$^-$ mutants lacking coenzyme QH_2-cytochrome c reductase activity; some of them lack also cytochrome c oxidase activity.

drug^r, mitochondrially inherited mutations leading to drug resistance.

ery^r, mitochondrially inherited mutations conferring resistance to erythromycin.

op1, nuclear mutations conferring inability to grow on non-fermentable substrates; *op1* ρ^- cells are inviable.

CFU, colony-forming units.

(iv) *Isolation of* mit$^-$ *mutants*

Strains carrying the *op1* mutation do not grow on non-fermentable substrates: for growth to proceed, some as yet unknown mitochondrial functions are required, so that ρ^- mutations are lethal in *op1* cells (Kovacova *et al.* 1968). On the other hand, mutations in mitochondrial genes coding for subunits I, II and III of

cytochrome c oxidase (*oxi3*, *oxi1* and *oxi2* genes, respectively, Cabral *et al.* 1978) as well as in the *box* region (Kotylak & Slonimski, 1977) have no deleterious effect on these strains. Mutations in these genes, defined as ρ^+ *mit*$^-$ by Slonimski & Tzagoloff (1976), were identified according to Kotylak & Slonimski (1977). The general scheme of their isolation was as follows:

Cells were incubated in manganese medium or irradiated with u.v., and plated on YEPglu10 plates. After 4–5 days the colonies were transferred in a grid pattern onto plates with the same medium, and after an overnight incubation were replica-crossed with the ρ° tester. After 2–3 days the diploids were replicated on YEPglyc medium, and those which failed to grow were classified as *mit*$^-$ mutants. The mutants were subcloned, and 20–40 subclones again replica-crossed with the ρ° tester. To determine whether segregation of *mit*$^-$ from *mit*$^+$ alleles plays an important role in establishing of pure *mit*$^-$ clone, samples of mutagenized cells were grown in liquid manganese-free medium for 24 h (8–9 cell divisions) and tested as above (see Tables 2–4).

(v) *Identification of the* mit$^-$ *mutants*

This was based on restoration of the *mit*$^+$ phenotype by known ρ^- testers (Table 1).

(vi) *Induction of* ery^r *mutants and* mit$^+$ *revertants*

The manganese concentration and inocula were as those described above. Other methods are described in the legend to Table 6, or have been described previously (Putrament, Baranowska & Prazmo, 1973; Putrament *et al.* 1975).

(vii) *Induction of* mit$^-$ *mutants with ultraviolet light*

Cells of the *op1* strain were prepared as for manganese mutagenesis and irradiated in water at a cell density of $1–5 \times 10^6$/ml with a UV HND 30 (Osram) lamp. The dose rate incident on the cells was measured as 33 ergs/mm^2. The irradiated cells were plated on YEPglu10 plates.

RESULTS

(1) *Induction of mit*$^-$ *mutations*

The data presented in Table 2 shows that in u.v.-irradiated cells the frequency of *mit*$^-$ mutations increased by one order of magnitude. Manganese proved to be much more efficient. The frequencies of *mit*$^-$ mutations depended neither on the concentration of the cation, nor on cell divisions taking place during mutagenic treatment (cf. Table 3). They increased about twice, however, after 48 h incubation, as compared with 24 h. When after 24 h treatment the cells were allowed to undergo 8–9 divisions in manganese-free medium the frequencies of *mit*$^-$ mutations did not change, as compared with the values found after direct plating.

Upon subcloning and retesting, the majority, but not all, *mit*$^-$ clones proved to contain no *mit*$^+$ cells.

The experimental procedure applied to induce *mit*$^-$ mutations with u.v. light did not allow segregation of *mit*$^-$ and *mit*$^+$ alleles. As will be shown below, during treatment with 8 and 10 mM-manganese the cells underwent 1–3 divisions, yet *mit*$^-$ mutants were recovered. It seems, therefore, that segregation of *mit*$^-$ and *mit*$^+$ alleles plays an unimportant role in the origin of homoplasmic *mit*$^-$ clones from mutagen-treated cells.

Table 2. *Induction of* mit$^-$ *mutations by manganese and u.v.*

	Incubation time in manganese medium			
	24 h		48 h	
Mn^{2+} (mM)	Colonies tested	Per cent *mit*$^-$	Colonies tested	Per cent *mit*$^-$
	Direct plating			
0 (spontaneous)	9716	0·04 (0·01–0·06)	—	—
4	1007	1·2 (0·6–1·9)	1067	2·1 (1·4–3·2)
6	1094	0·8 (0·4–1·5)	1186	2·5 (1·7–3·6)
8	1299	1·4 (0·8–2·2)	1041	2·4 (1·5–3·5)
10	1149	0·7 (0·3–1·3)	1063	2·2 (1·4–3·2)
	With 24 h post-incubation in liquid manganese-free medium			
8	472	1·5 (0·6–3·0)	—	—
10	875	0·7 (0·2–1·5)	—	—
u.v.	1339	0·5 (0·2–1·0)	—	—

mit$^-$ mutants of strain 777-3A were identified as described by Kotylak & Slonimski (1977). DT15 was used as a ρ^0 tester strain. Over 99 % of colonies tested formed diploids in replica-crosses with the tester strain. A single dose of u.v., 1980 ergs/mm^2, was used. It gave > 1 % cell survival. Confidence intervals of 95 % of the *mit*$^-$ frequencies are given in parentheses. The differences between the values found after 24 and 48 h of mutagenic treatment with manganese are statistically significant only for 6 and 10 mM-manganese.

(2) *Cell divisions and survival of* ρ^+ cells during and after manganese treatment

Manganese added to the growth medium affects the cells in a number of different ways. First, it slows down cell division. Mitochondrial mutations can be induced even when the cells practically do not divide (Putrament *et al.* 1977). Thus, at proper concentration the cation can serve not only as a mutagenic agent, but also as an agent which inhibits cell division, thus preventing the segregation of *mit*$^-$ from *mit*$^+$ alleles. Second, manganese may exert a non-specific toxic effect, so that often, although not always, some of the cells become inviable. Our unpublished observations indicate that ρ^- petites are more sensitive to this toxic effect than ρ^+ cells. Third, *ery*^{r} presumed point mutations are induced after 4–6 h treatment, while ρ^- mutations are induced only after at least 12 h treatment (Putrament *et al.* 1975). The purpose of the experiments described below was to establish how different concentrations of manganese inhibit cell division, to estimate its non-specific toxic effects and to determine the frequency of induction of ρ^- mutations.

The cells treated with 4 and 6 mM-manganese underwent several divisions during 48 h incubation (Table 3). Cells treated with 8 and 10 mM-manganese divided only 2–3 times during 48 h incubation, while after 24 h incubation they divided only once, and did not separate from each other.

If such pairs or bigger clumps of cells contained *mit*$^-$ and *mit*$^+$ cells, the mutants would never be recovered even if cell divisions were not required for segregation

Table 3. *Doublings of colony-forming units during* 24 *and* 48 *h incubation in manganese medium*

Strain:	777–3A, *op1*		AB4–11D, *OP*	
Mn^{2+} (mM)	24 h	48 h	24 h	48 h
	Direct plating			
0 (control)	9		9	
4	2·3	6·7	2·0	7·0
6	1·0	3·5	1·0	3·5
8	0·2	2·0	0·5	2·0
10	0·0	0·7	0·5	1·0
With 24 h post-incubation in liquid manganese-free medium				
8	8·2	—	7·3	—
10	9·0	—	6·5	—

The cells were treated as described in Materials and Methods. The CFUs counted consisted of 1–3 cells after incubation with 8 and 10 mM and several cells after incubation with 4 and 6 mM-manganese. The doublings of CFU after 48 h incubation refer to the number of doublings which the cells underwent starting with time 0. The results for strain 777-3A, *op1* represent the mean from five experiments for 4–8 mM-manganese, and two experiments for 10 mM-manganese, while those for strain AB4-11D, *OP* are from a single experiment in which the survival of CFU was 100%, and over 200 colonies were tested.

Table 4. *Survival of colony-forming units in strain* op1 *and of the* ρ^+ *CFU in strain* OP

	op1 CFU survival (%) after incubation for		*OP* % survival of ρ^+ cells after incubation for	
Mn^{2+} (mM)	24 h	48 h	24 h	48 h
	Direct plating			
0	100	100	92	87
4	17 ± 9·6	0·9 ± 0·3	59	2·4
6	25 ± 14	0·9 ± 0·8	62	9·0
8	35 ± 21	0·9 ± 0·7	75	24·0
10	35 ± 11	0·8 ± 0·3	77	33·0
With 24 h post-incubation in liquid manganese-free medium				
8	9 ± 3		14	
10	12 ± 5		20	

All data are from the experiments described in the legend to Table 2. The percentages of surviving ρ^+ cells (pure ρ^+ and sectored ρ^+ and ρ^- colonies) in strain AB4-11D are corrected for spontaneous ρ^- mutants.

of *mit*$^-$ from *mit*$^+$ alleles. If, on the other hand, the clumps contained *mit*$^-$ and ρ^- cells, the latter would be inviable in the strain carrying the *op1* nuclear mutation, so that only *mit*$^-$ mutants would grow into colonies.

It was important, therefore, to estimate the induction frequency of ρ^- mutations in the *op1* strain under conditions of different manganese concentrations and times of incubation in the manganese medium. Estimation of cell survival of the *op1* strain was not sufficient because it would be impossible to distinguish cell lethality resulting from ρ^- mutations and that resulting from the non-specific toxic effects of the cation. Therefore for the measurements of ρ^- induction, two *OP* strains otherwise isogenic with the *op1* strain were used.

When the data for the number of cell divisions of strain *op1* (Table 3) are compared with those for survival (Table 4), it is clear that a high percentage of cells lost their viability after a few divisions in manganese-containing medium. Exactly the same phenomenon was observed when the *op1* strain was incubated with ethidium bromide: ρ^- mutations were induced, and the cells were capable of undergoing only a few residual divisions.

A total of seven experiments were performed with the *OP* strains AB1-4A/8 and AB1-11C (Table 1). In all experiments the percentages of pure ρ^- clones decreased with the increased concentrations of manganese. The results of one of the experiments are given in Table 3. The data are expressed as per cent survival of the ρ^+ cells rather than induction of ρ^- mutations, so that the cell survival of the *op1* strain (i.e. cells which escaped either induction of ρ^- mutations or non-specific toxic effects of manganese) can be compared with the numbers that remained ρ^+ in strain *OP*. Survival of the ρ^+ cells in strain *OP* was higher than cell survival of strain *op1*. One can infer, therefore, that a large fraction of cells of strain *op1* lost viability as a result of ρ^- mutations, but the non-specific toxicity of the cation was also in part responsible for the total decrease of cell survival in this strain.

When the cells after incubation for 24 h in 8 and 10 mM-manganese medium were allowed to grow for the next 24 h in standard medium and then plated, the survival of CFU in *op1* strain and that of ρ^+ cells in *OP* strain was lower than the respective values found after direct plating. This suggests strongly that after 24 h incubation in manganese media a considerable part of surviving ρ^+ cells in both strains segregated out ρ^- progenies.

Thus, during treatment with manganese the cells underwent only a few divisions, and most of the progeny were inviable in the *op1* strain, mainly as a result of ρ^- mutations.

(3) *Growth rates of* ρ^+ mit$^+$, ρ^+ mit$^-$ *and* ρ^- *isogenic strains*

If the second assumption concerning the origin of pure *mit*$^-$ clones is correct (see Introduction), the ρ^+ *mit*$^-$ phenotype should have a selective advantage over ρ^-. The results shown in Table 5 indicate that this is true at least as regards the growth rates of ρ^+ *mit*$^-$ and ρ^- cells.

(4) *Induction of* ery^r *mutation and* mit$^+$ *reversion*

The absence of dose-dependence in the induction of *mit*$^-$ mutations (Table 2) strikingly contrasted with our previous findings (Putrament *et al.* 1973) on induction of *ery*^{r} mutations by manganese. Since the difference could be due to strain specificity, induction of *ery*^{r} mutations and reversion of a *mit*$^-$ mutant *oxi3*$^-$ were estimated in a haploid and a diploid strain, respectively, both isogenic with strain 777-3A used for induction of *mit*$^-$ mutations.

Table 5. *Growth rates of isonuclear* ρ^+ mit$^+$, ρ^+ mit$^-$ *and* ρ^- *diploid strains*

	Division time (h) in medium:	
Diploid	YEPglu2	Minimal, 2% glucose
ρ^+ *mit*$^+$	1·8	2
ρ^+ *mit*$^-$	2·0	2·3
ρ^-	3·3	8·3

The strain ρ^+ *mit*$^+$ is a diploid from cross 777-3A × AB1-4A/8 ρ°, ρ^+ *mit*$^-$ – from cross 777-3A *mit*$^-$ V2B1 (oxi3$^-$) × AB1-4A/8 ρ°. This diploid was grown overnight in YEPglu2 with 50 μg/ml ethidium bromide, and a ρ^- (or ρ°) mutant was isolated. For the experiment the cells were pre-grown in YEPglu to a mid-exponential phase, and inocula (ca. $1 \times 10^4 - 8 \times 10^4$ cells) were added to the media as indicated. After 20 h incubation at 28 °C with shaking the cells were counted, and division rates calculated.

Table 6. *Induction by manganese of* ery^r *mutations and* oxi3$^- \rightarrow +$ *reversions: expressed as mutants/revertants per* 10^5 ρ^+ *survivors after* 24 *and* 48 *h incubation*

	ery^r mutants		*oxi3*$^+$ revertants	
	24 h	48 h	24 h	48 h
	Direct plating			
0 mM-Mn^{2+}	0·2	—	0·9	—
4	69	124	59	26
6	116	371	122	149
8	153	325	219	125
10	221	209	415	62
Plating after 24 h post-incubation in liquid, non-selective manganese-free medium				
8	44		7	
10	39		8	

Induction of *ery*^{r} mutations was determined in strain AB1-4A/8. For induction of *mit*$^+$ revertants a *mit*$^-$ mutant V281 (induced with 10 mM-manganese, 24 h treatment) was synchronously crossed with strain AB1-4A/8 ρ°. During 24 h incubation in manganese-free media the surviving haploid cells underwent about 9 divisions, and diploids 12 divisions. For further experimental details see Materials and Methods.

The results show that after 24 h mutagenic treatment the frequencies of both types of mutations were strictly dependent on manganese concentrations (Table 6). After 48 h treatment there was a further increase in the frequencies of *ery*^{r}, but not of *mit*$^+$, mutations.

When the cells after incubation for 24 h in 8 and 10 mM-manganese medium were

https://doi.org/10.1017/S0016672300020607 Published online by Cambridge University Press

allowed to grow for the next 24 h in standard medium and then plated, the frequencies of *ery*^{r} and *mit*$^+$ mutations fell sharply below the values found in samples which were plated directly after mutagenic treatment (Table 6, last two rows). The possible meaning of these data will be analysed in the Discussion.

(5) *Segregation of* mit$^-$ *and* mit$^+$ *alleles in diploids*

In order to test the third possibility concerning the origin of *mit*$^-$ clones (see Introduction) nine mutants isolated from cells mutagenized with 8 or 10 mM-manganese for 24 h were crossed with the isogenic strain ρ^+ AB1-4A/8 and mitotic segregation of *mit*$^-$ and *mit*$^+$ alleles in diploids was tested. In view of the fact that the strains crossed were isonuclear and isomitochondrial (except for the *op1* and *mit*$^-$ mutations) no bias or asymmetry of the output of the *mit*$^-$ and *mit*$^+$ alleles would be expected in random samples of the diploid progenies of these crosses (Dujon, Slonimski & Weill, 1974) unless the *mit*$^-$ mutations themselves led to the bias. Indeed, in diploid progeny involving four *mit*$^-$ mutants, the number of *mit*$^-$ and *mit*$^+$ diploids were equal (Table 7). In crosses involving four other

Table 7. *Segregation of* ρ^+ mit$^+$ *and* mit$^-$ *alleles in diploids from* mit$^+$ × mit$^-$ *crosses*

		Number of colonies			
mit$^-$ No.	Mutant	*mit*$^+$	*mit*$^-$	Total ρ^+	Per cent ρ^-
V320	*oxi2*$^-$	161	143	304	7
V325	n.i.	90	199*	289	8
V324	*oxi3*$^-$	268	179*	447	4
V342	*oxi3*$^-$	153	177	390	4
V273	*oxi2*$^-$	153	116	269	8
V272	*oxi1*$^-$	148	201*	349	14
V235	*oxi2*$^-$	83	211*	294	13
V234	*oxi2*$^-$	119	104	223	7
V319	*oxi3*$^-$	214	0	214	44

Each mutant was synchronously crossed with strain AB1-4A/8 ρ^+ and the zygotes were allowed to grow for 3 days in minimal medium with 10% glucose. Then the diploids were plated on glucose minimal medium and after 3 days incubation the clones were replicated on YEPglyc dishes. *mit*$^-$ were classified as colonies which did not grow on glycerol, and which on minimal medium had diameters similar to those of *mit*$^+$ colonies. ρ^- were classified as very small colonies, none of which could grow on glycerol (see Table 5). The mutant V325 was not restored by any of the ρ^- testers (Table 1). n.i., not identified.

* Deviation from 1:1 ratio significant at 95% confidence limit.

mutants a bias was observed due either to more than 50% being *mit*$^-$ diploids, or to more than 50% being *mit*$^+$ diploids. In a cross involving one mutant only two types of diploid cells were found: big colonies which grew on glycerol medium and therefore were *mit*$^+$, and very small colonies, which were classified as ρ^- rather than *mit*$^-$ for the reasons described above (section 3). Thus, out of 9 mutants tested only two had a tendency of preferential *mit*$^+$ → *mit*$^-$ conversion, but even these mutants did not convert all *mit*$^+$ alleles present in the zygotes into *mit*$^-$.

DISCUSSION

The main purpose of this work was to see which of the three possible processes discussed in the Introduction might be involved in the origin of homoplasmic *mit*$^-$ clones. The relevant data can be summarized as follows: Manganese treatment and u.v. irradiation increased the frequencies of *mit*$^-$ mutations by 1–2 orders of magnitude (Table 2). The mutants recovered from mutagenized cells are unlikely, therefore, to be those already present in yeast cells before mutagenic treatment. The mutants induced with 8 and 10 mM-manganese, and those induced with u.v. light were recovered under conditions which practically entirely prevented segregation of *mit*$^-$ and *mit*$^+$ alleles (Table 3). The majority of *mit*$^-$ clones contained no *mit*$^+$ cells. When the mutagenized cells were allowed to undergo 8–9 divisions prior to screening the *mit*$^-$ mutants the frequencies of the latter did not increase (Table 2). The induction of *mit*$^-$ mutations by manganese was accompanied by loss in viability of the cells of the strain carrying *op1* mutation; this must, at least in part, be due to induction of ρ^- mutations (Table 4, compare survival of *op1* and of ρ^+ *OP* cells). Thus, there seems to be a positive correlation between induction of ρ^- mutations, and either induction, or recovery of *mit*$^-$ mutations. At the level of whole cells the *mit*$^+$ phenotype has a selective advantage over *mit*$^-$ in growth rate, but *mit*$^-$ has a strong selective advantage over ρ^- in this respect (Table 5). In strain 777-3C *op1*, from which the *mit*$^-$ mutants were isolated, the cells carrying a *mit*$^-$ mutation can survive, while pure ρ^- cells are inviable, so that the selective advantage of *mit*$^-$ over ρ^- phenotype is absolute.

In crosses *mit*$^-$ × *mit*$^+$ no unidirectional *mit*$^+$ → *mit*$^-$ conversion was observed (Table 7). It seems, therefore, that the third pathway for the formation of homoplasmic *mit*$^-$ clones described in the Introduction can be ruled out. Our data are insufficient to exclude the possibility that during treatment with 4 and 6 mM-manganese some homoplasmic *mit*$^-$ clones originated by segregation from heteroplasmic cells carrying *mit*$^+$ and *mit*$^-$ alleles. On the other hand, no such segregation could occur during treatment with 8 and 10 mM-manganese, because even after 48 h treatment the cells could undergo far too few divisions for segregating the *mit*$^+$ and *mit*$^-$ alleles (Birky, 1978). Of course, no segregation of the two alleles was involved in induction of *mit*$^-$ clones by u.v.

Thus, the main pathway of the origin of homoplasmic *mit*$^-$ clones following either u.v. irradiation or manganese treatment seems to be intracellular selection from cells in which at some period there is a single (or very few) essentially intact mitochondrial genome(s) carrying a *mit*$^-$ mutation, while the remaining genomes are irreversibly damaged. If this conclusion is correct, then in strains carrying the wild type, *OP* allele, the *mit*$^-$ mutants will be recovered from colonies containing *mit*$^-$ and ρ^- sectors. Our unpublished observations suggest that this is indeed the case.

After 24 h treatment the frequencies of *ery*^{r} mutations, and reversion of *oxi3*$^-$ mutation increased with the increase of manganese concentrations (Table 6). (Longer incubation in manganese media gave erratic results.) However, no such

dose-dependence was observed in the frequencies of *mit*$^-$ mutants. Incubation of mutagenized cells in non-selective medium prior to plating led to a considerable decrease of *ery*^{r} and *oxi3*$^+$ mutant cells, but had no such effect on the *mit*$^-$ mutants found. The percentage of whole ρ^- colonies were positively correlated with cell divisions either during treatment with 4 and 6 mM-manganese, or during incubation in standard medium of cells treated with 8 and 10 mM-manganese (Table 4).

We can propose only a tentative interpretation of some of these results. The induction of reversion of the *oxi3*$^-$ mutation was dose-dependent in exactly the same way as was induction of *ery*^{r} mutations. Therefore pure *ery*^{r}, as well as *oxi3*$^+$ revertant clones are formed after the cells are plated on selective media. On these media the ρ^+ *ery*^{r} and ρ^+ *oxi3*$^+$ mutations have a selective advantage not only over the respective parental ρ^+ *ery*^{r} and ρ^+ *oxi3*$^-$ phenotypes, but also over the ρ^- phenotype. Thus, all, or nearly all *ery*^{r} and *oxi3*$^+$ mutations induced are recovered on selective media as pure mutant clones. On the other hand, *mit*$^-$ mutants, having a selective advantage only over ρ^-, are recovered only from some cells, as discussed previously. It seems possible that the induction of *mit*$^-$ mutations increases with the increased concentrations of manganese, as is the case with the induction of *ery*^{r} and *oxi3*$^+$ mutations. But the fraction of the *mit*$^-$ mutants recovered is higher under conditions favouring induction of ρ^- mutations (at lower concentrations of the cation), and lower under conditions limiting induction of ρ^- mutations (at higher concentrations of the cation). In other words, the apparent absence of dose-dependence in the frequencies of *mit*$^-$ mutations may be the effect of induction of ρ^- mutations on the recovery of the *mit*$^-$ mutant clones. The latter may be increased under conditions favouring induction of ρ^- mutations.

This work was supported by the Polish Academy of Science under project 09.7.2.1.

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