ON THE MECHANISM OF PETITE GENESIS IN YEAST IV. BIOCHEMICAL CHARACTERIZATION OF A CONDITIONAL CYTOPLASMIC MUTANT PRODUCING PETITES AT RESTRICTIVE TEMPERATURE

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SUMMARY: A thermosensitive mutant, tsm8, of S. cerevisiae was isolated which is converted into rho$^-$ cells within 6-7 generations when grown in glucose medium at the non-permissive temperature (36°C). In glycerol at 36°C, growth stops after one more generation. Although some respiratory and energy conserving functions decrease only gradually after the shift from permissive (23°C) to non-permissive (36°C, glycerol) conditions, the labelling pattern of mitochondrially synthesized protein responds rapidly to the shift, as low molecular weight peptides emerge at the expense of the proteins normally synthesized in mitochondria.

INTRODUCTION

The mechanism of petite mutation in Saccharomyces cerevisiae is unknown. It is unlikely that only one mechanism of petite induction exists as a variety of inducing agents acting in different ways on mitochondria (intercalating dyes, chloramphenicol or heat) are effective in petite induction, and also because the petite cells formed by different inducers vary in stability, presence and genetic information of mitochondrial DNA (rho$^-$ or rho^o). The mutation appears to be a multiple step process the initial step(s) of which is (are) reversible (1,2), whereas the manifested mutation does not revert at all but rearrangement and loss of genes on mitochondrial DNA may continue to occur. Glucose repression or the presence of nalidixic acid provide a certain protection against petite formation by ethidium bromide (3,4). Because of the complexity of this process it may be helpful to use conditional mutants to study petite induction. We report here a study of a mutant, tsm8, which is highly efficient in producing petites. In these the presumptive early steps in the process of induction are reversible.

MATERIALS AND METHODS

The haploid thermosensitive mutant strain tsm8 was isolated and initially characterized as has been described previously (5). For the experiments described here mainly the isonuclear diploid strains SM 551 [tsm8] and SM 552 [TSM8] were used. For measurement of the kinetics of respiratory enzyme activity cells were grown in a stirred fermenter (Chemap, Switzerland) at 80% oxygen satura-

tion on 5% glycerol (or 6% glucose), 1% yeast extract (Difco), 0.5% Bacto peptone (Difco), initially at 23°C until a titer of $5\text{-}8\text{x}10^7$ was reached. Then the temperature was raised to 36°C and the cells harvested at the times indicated. Cells were broken by shaking with glass beads (Braun MSK shaker, 10 sec, cooling with liquid CO_2) and mitochondria isolated by successive differential centrifugation as described earlier (6).

For measurement of oxidative phosphorylation and mitochondrial protein synthesis cells were grown in flasks on 3% glycerol at 23°C (other conditions as above) with vigorous aeration and harvested at a titer of $2\text{-}4\text{x}10^7$. For measurement of oxidative phosphorylation mitochondria were prepared by lysis of spheroplasts (7). Respiratory control ratios and P/O values were determined from the respiratory state 3/state 4 transitions as defined by Chance and Williams (8). The labelling and SDS polyacrylamide gel electrophoresis of the products of mitochondrial protein synthesis was essentially as published recently (9). Special incubation conditions are given in the legends to the figures.

RESULTS

Mutant tsm8 grows normally at 23°C both on fermentable and non-fermentable substrate (10). When grown on glucose at 36°C cells are converted into rho^- cells within 6-7 generations (Fig. 1). The decrease in rho^+ cell frequency follows simple dilution kinetics, which extrapolates in agreement with the assumption of about 50 copies of mitochondrial DNA per rho^+ cell. This result which will be published elsewhere in detail, is further substantiated by the fact that both the ability of the mutant to transmit mitochondrial markers to zygotes and the recombination frequency follow nearly the same kinetics at the elevated temperature (11). This indicates that mitochondrial DNA most probably is lost as a whole, unfractionated.

Fig. 2 shows that part of the enzymes of the respiratory chain of glucose grown cells decrease rapidly in enzymatic activity during growth at 36°C when correlated with the number of rho^+ cells remaining in the culture (Fig. 2A), whereas other respiratory enzymes stay at a relatively constant level (Fig. 2B). It is evident from a comparison of the two types of enzymes that those which are unstable contain mitochondrially synthesized components, whereas the others probably do not. Within the error of the experiment the concentration of oligomycin necessary for half-inhibition of mitochondrial magnesium-dependent ATPase does not decrease in mitochondria derived from cells grown at restrictive temperature. The enzymatic activities of NADH- and succinate : cytochrome c oxidoreductase and of cytochrome c oxidase decrease

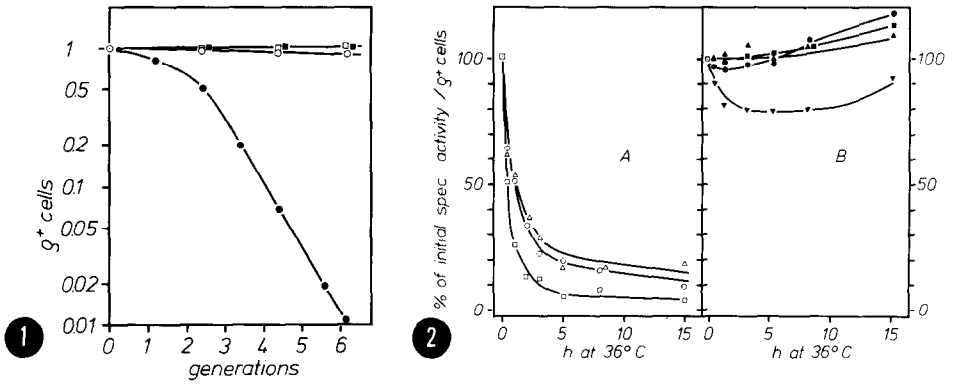

Fig. 1: Decrease in rho$^+$ cells in glucose medium at 36°C. ● glucose, 36°C, o glucose, 23°C; ■ glycerol, 36°C, □ glycerol, 23°C. In all cases cells were grown in the medium indicated and plated on glucose agar at the times given, incubated at 23°C, and then replica-plated on glucose (36°C), glycerol (23°C and 36°C).

Fig. 2: Time-dependent decrease in enzymatic activities. Cells were grown in glucose medium at 23°C, the temperature raised to 36°C, cells harvested at times indicated and mitochondria prepared and assayed as described in METHODS. 2A: □ cytochrome c oxidase; o NADH : cytochrome c oxidoreductase; Δ succinate : cytochrome c oxidoreductase. 2B: ● NADH : menaquinone oxidoreductase; ■ succinate : menaquinone oxidoreductase; ▼ oligomycin sensitive ATPase; ▲ concentration of oligomycin necessary for half-inhibition of ATPase.

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to below 10% of the initial value within 2 generations. These rates are slower than one might expect for a thermolabile function.

In Table I respiratory control ratios and P/O values of wild type (average of two preparations) and mutant tsm8 (average of 3 preparations) are compared at 23°C and 36° C, respectively. The cells were grown on glycerol at 23°C in each case.

From Table I it is evident that at the average the RC ratios are lower in the mutant than the respective values obtained with the wild type, both at 23°C and 36°C. But also the RC values of the wild type are affected by the higher temperature. At 36°C the decrease both in RC and P/O ratios is much more pronounced in the mutant, but respiratory control is preserved with all substrates tested at the non-permissive temperature, even after 20 min of preincubation and under none of the conditions tested does the P/O value drop to zero.

Fig. 3 reveals that both mitochondrial and cytoplasmic protein syntheses are affected by the temperature shift. Cells were

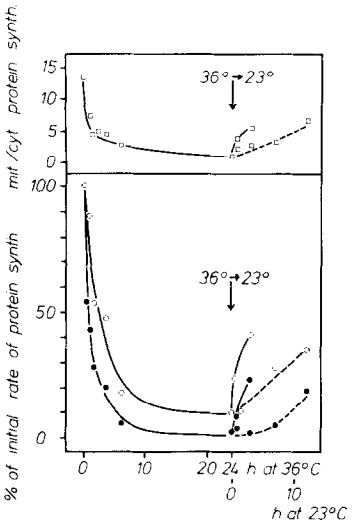

Fig. 3: Time-dependent decrease in cytoplasmic and mitochondrial protein synthesis. Cells were grown at 23°C in glycerol, harvested, washed, resuspended in glycerol synthetic medium at 36°C, and labelled with [^{3}H] leucine at the times indicated in the presence of cycloheximide (200 µg/ml, ●) or erythromycin (4 mg/ml, o), precipitated with trichloroacetic acid, washed on filters and radioactivity counted in toluene, PPO (5 g/l) and dimethyl POPOP (300 mg/l). Top figure shows the ratio of mitochondrial to cytoplasmic protein synthesis (□).

TABLE I Respiratory control ratios and P/O values of mutant and wild type

Substrate:	wild type				mutant tsm8			
	23°C		36°C		23°C		36°C	
	RC	P/O	RC	P/O	RC	P/O	RC	P/O
NADH	4.2	1.8	3.0	1.6	2.8	1.6	1.6	1.0
malate + Pyruvate	4.0	1.8	3.1	1.7	2.9	1.7	1.7	1.4
ketoglut.	4.9	3.2	3.9	3.2	3.9	3.1	2.2	2.3
succinate	2.1	1.5	1.6	1.4	1.5	1.5	1.2	0.7
ascorbate	1.9	1.1	1.6	0.7	1.5	0.8	1.1	0.4

About 0.1 mg mitochondrial protein was incubated in a buffer containing 30 mM potassium phosphate, 10 mM Tris, 10 mM maleate, 0.6 mM mannitol, 0.05% bovine serum albumin, pH 6.5, in a thermostated, closed vessel of 2 ml volume. Oxygen consumption was measured with a Clark type electrode. P/O ratios were calculated according to Chance and Williams (8). Final substrate concentrations were: 2 mM NADH, 5 mM malate, 5 mM pyruvate; 8 mM ketoglutarate; 10 mM succinate; 6.5 mM ascorbate; 0.05 mM ADP.

aerated at 36°C with glycerol as carbon source. Under these conditions the cells continue to grow only for one more generation. The rate of intrinsic mitochondrial protein synthesis decreases more rapidly than its cytoplasmic counterpart. After 6 h, 10% of the original rate of mitochondrial protein synthesis is preserved and there is still significant synthesis after 24 h. Moreover, this decrease is reversed by switching the cells back to 23°C in the presence or absence of glucose. This indicates that also the mitochondrial ribosomes do not involve a thermolabile function. Fig. 4 shows that the mitochondrial translation products synthesized at the elevated temperature are not the same molecular weight as those formed under permissive conditions. When cells are grown on galactose for one generation (Fig. 4A) at the elevated temperature and then labelled in the presence of cycloheximide, the SDS polyacrylamide gel pattern of mitochondrially synthesized proteins changes drastically. A bulk of low molecular weight species varying in size emerges at the expense

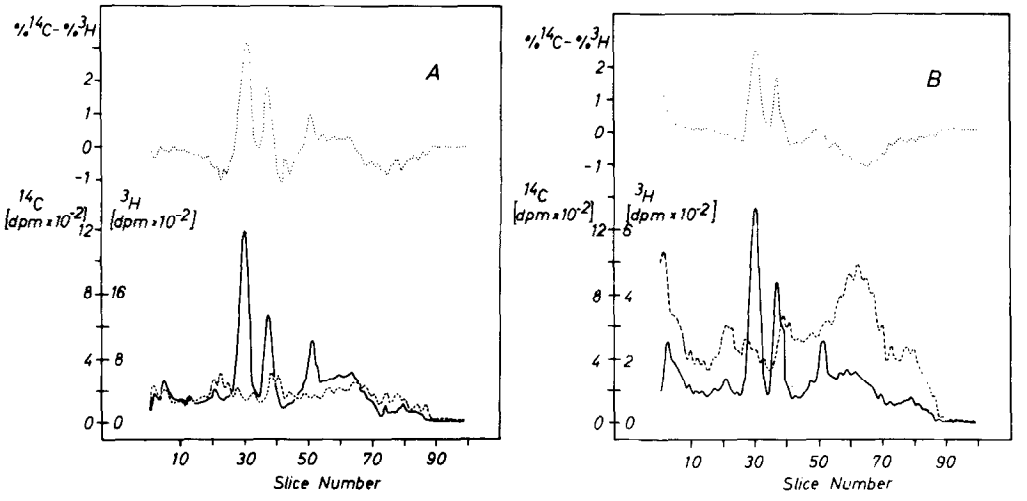

Fig. 4: Double labelling experiment of mutant cells labelled at 23°C and 36°C. Cells were grown at 23°C in 3% galactose, harvested, washed, one portion resuspended in 1.5% galactose synthetic medium at 23°C (——, [14C] leucine, isoleucine, valine, phenylalanine, presence of cycloheximide), and the other at 36°C and grown for 1 more generation (Fig. 4A) or about 5 more generations (Fig. 4B) and then labelled (----, [3H] leucine, isoleucine, valine, phenylalanine, presence of cycloheximide). Half of the mitochondrial protein labelled at 23°C was combined with each of the two portions labelled at 36°C, coëlectrophoresed, sliced and counted as described previously (9).

of the high molecular weight proteins comprising bands I, II and III of cytochrome c oxidase. Whether the low molecular weight bands progressively emerging after prolonged incubation at 36°C (about 5 generations, Fig. 4B) still contain some of the proteins synthesized at permissive temperature, or degradation products of normally synthesized proteins or nonsense proteins, cannot be decided. The fact is, however, striking that at the restrictive temperature a variety of new mitochondrial translation products is observed which are not synthesized under permissive conditions and that, on the other hand, the protein components of cytochrome oxidase synthesized in mitochondria are no longer labelled at the restrictive temperature.

DISCUSSION

Handwerker et al. (5) and Schweyen et al. (12-14) have shown that the tsm8 mutation which gives rise to petite cells at the non-permissive temperature is mitochondrially inherited. They also provided genetic evidence that the mutation does not affect 23S and 16S mitochondrial rRNA's, firstly, because the locus of the tsm8 mutation maps differently from the ribosomal rRNA's and, secondly, because the labelling pattern of mitochondrially synthesized proteins is altered much faster than the response of the mitochondrial translation activity to the temperature shift occurs.

Mutants of a similar phenotype have been described by Butow et al. (15,16) and Storm and Marmur (17), however, the genetic evidence is poor in both cases and the altered function has not been identified. We also cannot provide unequivocal proof for the function which might primarily be changed. We can, however, exclude that this function is a constituent of the respiratory chain, the energy conserving system and the mitochondrial ribosomes (cf. also ref.11), because the response of these functions on the temperature shift is too slow to contain a thermolabile component per se. All of them as well as cytoplasmic protein synthesis are, however, influenced by secondary reasons (lack of energy, exhaustion of mitochondrial protein precursor pools) during longer periods at restrictive temperature.

The most obvious and most striking feature of the mutant is its property of synthesizing undefined proteins of variable low molecular weight at the non-permissive temperature. This behavior of the mutant may be closely related to the thermolabile

function. The alteration of the membrane structure may, then, secondarily lead to the petite formation. Four explanations may be considered for the emerging of low molecular weight proteins at the non-permissive temperature. First: The shift to the non-permissive temperature induces a protease not active in the wild type. Second: The peptides are precursors of the normal mitochondrial proteins which are integrated into the membrane but not oligomerized at the elevated temperature. The mutation would affect, then, a kind of organizer protein. Third: The peptides are translation products of shortened messengers. The mutation would concern mitochondrial RNA polymerase. And, fourth: The low molecular weight proteins are due to the erroneous action of a thermolabile mitochondrial tRNA occasionally leading to premature peptide chain termination.

Which of the alternatives is correct cannot yet be decided by biochemical methods. There is, however, no genetic evidence available for the existence of mitochondrially coded proteases, organizer proteins or subunits of mitochondrial RNA polymerase, whereas, on the other hand, the coding of quite a variety of tRNA's on the mitochondrial genome in yeast has been documented (18).

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