We have isolated a thermosensitive mutant which is transformed into a population of cells devoid of mitochondrial DNA (rho') cells at 35 °C and is deficient in mitochondrial (mt) DNA polymerase activity. A single recessive nuclear mutation (mip1) is responsible for rho' phenotype and mtDNA polymerase deficiency in vitro. At 25 °C (or 30 °C) a dominant suppressor mutation (SUP) masks the deficiency in vivo. The meiotic segregrates (mip1 SUP) which do not harbor the suppressor have a rho' phenotype both at 25 and 35 °C. They have no mtDNA polymerase activity, in contrast with MIP rho' mutants of mitochondrial inheritance which do exhibit mtDNA polymerase activity. In the thermosensitive mutant (mip1 SUP), the replication of mtDNA observed in vivo at 30 °C is completely abolished at 35 °C. In the meiotic segregants (mip1 sup), no mtDNA replication takes place at 30 and 35 °C. The synthesis of nuclear DNA is not affected. DNA polymerases may have replicative and/or repair activity. There is no evidence that mip mutants are deficient in mtDNA repair. In contrast the MIP gene product is strictly required for the replication of mtDNA and for the expression of the mtDNA polymerase activity. This enzyme might be the replicase of mtDNA.

A DNA polymerase γ is present in mitochondria from higher eukaryotes (1-4). This class of enzyme is characterized by resistance to aphidicolin (for a review, see Ref. 5) and marked preference for poly(rA)-oligo(dT) as a primer template. However, the mitochondrial DNA polymerase characterized in yeast (6, 7) is unable to use the template poly(rA)-oligo(dT) and prefers activated DNA and poly(dA)-oligo(dT) as substrates. Therefore, this enzyme is not a polymerase γ. It is not related to the yeast nuclear polymerases from which it can be distinguished by its requirement for high Mg2+ concentrations (50 mM) and by its inability to use Mn2+ (7-9). The lack of mutants has hampered the elucidation of the physiological function of this mtDNA polymerase. In other words, it remains unknown whether the enzyme has a replicative and/or a repair function. In the present work, we characterize a nuclear mutant which exhibits a thermosensitive induction of cytoplasmic petites devoid of mtDNA. This mutant is deficient in mtDNA polymerase activity and unable to replicate mtDNA at the restrictive temperature.

**MATERIALS AND METHODS**

**Media**—The following solid media were used: YD (2% glucose, 2% yeast extract, 1% KAT, 2% agar); YG (2% glucose, 2% yeast extract, 1% KAT, 2% agar); WO (0.7% yeast nitrogen base Difco, 2% glucose, 2% agar Difco) supplemented with required amino acids when necessary. Liquid media contained 1% yeast extract KAT, 1% glucose, or 3% ethanol.

**Strains**—Saccharomyces cerevisiae D273-10B/A1 (or isogenic derivatives) and D225-5A were the parental strains. The genotypes and origins of the strains are given in Table I.

**Preparation of Mitochondria and Measurements of mtDNA Synthesis and mtDNA Polymerase Activity**—The strains grown in 1-3 liters of complete medium containing 1% glucose or 3% ethanol were harvested at the end of the exponential phase of growth. The cells were transformed into spheroplasts by treatment with β-mercaptoethanol followed by cell-wall digestion with zymolyase 20T (20,000 units/ml). After lysis of spheroplasts, the mitochondria were transformed into spheroplasts by treatment with β-mercaptoethanol followed by cell-wall digestion with zymolyase 20T (20,000 units/ml). After lysis of spheroplasts, the mitochondria were isolated by differential centrifugation, immediately frozen in liquid nitrogen, and stored at −70 °C. The mtDNA of intact isolated mitochondria was labeled with [methyl-3H]dTTP in a medium containing 0.5 mM mannitol, 25 mM Tris-HCl, 10 mM imidazole, 25 mM potassium phosphate, 17 mM MgCl2, 20 mM dATP, dCTP, dGTP, 5.8 μM dTTP (pH 6.8). The incorporation rate was estimated by the radioactivity present in acid-insoluble material as reported (14), except that Whatman GF/C filter paper was used instead of Whatman No. 3MM paper. mtDNA polymerase was extracted by mechanical shaking in the presence of 0.4 M NaCl, and its activity was measured by estimation of the incorporation rate of [methyl-3H]dTTP in activated salmon sperm DNA as reported (7).

**Labeling of mtDNA in Vivo**—After an overnight preculture at 30 °C in 5 ml of 1% glucose, 1% yeast extract, cells were harvested, washed 2 times with water, and inoculated in a medium containing 5 ml of 1% glucose and 0.1% yeast extract previously dephosphorylated (15).
The strains were preincubated for 1 h at 30 °C (permissive temperature) or 35 °C, centrifuged, and reinoculated into 5 ml of a fresh medium containing 1% glucose and 0.5% yeast extract (Difco). The cells were harvested, washed 3 times with 7 ml of 50 mM cooled sodium phosphate (4 °C), pH 6.5, and stored at 4 °C overnight. Spheroplasts and mitochondria were isolated as reported above, and mtDNA was purified as previously described (16). After restriction with the indicated enzymes, the mtDNA was electrophoresed in a 1% agarose gel (50 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 8.0). The gel was dried up and subject to autoradiography.

RESULTS

Rationale—The strategy used for the isolation of mutants deficient in mtDNA polymerase activity was based on the following prediction: if the enzyme is responsible for the replication of mtDNA, the mtDNA molecules of a mutant should be rapidly diluted out after a few generations, so that the resulting colony is mostly composed of rho0 cells. Moreover, thermosensitive mutants should maintain a normal mitochondrial genome at 25 °C and give rise to a population of rho0 cells at the restrictive temperature (35 °C).

Isolation of Thermosensitive Mutants (tsp0)—From three independent ethylmethanesulfonate mutageneses of S. cerevisiae D273-10B/A1, 360 clones out of 240,000 survivors could grow on glycerol at 25 °C but not at 35 °C. In only 31 clones, the lack of growth at 35 °C was due to a massive production of cytoplasmic petites (or rho0). Finally 12 of them harbored the thermosensitive rho0 phenotype (tsp0) and were selected. They were recessive and belonged to 11 complementation groups.

Synthesis of mtDNA in Isolated Mitochondria—In order to determine whether the thermosensitive phenotype in vivo was due to a deficiency in mtDNA synthesis, a kinetic study of incorporation of labeled dTTP into DNA of intact isolated mitochondria was carried out in the parental strain and tsp0 mutants at 25 and 35 °C. Aphidicolin (17) was added to all assays in order to eliminate interference with nuclear contamination. Indeed we found that this potent inhibitor of DNA polymerase α (5), and more especially of yeast nuclear polymerases (18), does not inhibit the mtDNA polymerase of yeast up to concentrations of 50 µg/ml (data not shown). In this respect, yeast mtDNA polymerase resembles the mtDNA polymerase γ of higher eukaryotes (5). The strain tsp071 was the only mutant deficient in mtDNA synthesis (Fig. 1). The low level of synthesis observed at 25 °C was even lower at 35 °C, in agreement with the thermosensitive phenotype in vivo.

Mitochondrial DNA Polymerase Activity in the Mutant tsp071—In order to elucidate whether the deficiency in mtDNA synthesis was due to a mutation affecting mtDNA polymerase activity or another protein involved in the replication complex, the mtDNA polymerase was extracted from mitochondria and assayed in the presence of activated DNA as a substrate. As shown in Fig. 2, the mtDNA polymerase activity of the parental strain was higher at 35 °C than at 25 °C. In most mitochondrial extracts of the mutant, no activity was detected at 25 °C or at 35 °C, although in vivo, the mutant could grow on glycerol at 25 °C. However, in the preparation shown in Fig. 2, a very low polymerase activity resistant to aphidicolin (less than 10% of that of the wild-type strain) was detected at 25 and 35 °C in the presence of 50 µg/ml aphidicolin, dissolved in ethanol. The final concentration of ethanol in the assay was 1% and had no inhibitory effect.

The strain tsp071 was stored at 35 °C, centrifuged, and reincubated into 5 ml of a fresh medium containing 1% glucose and 0.5% yeast extract (Difco). The cells were harvested, washed 3 times with 7 ml of 50 mM cooled sodium phosphate (4 °C), pH 6.5, and stored at 4 °C overnight. Spheroplasts and mitochondria were isolated as reported above, and mtDNA was purified as previously described (16). After restriction with the indicated enzymes, the mtDNA was electrophoresed in a 1% agarose gel (50 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 8.0). The gel was dried up and subject to autoradiography.

Mitochondrial DNA Polymerase Activity in the Mutant tsp071—In order to elucidate whether the deficiency in mtDNA synthesis was due to a mutation affecting mtDNA polymerase activity or another protein involved in the replication complex, the mtDNA polymerase was extracted from mitochondria and assayed in the presence of activated DNA as a substrate. As shown in Fig. 2, the mtDNA polymerase activity of the parental strain was higher at 35 °C than at 25 °C. In most mitochondrial extracts of the mutant, no activity was detected at 25 °C or at 35 °C, although in vivo, the mutant could grow on glycerol at 25 °C. However, in the preparation shown in Fig. 2, a very low polymerase activity resistant to aphidicolin (less than 10% of that of the wild-type strain) was detected at 25 °C. This activity seemed to be thermosensitive, since it was decreased at 35 °C. The possibility that the lack of incorporation of [3H]dTTP into activated DNA was due to an increased degradation of the substrate by the mitochondrial deoxyribonucleases (19–21) was excluded. Indeed the DNA degradation activity present in the mitochondrial extracts was low and similar in the mutant and wild-type strain (data not shown). The loss of DNA polymerase activity in the mutant mitochondrial extracts might be...
due to the high lability of the enzyme.

Genetics of the Mutant tsp\(^6\)71—The mutant tsp\(^6\)71 was able to grow on glycerol medium at 25°C. The time required for cell division was 6 h instead of 3 h in the parental strain. The spontaneous production of rho\(^-\) mutants was about 1% at 25°C and 100% at 35°C. In these conditions, the production of rho\(^-\) was lower than 1% in D273-10B/A1. The diploids issued from the cross of the mutant tsp\(^6\)71 with the wild-type strain AF1-22B had a wild-type phenotype, indicating that the mutation was recessive.

A meiotic isogenic segregant (AF1-22B) issued from the cross of the mutant tsp\(^6\)71 with a wild-type strain and exhibiting the thermosensitive phenotype was used in the genetic analyses. The meiotic progeny issued from the cross between the mutant AF1-22B and the wild-type strain D225-5A was analyzed for its ability to grow on glycerol at 25 and 35°C (Table II, cross AF6). In addition to the two parental phenotypes, a new phenotype was observed among the meiotic segregants. Some of them were unable to grow on glycerol both at 25 and 35°C. Moreover, they were composed of an entire population of rho\(^-\) cells at both temperatures. Out of 32 tetrads, only five exhibited a 2:2 segregation of the parental phenotypes. In 9 tetrads, the thermosensitive phenotype of the mutant disappeared. In contrast, these tetrads exhibited a 2:2 segregation of the rho\(^-\) phenotype both at 25 and at 35°C. Moreover, in 18 tetrads, all types of spores were present with 2 wild-type spores, 1 tsp\(^6\) spore, and 1 rho\(^-\) spore. These data suggested that the tsp\(^6\) phenotype was produced by mutations in two unlinked nuclear genes. One mutation, assigned to the locus mip1 (mip for mitochondrial polymerase) should be responsible for the inability to grow on glycerol (rho\(^-\) phenotype), and the second mutation should be a suppressor masking the deficiency at 25 but not at 35°C. Therefore, the original isolate tsp\(^6\)71 should have the genotype mip1\(^{SUP}\). The ability to grow on glycerol of the diploids issued from a cross mip1\(^{SUP}\) \(\times\) mip1\(^{Sup}\) indicated that the suppressor was dominant.

Several crosses were performed to ascertain the involvement of two nuclear genes in the expression of the tsp\(^6\) phenotype (Table II, crosses AF24 and AF12). Cross AF24 clearly showed that the mutation mip1 segregated 2:2 and was, therefore, the result of a mutation in a single nuclear gene. Similarly, cross AF12 showed that the suppressor mutation was located in one nuclear gene. Tetrad analysis and modification of phenotypes during storage of the strains at 4°C for several months showed that the suppressor mutation was rather unstable, especially in the MIP genetic background (data not shown).

Cosegregation of the rho\(^-\) Phenotype and DNA Polymerase Activity—The DNA polymerase activity was measured in the mitochondrial extracts from the meiotic segregants of tetrads representative of parental ditypes, nonparental ditypes, and tetratypes. A clear 2:2 segregation of the DNA polymerase activity was observed, as well as a perfect cosegregation of this trait with the rho\(^-\) phenotype observed at 25 and/or 35°C (Fig. 3). It was verified that the deficiency in the mtDNA polymerase activity from the meiotic segregants exhibiting rho\(^-\) phenotype both at 25 and 35°C was not the consequence of the loss of the mitochondrial genome. Indeed, in a rho\(^-\) mutant of mitochondrial inheritance with a wild-type MIP nuclear genome, obtained by ethidium bromide mutagenesis, a significant mtDNA polymerase activity was measured (Fig. 4). Therefore, the simplest conclusion was that the inability of the cells to grow on glycerol, their rho\(^-\) phenotype, and their deficiency in mtDNA polymerase activity were the result of a mutation in a single nuclear gene (mip). The loss of the mitochondrial genome was produced by the deficiency in mtDNA polymerase activity.

Replication of mtDNA in Vivo—The mtDNA polymerase

![FIG. 2. Mitochondrial DNA polymerase activity in mitochondrial extracts from D273-10B/A1 and the mutant tsp\(^6\)71 grown with ethanol as carbon source. [3H]dTTP incorporation into acid-insoluble fraction was measured at 25 and 35°C in the presence of activated salmon sperm DNA as substrate and 50 µg/ml aphidicolin.](image-url)
characterized in yeast mitochondria might be a replicative and/or a repair enzyme. In order to determine whether the MIP gene is strictly required for the replication of mtDNA, the level of $^{32}$Pphosphate incorporated into mtDNA in vivo was measured after cell incubation at permissive temperature (30°C) and at 35°C. The autoradiographies of restriction fragments of labeled mtDNA separated by agarose gel electrophoresis show that at the permissive temperature, a normal replication of the mtDNA from the mutant tsp071 (mip SUP) occurred (Fig. 5A). However, at the restrictive temperature, no newly replicated mtDNA was detected in the mutant tsp0 (Fig. 5B). In the rho' mutant (mip sup), no replication of mtDNA was observed (Fig. 5A). These results strongly suggested that the replication of the mtDNA is prevented at 30 and 35°C in the mip mutant and only at 35°C in the mip SUP mutant. However, in order to eliminate the possibility that the lack of radioactivity into mtDNA was due to the degradation of newly synthesized mtDNA by the mitochondrial deoxyribonucleases (19-21), the following experiment was performed. In a first step, the cells of the wild-type strain and tsp071 mutant were incubated with $^{32}$Pphosphate for 5 h at the permissive temperature, and after an extensive washing with a large excess of unlabeled phosphate, they were further incubated at 35°C for an additional 5 h. No degradation of the newly synthesized mtDNA was detected upon incubation at 35°C in the wild-type strain and the mutant tsp071 (Fig. 5C). The stability of the mitochondrial DNA of the mutant tsp071 corroborated the finding that the mitochondrial deoxyribonuclease activities solubilized from the inner membrane with Triton X-100 and measured as reported (20) were similar in the mutant and wild-type strain (data...
not shown). These results show that the MIP gene is required for the replication of mtDNA and that the mtDNA polymerase characterized by Wintersberger and Blutsch (7) is necessary for the replication of mtDNA. Most likely this enzyme is the replicase of the mtDNA.

The MIP gene product does not interfere with the replication of nuclear DNA, as shown by the CsCl nuclear DNA gradients obtained with the mutant grown for 6 h at 35 °C in the presence of [14C]uracil (data not shown).

**DISCUSSION**

In the present work, we have identified a nuclear mutation mip which elicits the loss of the mitochondrial genome, giving rise to a population of rho− cells. The mip rho− nuclear mutants do not exhibit any mtDNA polymerase activity resistant to aphidicolin, in contrast with MIP mitochondrial rho− mutants which possess a significant polymerase activity. In the original mutant tsp′71, the phenotype induced by the mip mutation is masked in vivo by a dominant suppressor mutation (SUP) at 25 °C but not at 35 °C. The SUP mutation is nuclear and unlinked to the mip mutation. In this thermosensitive mutant, the synthesis of mtDNA in isolated mitochondria and the mtDNA polymerase activity are not detected at 35 °C and remain very low at 25 °C, suggesting an extreme lability of the enzyme.

The mtDNA polymerase studied in the present work might have a replicative and/or a repair function. The induction rate of cytoplasmic petioles after UV or γ-rays irradiation is similar in the wild-type strain and in the mutant tsp′71. In addition, the rate of spontaneous point mutations of mtDNA is equivalent in the wild-type strain and in the mutant (data not shown). Therefore, there is no evidence that the product of the MIP gene is involved in the repair of yeast mtDNA. In contrast, experiments of mtDNA labeling in vivo have unambiguously demonstrated that the mip mutation prevents mtDNA replication either at 25 and 35 °C in the absence of the suppressor mutation or at 35 °C only in the presence of the suppressor. In the same conditions, the level of the synthesis of nuclear DNA is normal in mip mutants. These results show that the product of the MIP gene is strictly required for the replication of mtDNA and suggest that the polymerase characterized in 1970 by Wintersberger and Wintersberger (6) is the yeast mitochondrial replicase. They also show that the replication of mtDNA in yeast exhibits a high degree of autonomy in regard to nuclear replication events. This conclusion corroborates several previous reports about the partial level of interdependence and coordinate regulation of the replication of mitochondrial and nuclear DNA. In a few cdc mutants (cdc4, cdc7, cdc28) defective in the initiation of nuclear DNA replication, the replication of mtDNA is not blocked, while it ceases in mutants defective in nuclear DNA chain elongation (cdc8, cdc21) (22). In addition, in the nuclear mutant tpi (23, 24), as well as in our mutant tsp′71, the replication of mtDNA is specifically arrested in certain growth conditions, without interfering with the replication of nuclear DNA.

A final question has to be answered: does the MIP gene code for the mtDNA polymerase itself (or for a polypeptide belonging to the enzyme complex)? The fact that the synthesis of the mtDNA in isolated mitochondria and the DNA polymerase activity in mitochondrial extracts are thermosensitive suggests that the MIP gene might be the structural gene of the yeast mtDNA polymerase. Whatever the final answer is, the MIP gene is the first eukaryotic identified gene which directly controls the activity of the mitochondrial DNA polymerase.

**Acknowledgments**—This work was carried out in the laboratory of Dr. A. Goffeau. We thank him for encouragements and critical reading of the manuscript. Dr. A. H. Todd from Imperial Chemical Industries is gratefully acknowledged for the gift of aphidicolin.

**REFERENCES**

1. Bolden, A., Noy, G. P., and Weisbach, A. (1977) *J. Biol. Chem.* 252, 3351–3356
2. Bertazzoni, U., Scovassi, A. I., and Brun, G. M. (1977) *Eur. J. Biochem.* 81, 237–248
3. Tarrago-Litvak, K., Desgranges, C., Araya, A., and Litvak, S. (1979) *Eur. J. Biochem.* 93, 271–278
4. Scovassi, A. I., Wicker, R., and Bertazzoni, U. (1979) *Eur. J. Biochem.* 100, 491–496
5. Spadari, S., Sala, F., and Pedrally-Noy, G. (1982) *Trends Biochem. Sci.* 7, 29–32
6. Wintersberger, U., and Wintersberger, E. (1970) *Eur. J. Biochem.* 13, 20–27
7. Wintersberger, U., and Blutsch, H. (1976) *Eur. J. Biochem.* 68, 199–207
8. Wintersberger, U. (1974) *Eur. J. Biochem.* 50, 197–202
9. Chang, L. M. S. (1977) *J. Biol. Chem.* 252, 1873–1880
10. Foury, F., and Goffeau, A. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 6529–6533
11. Ogurt, H., St. John, R., and Nagai, S. (1987) *Science* 125, 928–929
12. Slonimski, P. P., and Tzagoloff, A. (1976) *Eur. J. Biochem.* 61, 27–41
13. Ephrussi, B., de Margerie-Hottinger, H., and Roman, H. (1965) *Proc. Natl. Acad. Sci. U. S. A.* 41, 1065–1071
14. Foury, F. (1982) *J. Biol. Chem.* 257, 781–787
15. K lootwijk, J., Klein, I., and Grivell, L. A. (1975) *J. Mol. Biol.* 97, 337–350
16. Foury, F., and Kolodny, J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 5345–5349
17. Brundrett, K. M., Dalziel, W., Hesp, B., Jarvis, J. A. J., and Neidle, S. (1977) *J. Chem. Soc. D. Chem. Commun.* 1027–1028
18. Badaracco, G., Capucci, L., Plevani, P., and Chang, L. M. S. (1982) *J. Biol. Chem.* 257, 10720–10726
19. Jacquemin-Sablon, H., Jacquemin-Sablon, A., and Paoletti, C. (1979) *Biochemistry* 18, 119–127
20. Foury, F. (1982) *Eur. J. Biochem.* 124, 253–259
21. Rosamond, J. (1981) *Eur. J. Biochem.* 120, 541–546
22. Newton, C. S., and Fangman, W. L. (1975) *Cell* 5, 423–426
23. Rubin, B. Y., and Blamire, J. (1977) *Mol. Gen. Genet.* 156, 41–47
24. Rubin, B. Y., and Blamire, J. (1979) *Mol. Gen. Genet.* 169, 41–47