Influence of the Nuclear Gene *tmp3* on the Loss of Mitochondrial Genes in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae tmp3* mutant is deficient in the mitochondrial enzyme complex that participates in the formation of one-carbon-group-tetrahydrofolate coenzymes, serine transhydroxymethylase, dihydrofolate reductase, and thymidylate synthetase, thus leading to multiple nutritional requirements of dTMP, adenine, histidine, and methionine. The *tmp3* mutant quickly loses its mitochondrial genome even when grown on fully supplemented medium or on a high concentration of 5-formyl tetrahydrofolate, which replaces all the four requirements. A study of the loss of the mitochondrial genome by following several mitochondrial genetic markers showed that there was a preferential specific loss of a large region of the mitochondrial genome, covering *mit ts983*, *E⁺*, *C⁺*, and *mit ts982* up to *O_I*, and retention of the region of *P⁺* and *mit tscs1297*. A kinetic study showed that there was a preferentially rapid loss of the region covering the *mit⁺* alleles *ts983* to *tscs902* at the rate of 10% per generation.

Inhibition of DNA synthesis in *Saccharomyces cerevisiae* cells, either by limitation of the precursor supply (4) or by deficiencies in other functions needed for its synthesis, such as *cdc8* (19), results in the formation of respiratory-deficient cells.

The loss of mitochondrial DNA and the formation of respiration-deficient strains in *S. cerevisiae* grown in the presence of antifolate drugs (5, 6, 22, 23) is due to the limitation of tetrahydrofolate formation and, hence, of 5,10-methylene tetrahydrofolate and 10-formyl tetrahydrofolate needed for the biosynthesis of both DNA and protein.

A similar formation of respiratory-deficient cells by mutations leading to a limitation in the formation of thymidylate was described in mutants bearing *tmp1* allelic to *cdc21* (4, 19) in *fol* mutants (15), and in *tmp3* mutants (16).

There are two types of mutants which are deficient in dTMP formation. The first one, *tmp1*, is deficient in thymidylate synthetase (2, 12). Here the newly isolated mutants are [*rho*⁻] as a consequence of the isolation procedure which uses antifolate drugs (4, 5), but [*rho*⁺] strains can be constructed (12, 15). *cdc21* [*rho*⁺] strains continue to produce respiratory-deficient cells at a high rate, even at the permissive temperature (19), showing the mitochondrial mutator effect of the presence of the *cdc21* nuclear gene.

Thymidylate starvation of a *tmp1* strain has been shown to lead to a rapid loss of mitochondrial DNA (1).

The other type of mutant is deficient in dTMP formation because of a deficiency in the formation of folate coenzymes leading to the multiple requirements of adenine, histidine, and methionine, as well as thymidylate. Two of these mutants, bearing, respectively, *fol1* and *fol2* (15), and in which the precise gene function lost is not known, are usually respiratory deficient but can give some *fol1* segregants which are respiratory sufficient when crossed to [*rho*⁺] strains and maintained on 5-formyl tetrahydrofolate.

The remaining type of *fol* mutant, that bearing *tmp3* (16, 24, 25), has the same multiple requirements as the other *fol* mutants (adenine, histidine, methionine, and thymidylate), which can be replaced by a high concentration of 5-formyl tetrahydrofolate. This mutant belongs to one of the groups of complementation of *fol* mutants (Luzzati and Zelikson, unpublished data). The *tmp3* mutant is deficient in the mitochondrial enzymes serine transhydroxymethylase, dihydrofolate reductase, and thymidylate synthetase (25). Until now no stable respiratory-sufficient segregants were obtained, even if *tmp3* strains were crossed to [*rho*⁺] strains and both sporulation of the diploid and germination of spores were conducted in the presence of 5-formyl tetrahydrofolate (or dTMP).

Spontaneous or induced cytoplasmic deficient mutants in *S. cerevisiae* are the result of exten-

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sive deletion of the wild-type [*rho*⁺] mitochondrial DNA, and the emerging [*rho*⁻] clones can retain any part of the mitochondrial genome (10). There is also a preferential deletion of a unique region carrying *oxi3*, *oliII*, irrespective of whether the deletions are spontaneous or mutagen induced (11, 14, 17, 20).

In the present study, we examined the loss of the mitochondrial genome in *tmp3* segregants obtained from *tmp*⁺*tmp3* [*rho*⁺] diploids, showing that the presence of the *tmp3* nuclear gene acts as a mutator even if all the nutritional requirements are supplied, either by a high concentration of 5-formyl tetrahydrofolate or by the sum of dTMP, adenine, histidine, and methionine, independent of the carbon source used. Further, we intended to determine whether there is a preferential specific loss of the mitochondrial genome different from a spontaneous [*rho*⁻] occurring in a *tmp*⁺ parental strain.

MATERIALS AND METHODS

Strains: nomenclature. The mutant described as *tmp3* (16, 24, 25) is deficient in one-carbon-group-folate coenzyme formation. This mutant can utilize 5-formyl tetrahydrofolate (folinic acid) at high concentrations, 100 to 200 mg/liter, to satisfy all the multiple nutritional requirements inherent to this mutation, i.e., dTMP, adenine, histidine, and methionine. The *tmp3* mutant complements one of the *fol* mutants described (15) and does not complement the other one (previously designated *tmp2-8*). Actually, *tmp3* might be allelic to *fol1* for the following indirect results: 23 tetrads analyzed (couple *ade3 tmp3*) gave 3 parental ditype, 5 nonparental ditype, and 15 tetratype, which, even with such a low sample, suggests the independence of the two loci. Recent data (18) on the genetic map of *S. cerevisiae* showed that *fol2* is linked to *ade3* (38.5 centimorgans). So, if *tmp3* does not complement one of the two *fol* mutants and it is independent of *ade3*, it might belong to the same complementation group as *fol1*. For the sake of clarity in this paper we have used the *tmp3* nomenclature. *tup4-55* is one of the dTMP permeability genes (22).

A list of strains is summarized in Table 1.

Media. Y medium contained 1% yeast extract, 0.2% (NH₄)₂SO₄, 0.1% KH₂PO₄, and 0.05% MgSO₄. The carbon source was 1% glucose (YG), 2% glycerol (YGly), or 1% lactate (Ylac) at pH 4.8. For growth of *tmp3* mutants, 60 mg of dTMP and 10 mg of adenine per liter were added to the Y medium (YGT), or 5-formyl tetrahydrofolate (folinic acid) (YGF) was added at 100 mg/liter in the presence of adenine or at 200 mg/liter without adenine. Antibiotic-containing media were as described by Bolotin-Fukuhara et al. (3). Minimal medium, sporulation medium, and tetrad analysis were as described before (16).

Random spore analysis of *tup4 tmp3* strains. Sporulation of diploids was induced in liquid medium (containing dTMP, 120 mg/liter, or folinic acid, 200 mg/liter), and after 2 to 3 days at 28°C, 90 to 95% asci were obtained. The asci suspension was washed and suspended in 0.1 M Tris-hydrochloride buffer (pH 8.8) containing 0.4 mg of dithiothreitol and 20 mg of

Helicase (Industrie Biologique Française) per ml. After 2 h of incubation at 30°C in a rotary shaker, asci were washed twice in water and then mixed for 1 min at the maximum speed of a Cyclomixer (Clay Adams) to release ascospores. Spores were resuspended in water and plated at a convenient dilution onto YG, YGly, or YL medium supplemented with dTMP or folinic acid. The colonies obtained from the germinating spores were replicated onto a series of plates as follows: a plate containing the same medium as the original mother plate (first transfer plate); a second one containing neither dTMP nor folinic acid, to score for *tmp*⁺ clones; a third one containing minimal glucose medium, to score for possible remaining diploids; and two complete media, the same as the original mother plate but containing a lawn of [*rho*⁺] antibiotic-sensitive tester strains, one α and the other α , to allow formation of respiratory-sufficient diploids and expression of hidden antibiotic resistance markers carried by the segregated spores. The last two plates were incubated overnight at 28°C, replicated onto glucose minimal medium for protophic isolation of diploids, and afterwards replicated onto a series of glycerol antibiotic testing media for detection of antibiotic resistance markers (8).

In each experiment about 800 spore clones were checked; the remaining diploids were less than 0.5%. a *tmp3* spore segregants were picked from the mother plate (or the first transfer plate, depending on the experiment), suspended in a small amount of water, and spotted onto 11 YGT (or YFG) plates. On each spot a drop of a fresh culture of one of the *mit*⁻ α tester strains (nine strains) or an α [*rho*⁰] or α [*rho*⁺] antibiotic-sensitive strain was added. After overnight growth at 28°C, each plate was replicated onto glucose minimal medium to select diploids; plates were later replicated onto glycerol-containing plates and incubated at both permissive and restrictive temperatures for detection of *mit*⁺ alleles (3). We also conducted a control check of the diploids obtained by crossing the spore segregants to both α [*rho*⁰] and α [*rho*⁺] antibiotic-sensitive strains for growth ability on glycerol and retention of antibiotic resistance markers.

Study of the kinetics of loss of the mitochondrial genome markers during growth of spore descendants. LZ80 diploid, antibiotic sensitive and constructed by a cross of a *tmp3 tup*⁺ strain and a *tmp*⁺ *tup4-55* one, was sporulated in the presence of folinic acid. The dissected tetrads were germinated in glucose-folate medium (YGFy), and the 3-day-old colonies were resuspended in 0.15 ml of the same medium in a multiwell dish and analyzed by a spot test for distribution of nuclear markers, mating type, and growth on YGlyF, YLacF, YGF, YGT, and YG. A *tmp3 tup*⁺ strain will grow on YGF but not on YGT, whereas a *tmp3 tup4-55* strain will grow on both media. Spore descendants having an α mating type and one of the following genotypes: *tup4-55 tmp3*, *tup*⁺ *tmp3*, or *tup4-55 tmp*⁺—were inoculated (at about 10⁶ cells per ml) into 6 ml of YGF liquid medium in an Erlenmeyer flask with a side tube. Growth was followed by turbidimetry (Klett colorimeter, filter 54). Samples were taken at zero time, at intervals of 1 h during logarithmic growth, and at stationary phase (two samples), and checked for growth on plates containing glycerol (YGlyF), lactate (YLacF), glucose (YGF), and YG without folinate to score for possible reversions of

TABLE 1. List of strains

Strain	Genotype		Origin and reference
	Nuclear	Mitochondrial	
LZ22-56B	α <i>tup4-55 lys1</i>	$[\rho^+]$	Our laboratory
LZ22-56C	α <i>tup4-55 tmp3 ade2</i>	$[\rho^+]$ (<i>gly⁻ lac⁺</i>) ^a	Isolated and maintained on lactate
LZ37-7C	α <i>tup4-55 tmp3 ser1 leu1</i>	$[\rho^-]$	Our laboratory
LZ39-10C	α <i>tup4-55 tmp3 ser1</i>	$[\rho^-]$	Our laboratory
LZ39-10B	α <i>tup4-55 ade3-10 ser1</i>	$[\rho^+]$	Our laboratory
LZ61/10-1C	α <i>tup4-55 tmp3 ser1 leu1</i>	$[\rho^-]$ P ^r	Derived from cross LZ37-7C $\times$ MH41-137A/4
LZ62/6-2D	α <i>tup4-55 tmp3</i>	$[\rho^-]$ P ^r	Derived from cross LZ37-7C $\times$ MH32-10A
LZ80-1A	α <i>tup4-55 tmp3 lys1 leu1</i>	<i>lac⁺ gly⁺</i> , becoming $[\rho^-]$ ^b	Our laboratory
LZ80-2D	α <i>tup4-55 lys1 leu1</i>	$[\rho^+]$	Our laboratory
LZ80-3A	α <i>tmp3 leu1</i>	<i>lac⁺ gly⁻</i> , becoming $[\rho^-]$ ^b	Our laboratory
LZ80-3C	α <i>tup4-55 lys1</i>	$[\rho^+]$	Our laboratory
LZ80-4D	α <i>tmp3 lys1</i>	<i>lac⁺ gly⁺</i> , becoming $[\rho^-]$ ^b	Our laboratory
LZ80-7A	α <i>tmp3 leu1</i>	$[\rho^-]$	Our laboratory
LZ80-7D	α <i>tmp3 leu1</i>	<i>lac⁺ gly⁻</i> , becoming $[\rho^-]$ ^b	Our laboratory
LZ80-11C	α <i>tup4-55 tmp3</i>	$[\rho^-]$	Our laboratory
Tester strains			
MH41-137 A/4	α <i>ade2 trp2</i>	$[\rho^+]$ C ₃₂₁ E ₅₁₄ OI ₁₄₅ P ₄₅₄ ^r	(3)
MH32-10A	α <i>his1 trp2</i>	$[\rho^+]$ C ₃₂₁ E ₅₁₄ OI ₁₄₅ P ₄₅₄ ^r	(3)
DG 3-115	α <i>ura1</i>	$[\rho^+]$ C ₃₂₁ E ₂₂₁ OI _{L7/1}	(13)
FF1210-6C	α <i>ura1</i>	$[\rho^+]$ C ^s E ^s OI ^s P ^s	(3)
194-5C	α <i>ade6 his4 ura1</i>	$[\rho^+]$ Ab ^{sc}	Our laboratory
LZ22-56B/E5	α <i>tup4-55 lys1</i>	$[\rho^0]$	Our laboratory
FF1210-6C/[ρ^0]	α <i>ura1</i>	$[\rho^0]$	(3)
FF1210-6C/324	α <i>ura1</i>	<i>ts324 cob</i> , subgroup 2	(3)
FF1210-6C/360	α <i>ura1</i>	<i>ts360 oxi_{III}</i> subgroup D3	(3)
FF1210-6C/628	α <i>ura1</i>	<i>ts628 oxi_i</i>	(3)
FF1210-6C/902	α <i>ura1</i>	<i>tscs902</i> between C and E	(3)
FF1210-6C/975	α <i>ura1</i>	<i>tscs975 oxi_{III}</i> subgroup D2	(3)
FF1210-6C/982	α <i>ura1</i>	<i>ts982</i> near <i>met1</i> tRNA	(3)
FF1210-6C/983	α <i>ura1</i>	<i>tscs983</i> between E and OI	(3)
FF1210-6C/984	α <i>ura1</i>	<i>ts984</i> near <i>met1</i> tRNA	(3)
FF1210-6C/1297-4C	α <i>ura1</i>	<i>tscs1297-4c</i> near P	(3)

^a As $[\rho^-]$ we designed a strain which could not grow on either respiratory substrate, glycerol (*gly⁻*) or DL-lactate (*lac⁻*).

^b These strains, when obtained immediately after germination, could grow on glycerol or lactate but quickly lost this capacity and became $[\rho^-]$.

^c Ab^s, Antibiotic sensitive.

tmp3 strains. Besides the growth capacities, all samples were checked for respiratory complementation capacity at the restrictive temperature by crossing with temperature sensitive (*ts*) or cold-sensitive (*cs*) *mit⁻* tester strains: ca. 10⁵ cells were spotted onto YGF plates and mated immediately with ca. 10⁶ cells of each of the nine α *mit⁻* tester strains and with controls of both α $[\rho^0]$ and α $[\rho^+]$ antibiotic-sensitive tester strains. After an overnight growth of the mated samples, they were replicated onto glucose minimal medium for prototrophic selection of diploids. The diploid patches obtained were suspended in water

and mass-checked (ca. 10⁶ cells) for growth on glycerol at the permissive and restrictive temperatures. Random analysis of the diploid progeny of different samples along the growth curve was conducted. Diploid suspensions were diluted and plated onto glycerol medium (ca. 100 colonies per plate) and incubated at the permissive temperature. At least 800 colonies of each diploid were replicated from the permissive temperature-incubated plates onto glycerol plates and were incubated at the restrictive temperature, 20 or 36°C, to study the percentage of diploids retaining the *mit⁺* alleles.

RESULTS

Loss of respiratory capacity in *tmp3* mutants. *tmp3* mutants or *tmp3* segregants obtained from heterozygous diploids are usually respiratory deficient. Stable *tmp3* strains maintained on glucose-thymidylate medium (YGT) are [*rho*⁻], but can retain some of their mitochondrial DNA (Table 2). Apart from the mitochondrial genetic evidence, an ultracentrifugation analysis of mitochondrial DNA (unpublished data) of *tmp3* strains showed the presence of a very small amount of light (density, 1.675 g/cm³) mitochondrial DNA.

tmp3 segregants such as LZ22-56C, obtained by sporulation of heterozygous diploids and germination of spores on glycerol as carbon source (YGlyT) instead of the usual glucose, and maintained on lactate medium, lost their ability to grow on glycerol but continued to grow very slowly on lactate (with a generation time of 24 h). These segregants behaved as a [*rho*⁺] strain, *gly*⁻; when mated to a [*rho*⁰] strain, the diploids issued were respiratory sufficient and grew on glycerol. The same was evident from the results on the transmission of mitochondrial antibiotic markers (Table 2).

tmp3 segregants from a diploid heterozygous for *tmp3* and homoplasmic for the resistance to the four usual antibiotics (LZ61 or LZ62) were also respiratory deficient but showed some transmission of mitochondrial markers (Table 3).

tmp3 tup4-55 spore descendants became [*rho*⁻] soon after their isolation even if supplemented with high concentrations of 5-formyl tetrahydrofolate or thymidylate; they failed to grow on glycerol and lactate (if not maintained on lactate) and lost rapidly their mitochondrial antibiotic resistance markers (Table 3). Even when *tmp3* spores were tested as soon as possible after their isolation, there was a large variation among the different *tmp3* spores as to their transmission of the antibiotic resistance markers. The only common feature was a preferential retention of the paromomycin resistance mark-

er. *tmp*⁺ spores kept all their antibiotic resistance markers when [*rho*⁺]; the few spontaneous [*rho*⁻] *tmp*⁺ spores obtained lost all their antibiotic resistance markers to the same extent, or even preferentially (for example, spore LZ61-3, Table 3) lost the paromomycin resistance marker.

A summary of the retention of *mit*⁺ alleles and antibiotic resistance alleles in *tmp3* and *tmp*⁺ spores isolated by the random spore isolation technique and tested as soon as possible is depicted in Fig. 1. *tmp3 tup4-55* spores quickly lost part of their mitochondrial genome (22 of 24 spores) when isolated on glucose and dTMP or folinate medium; *tmp*⁺ wild-type strains (it is not known whether *tup*⁺ or *tup4-55*) however, retained their mitochondrial genome. Some *tmp*⁺ strains accumulated deletions in various regions.

The presence of a *tup* gene (we used *tup4-55* in the present study) is needed for the permeation of dTMP into the yeast cell, permitting its utilization for the biosynthesis of DNA in *tmp3* mutants. The *tmp3* mutant is defective in the formation of one-carbon-group-tetrahydrofolate, and it can grow well on media supplemented with high concentrations of folinic acid. Since *tmp3*-bearing strains do not need the presence of the *tup* gene for growth on folinic acid, a study of the influence of *tmp3* or *tup4-55* on the transmission of the mitochondrial genome is possible.

Respiratory-sufficient diploids heterozygous for both *tmp3* and *tup4-55* were sporulated, and spores were germinated on glucose-folinate medium. *tmp3* and *tup4-55* segregated independently.

Some *tmp3* spores (whether *tup4-55* or *tup*⁺), tested as soon as possible (after ca. 20 to 25 generations), still grew on glycerol and lactate media; some grew only on lactate, and others did not grow on any respiratory substrates. They quickly lost their ability to grow on glycerol and lactate and lost their *mit*⁺ alleles a few generations later. The *tmp*⁺ *tup4-55* spore strain descendants retained both their ability to grow on

TABLE 2. Retention of mitochondrial antibiotic-sensitive alleles in stable *tup4-55 tmp3* and *tmp*⁺ strains

Antibiotic-sensitive strain	Tester strain	Growth of diploid on glycerol	% Retention of markers in diploid clones			No. of clones tested
			C ^s	E ^s	O ^s	
LZ39-10C (<i>tup4-55 tmp3</i>)	DG3-114 (Ab ^r)	+	2.5	0.8	0	540
	LZ22-56BE/5 ([<i>rho</i> ⁰])	-				
LZ39-10B (<i>tup4-55 tmp</i> ⁺)	DG3-115 (Ab ^r)	+	51.0	46.0	34.0	573
	LZ22-56BE/5 ([<i>rho</i> ⁰])	+				
LZ22-56C (<i>tup4-55 tmp3</i>) (maintained on lactate)	DG3-115 (Ab ^r)	+	48.0	41.0	28.5	675
	LZ22-56BE/5 ([<i>rho</i> ⁰])	+				

TABLE 3. Loss of antibiotic resistance alleles in *tmp3* segregants issuing from respiratory-sufficient, antibiotic-resistant *C⁺ E⁺ O₁⁺ P⁺* heterozygous diploids for *tmp3* and *tup4-55*^a

Spore and genotype	% Transmission of markers				No. of clones tested
	<i>C⁺</i>	<i>E⁺</i>	<i>O_I⁺</i>	<i>P⁺</i>	
<i>[rho⁻] tmp3 tup4-55</i>					
LZ61/10-1C	1.1	1.1	0	99.0	636
LZ62/6-2D	0	0	0	96.0	595
LZ61-2	1.1	1.2	0	56.0	1,030
LZ61-6	26.8	26.8	9.6	32.6	520
LZ61-9	0	0.2	0.1	8.0	1,025
LZ61-61	0.8	0.5	0	11.0	930
LZ61-72	0.2	0.2	0.1	96.0	1,050
LZ62-4	0	0.2	0	5.0	975
<i>[rho⁻] tmp⁺</i>					
LZ61-3	10.0	13.0	19.0	0	467
<i>[rho⁺] tmp⁺</i>					
LZ61/10-1D	28.0	28.0	25.0	21.0	565

^a The first two spores, LZ61/10-1C and LZ62/6-2D (as well as the other 12 spores), were obtained by the usual tetrad dissection and germination on glucose-dTMP medium (YGT); being α they were mated to the *[rho⁺]* antibiotic-sensitive α strain 194-5C. *tmp⁺ rho⁺* spores of the same tetrad retained all of the antibiotic resistance markers. The other spores were obtained by random spore isolation and were tested for their ability for transmission of antibiotic resistance markers as soon as possible after isolation. The isolated spores were of a mating type and were crossed to the α *[rho⁺]* Ab^s strain FF1210-6C.

glycerol and lactate and their *mit⁺* alleles. Five of the six *tmp3* strains tested had lost all their *mit⁺* alleles after the third transfer by replica plating (Fig. 2).

A further study of the kinetics of the loss of *mit⁺* alleles in *tmp3* strains and *tmp⁺* strains (Fig. 3) showed that during growth there is a progressive loss of *mit⁺* alleles in *tmp3* but not in *tmp⁺* strains. Among the *mit⁺* alleles tested, the first to be lost are *ts983* and *tscs902* alleles, while the region of the mitochondrial genome

which is mostly retained is that containing the paromomycin resistance allele and the closely linked *tscs1297-4c mit⁺* allele: less than 0.5% loss per generation. *mit⁺* alleles *ts983* and *tscs902* are lost at the frequency of 10% per generation (Fig. 4). This result is consistent with the kinetics of the rapid loss of growth ability on respiratory substrates such as glycerol. The same rapid loss of both *ts983* and *tscs902* was seen also in other *tmp3* strains tested, whether *tup4-55* or not.

FIG. 1. Mitochondrial genotype of *tmp3* and *tmp⁺* spores immediately after isolation. Spores were isolated by the random technique. Heavy line depicts the region of the mitochondrial genome maintained by this spore.

FIG. 2. Loss of *mit*⁺ alleles in spore descendants. Influence of further transfers, by replica plating onto glucose and 5-formyl tetrahydrofolate medium, on the ability to complement *mit tscs* mutants when mass mated. Solid line depicts the region of the mitochondrial genome maintained when all or most of the diploid population formed (>10³) retained the *mit*⁺ allele. Dotted line shows where only a small part of the population (10 to 30 diploid cells) retains the *mit*⁺ alleles.

The *tup*⁺ strains tested lost their *mit*⁺ alleles very slowly or not at all. The presence of the *tup4-55* gene had no influence on the [*rho*⁻] formation (as seen also in *tup3* strains). A highly mitochondrial mutable *tup*⁺ strain accumulating a large proportion of [*rho*⁻] cells during growth, such as LZ80-3C, was shown to lose its mitochondrial alleles more slowly than *tup3* strains. It loses all 11 *mit*⁺ alleles tested, almost to the same extent but for some preferential loss of *tscs1297-4c*, *ts360*, and *tscs975*. This strain, at zero time of the experiment summarized in Fig. 3B, already contained 24% [*rho*⁻] cells; 18% of the cells had no *tscs1297-4c* or *ts360* alleles, but only 4% lost the *tscs902* or *tscs983* alleles. During the experience summarized here, there was only slight loss of *mit*⁺ alleles. Many other *tup*⁺ strains tested showed preferential loss of the *P*^r (paromomycin resistance) and retention of the *O*^r (oligomycin resistance) markers.

A plot of the loss of all 11 *mit*⁺ *ts* alleles tested in diploid progeny of *tup3* strains show the preferential loss of several *mit*⁺ markers during growth, after one and two generations (Fig. 4), and at the same time complete retention the *P*^r and *mit*⁺ 1297-4c alleles.

DISCUSSION

The experiments summarized here show that the *tup3* mutant, which is defective in mitochondrial biosynthesis of one-carbon-group-tetrahydrofolate coenzymes and deficient in activities of the three mitochondrial enzymes serine transhydroxymethylase, dihydrofolate reductase, and thymidylate synthetase (25), quickly loses its mitochondrial DNA.

It was reported (1) that starvation of a *tup1* mutant for dTMP results in massive induction of cytoplasmic petites and increase of mitochondrial point mutations. Further work (19) showed that [*rho*⁻] production in *cdc21* (allelic to *tup1*) occurs even at the permissive temperature (23°C). Thus the deficiency in DNA precursor supply in *tup1* might be responsible for the [*rho*⁻] induction. The *tup3* mutant reported here loses its mitochondrial DNA even in the presence of high concentrations of the DNA precursors in which its biosynthesis is defective, i.e., adenine and dTMP. No increase in mitochondrial point mutations is visible when the kinetics of loss of *mit*⁺ alleles and antibiotic resistance markers is followed.

FIG. 3. Retention of mitochondrial *mit*⁺ alleles in *tmp3* and *tmp*⁺ spore descendants during logarithmic growth on glucose and 5-formyl tetrahydrofolate medium. (A) Growth curve: ○, *tmp*⁺ strain (LZ80-3C); ●, *tmp3* strain (LZ80-4D). (B) Retention of *mit*⁺ alleles by spore progeny sampled during growth of a *tmp3* strain, as analyzed by the restoration of growth ability on glycerol at the restrictive temperature of the diploids formed by mating to: △, *tscs1297-4c*; □, *ts360*; ▲, *ts628*; ▽, *ts984* and *ts982*; ■, *tscs975*; ●, *ts324*; ○, *tscs902* and *ts983*. Same as (B) but with a *tmp*⁺ strain.

FIG. 4. Retention of the different *mit*⁺ alleles in *tnp3* spore descendants (strain LZ80-4D) after one and two generations on glucose and 5-formyl tetrahydrofolate medium, as analyzed by the restoration of growth capacity on glycerol at the restrictive temperature of the diploids formed by mating to the different *ts cs* *mit*⁻ tester strains. ○, Zero control; ●, one generation; ●, two generations.

Mutant strains of *tnp1* can be maintained as [*rho*⁺] in the presence of high amounts of dTMP and on glycerol as carbon source (or at the permissive temperature for its temperature-sensitive allele, *cdc21* [12, 15]).

The *tnp3* mutant quickly loses both its ability to grow on glycerol and its mitochondrial DNA, even if primarily it was obtained as [*rho*⁺] by sporulation of a diploid heterozygous for *tnp3* and germination of spores on glucose or on a respiratory substrate such as glycerol or lactate in the presence of high amounts of either dTMP and adenine or folinic acid. A quasi-*[rho*⁺] state, as shown by persistence of mitochondrial antibiotic resistance markers (expressed only in diploids formed by mating to a [*rho*⁰] tester strain), can be achieved by maintaining some germinated spore descendants on DL-lactate, but growth is meager and slow. Transfer of these strains to conditions that permit active growth, such as glucose medium, results in rapid loss of mitochondrial DNA (and ability to grow on lactate).

It could be envisaged that in *S. cerevisiae* external dTMP cannot be used efficiently for mitochondrial DNA synthesis, but there is evidence that in the presence of *tup* genes, which permit the permeation of external dTMP into the cell, dTMP can be used for mitochondrial DNA synthesis (5).

The *tnp3* mutant has lost the mitochondrial counterparts of the three enzymes serine transhydroxymethylase, dihydrofolate reductase, and thymidylate synthetase (25). A study of the location of mitochondrial serine transhydroxymethylase in *S. cerevisiae* shows that it resides in the mitoplasm (partly in the inner membrane and mostly in the matrix; our preliminary data).

A precise model of the mechanism of the mitochondrial mutator effect of a *tnp3* nuclear gene is premature. A working hypothesis can be suggested, however.

It might be that *tnp3* lost a mitochondrial structural protein enabling import or fixation, or both, of the three enzymes into the mitochondria, allowing them to function intramitochondrially in the synthesis of C1-tetrahydrofolate, dTMP, and adenine and thus furnishing a local supply of precursors for the synthesis of DNA. Assuming that a close association between the DNA-synthesizing machinery and the enzymes providing the DNA precursors is needed, then if this association is not formed, the DNA-synthesizing machinery will be deranged. Some DNA will still be formed, especially the DNA region containing the paromomycin resistance marker (which is included as one of the putative origins of replication [9]). This is seen by the preferential maintenance of the genetic markers *P*^r and *mit*⁺ allele *tscs1297* and by the preferential deletion of the region beginning with *E*, *C*, and *tscs902* and continuing to *tscs983* and *O*_I. This is a quite different situation from the spontaneous and induced [*rho*⁻] mutants, in which there is a preferential loss in the region covering *oxi3*, *phol/O*_{II} and *mit175* (14), which is near the region mostly maintained in *tnp3* mutants. Once a deletion in mitochondrial DNA occurs, a rapid further fragmentation and loss of mitochondrial DNA continues, resulting, in most *tnp3* strains, in complete loss of mitochondrial DNA. From the results of the kinetics of loss of different *mit*⁺ alleles in *tnp3* mutants it may be calculated that at least 62 generations are needed for complete loss of alleles *tscs902* and *ts983*, and it must be much longer for the loss of the entire mitochondrial genome.

No simple diluting out of mitochondrial DNA molecules can explain this situation. Assuming that there are 50 to 100 mitochondrial DNA molecules per cell (8), dilution of mitochondrial DNA will lead to its complete disappearance in less than 10 generations.

It might still be possible that the loss of mitochondrial DNA is a secondary effect of the loss of mitochondrial protein synthesis. It was suggested (4) that the $[rho^-]$ character in *tup* and *tmp1* strains results from the selection procedure, which involves growing of the *S. cerevisiae* strains in the presence of inhibitors of dihydrofolate reductase, known to induce cytoplasmic petites acting primarily on mitochondrial protein synthesis (23). Since the *tmp3* strain is defective in mitochondrial formation of one-carbon-group-tetrahydrofolate and hence in 10-formyl tetrahydrofolate and initiation of protein synthesis, it could be that the mitochondrial protein synthesis is the primary event abolished. *tmp3* mutant spores, however, even when germinated on massive amounts of 5-formyl tetrahydrofolate as source of 10-formyl tetrahydrofolate, lose their mitochondrial DNA. It could be argued that tetrahydrofolate derivatives cannot be transported into the mitochondria, and only in situ formation could allow mitochondrial protein synthesis. As reported by Little and Haynes (15), however, 5-formyl tetrahydrofolate can enter the mitochondria, since these authors could get a $[rho^+]$ strain when a segregant of a strain heterozygous for the *fol* gene was germinated in the presence of a high amount of 5-formyl tetrahydrofolate.

In the present study no spore descendant of *tmp3* could be maintained as $[rho^+]$ (and growing well) even if massive amounts of 5-formyl tetrahydrofolate and respiratory substrate such as glycerol or lactate were used. Strains growing only slowly on lactate have been kept for many years; these strains still complement $[rho^0]$ strains, and the diploids obtained are respiratory sufficient. The slow growth on lactate but not on other respiratory substrates such as glycerol has also been observed for other recently isolated mutants (21).

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