J. Indian Inst. Sci., Jan.–Feb 1989, **69**, 89–96.
© Indian Institute of Science.

Replication and repair of mitochondrial DNA of *Saccharomyces cerevisiae*

D. S. PRADHAN, S. D. DESAI, K. G. CHETTY AND K. PASUPATHY
Biochemistry Division, Bhabha Atomic Research Centre, Bombay 400 085.

Received on January 4, 1988.

Abstract

This paper examines the replication and repair of yeast mitochondrial DNA during mitochondrial biogenesis.

Key words: Mitochondrial DNA, enzymology, biogenesis, replication, repair, respiration.

1. Introduction

The alternative life styles of *Saccharomyces cerevisiae* during growth under anaerobic and aerobic conditions have facilitated research on mitochondrial DNA functions. When anaerobically grown cells are subjected to aeration functional mitochondria are formed within 2 h of commencement of aeration. This transition process was found to be sensitive to a variety of physical and chemical agents and the primary lesion was found to be introduced in promitochondrial DNA[1–3]. Much of the work related to the enzymology of DNA replication is confined to the mammalian mitochondrial DNA[4–6]. In contrast to mammalian systems, the replication of mitochondrial DNA of *S. cerevisiae* has not been studied in detail. Yeast mitochondrial DNA is about 84000 base pair long, about five-fold larger than mammalian mitochondrial DNA[7], and hence its replication can be expected to be extremely complex. Mitochondrial DNA replication during anaerobic to aerobic transition was found to be crucial for the maturation of mitochondria and any damage to this replication process inhibited mitochondrial biogenesis[1,2]. In view of the extreme susceptibility of mitochondrial DNA during biogenesis in *S. cerevisiae* cells to DNA-acting agents, the modes of repair of mitochondrial DNA, in comparison with those of nuclear DNA repair are of crucial importance. The studies presented here were aimed at examining the replication and repair of yeast mitochondrial DNA during mitochondrial biogenesis.

2. Results and discussion

Table I depicts the results on mitochondrial biogenesis of anaerobic cells of *S. cerevisiae* during transition from anaerobic to aerobic state, which can be easily monitored as the

Table I
Respiratory adaptation in *S. cerevisiae*: effect of UV irradiation

Time of aeration of anaerobic cells (min)	Oxygen uptake of		
	Unirradiated cells aerated in dark	UV-irradiated cells aerated in dark	UV-irradiated cells aerated in light
	n moles of O_2/min/mg protein		
0	21	8	20
30	40	9	47
60	90	10	87
90	114	12	98
120	140	16	139
Aerobic cells	142	142	142

S. cerevisiae cells grown anaerobically were washed and suspended in phosphate buffer pH (7.0) to the cell density of 10^7 cells/ml. Portions of cell suspensions were exposed to UV (254 nm; 10 J/m^2). The irradiated and unirradiated cell suspensions were transferred to fresh aerobic growth medium and aerated in dark. In another experiment, the irradiated cell suspensions were aerated in visible light emitted by fluorescent lamp (370-800 nm). Aliquots of cell suspensions were withdrawn at different time intervals and the rate of substrate (succinate) oxidation was studied using a GME oxygraph[20].

increase in the rate of substrate oxidation using a GME oxygraph. This process was completely arrested by very low doses of UV radiation and has been shown to be due to lesions introduced in the DNA associated with promitochondrial structures of anaerobic cells[1]. During this respiratory adaptation, four promitochondria (per cell), each with a single copy of DNA are matured into 10-12 mitochondria (per cell) each containing 4-5 copies of DNA[8,9]. Mitochondrial DNA and RNA syntheses precede the maturation of mitochondria. This system of respiratory adaptation is hence ideal to study the mechanism of mitochondrial DNA synthesis.

Interestingly, it would seem from the results that almost all or perhaps important components of DNA replication machinery were synthesized *de novo* during transition of anaerobic cells to aerobic state.

As can be seen from Table II, induction of mitochondrial DNA polymerase was observable within the first 60 min of aeration. The enzyme activity was detected both in the cytoplasm and promitochondria.

Mitochondrial DNA polymerase was purified to electrophoretic homogeneity from aerobic cells. The properties of the enzymes are: it is Mg^{++}-dependent with an optimum pH at 7.5 and, surprisingly, lacks endonuclease activity. Antobodies to this enzyme were raised and cross-reactivity to the DNA polymerase associated with promitochondrial structures could be established (Table III).

Clayton *et al* have reported that in the mammalian mitochondrial system, H-strand DNA synthesis begins in the displacement loop region and appears to be primed by

Table II
Induction of mitochondrial DNA polymerase during respiratory adaptation

Time of aeration of anaerobic cells hr	p moles of TMP incorporated/ mg protein
0	4.5
$\frac{1}{2}$	52.0
1	45.0
Aerobic cells	5.0
Post-mitochondrial cytoplasm	2.5

Mitochondrial DNA polymerase was assayed as described by Wintersberger and Blutsch[21]. The assay mixture contained in a final volume of 0.125 ml: 0.05 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.002 M β-mercaptoethanol, 50 μM each of dGTP, dATP, dCTP and dTTP, 0.25 μCi of ^{3}HdTTP (specific activity 40 Ci/m mol) 13 μg of 'activated' calf thymus DNA and 25 μl protein of promitochondria or mitochondria or cytosol. Reaction mixture was incubated at 30°C for 15 min. Incorporation of labelled dTMP was assayed by precipitation of reaction products by 5% TCA containing 1% (w/v) sodium pyrophosphate. Acid insoluble material was collected on Whatman GF/C filter, washed with 10% TCA containing 1% sodium pyrophosphate followed by 5% TCA, alcohol:ether (1:1) and finally with ether and dried, and radioactivity measured.

Table III
Reactive efficiencies of antibodies against mitochondrial DNA polymerase with different polymerases

DNA polymerase	− antibodies	+ antibodies	% inhibition
	p moles of TMP incorporated/mg protein		
Yeast mitochondrial DNA polymerase	5.0	0.06	98.80
E. coli DNA polymerase	7.8	7.45	4.48
Rat nuclear DNA polymerase	0.071	0.070	1.40
Rat mitochondrial DNA polymerase	0.13	0.12	2.30

DNA polymerase assay was carried out essentially as described in the legend to Table II, except that to one set of experiments antibodies against mitochondrial DNA polymerase were added.

transcripts synthezied from a major promotor presumably catalysed by mitochondrial RNA polymerase[7,10,11]. L-strand DNA is synthesized on a single-stranded template and a

mitochondrial DNA primase was identified as the enzyme responsible for initiating L-strand DNA replication[12,13]. Yeast mitochondrial DNA is much larger (84 kb) as compared to mammalian mitochondrial DNA (17 kb). In spite of the enormous differences in their sizes, yeast mitochondrial DNA replication may be an RNA-primed bidirectional process similar to mammalian mitochondrial DNA replication. Recent work of Bernardi and group using petite mutants have identified at least seven ori sequences on yeast mitochondrial DNA based on the ori sequences studied in truncated DNAs in a variety of petite strains[14,15]. Of these, two seem to have nucleotide base-pair sequences similar to those found in mammalian mitochondrial DNA. Bernardi's work further suggested that ori sequences may lie within or in the vicinity of transcription-initiation sites[16]. Conceivably, therefore, it would seem that some at least of these ori sequences will function with RNA primers synthesized by RNA polymerase.

Induction of mitochondrial RNA polymerase during anaerobic-aerobic transition was also studied (Table IV). Methods have been described for purification of DNA polymerase and RNA polymerase from mitochondrial extract. However, it was necessary for our experiments that we obtain the two enzymes using the same mitochondrial extract. Hence a new method was developed in this laboratory that enabled co-purification of DNA and RNA polymerases from the same mitochondrial extract. The eluent from DEAE-cellulose column (at 0.2 M NaCl concentration) was tested for RNA polymerase activity and was further purified through another DEAE-cellulose column followed by glycerol gradient centrifugation. Mitochondrial RNA polymerase was purified thus to homogeneity and its properties were characterized.

It has been postulated that different enzymes (RNA polymerase and a primase) are involved in the RNA priming of the two nascent DNA chains and that the initiation of

Table IV
Induction of mitochondrial RNA polymerase during respiratory adaptation in S. cerevisiae

Time of aeration of anaerobic cell (h)	Activity of mitochondrial RNA polymerase p moles of ^{3}H-UMP incorporated/mg protein
0	0.144
$\frac{1}{2}$	2.87
1	1.63
Aerobic cells	0.92

Mitochondrial RNA polymerase was assayed as described by Levens *et al*[22]. The assay mixture in a final volume of 0.025 ml contained: 10 mM Tris HCl pH 7.9; 10 mM $MgCl_2$, 1 mg/ml of bovine serum albumin, 5% glycerol and 150 μM each of ATP, GTP and CTP; 1 μCi ^{3}H UTP (43 Ci/m mol), M 13 DNA 1 μg/assay. After incubation at 37°C for 20 min, reaction was terminated by the addition of 0.5 ml of 7% perchloric acid and 0.1 M sodium pyrophosphate. The reaction mixtures were then diluted with 5 ml of 0.1 M HCl and 0.01 M sodium pyrophosphate and filtered through Whatman GF/C filters, washed with 95% ethanol, dried and counted.

DNA synthesis of one strand is activated by transcription, and that of the other strand is primed by primase[11–13]. In view of a number of recent reports[5,17] which suggested that DNA polymerase could exist as polymerase-primase complex, this possibility was tested in the parent yeast mitochondrial system. Indeed yeast mitochondrial DNA polymerase was also found to have the enzyme activity capable of initiating DNA replication on single-stranded templates, like M 13 DNA and poly (dT) (results not shown). This could be due to the presence of primase responsible for initiation of DNA replication on one of the strands of mitochondrial DNA of *S. cerevisiae*.

2.1. *UV-induced damage and repair in mitochondrial DNA*

Respiratory adaptation (involving the induction of replication and transcription of DNA associated enzymes) was found to be sensitive to very low doses of UV irradiation[1]. The fragility of mitochondrial DNA can easily be shown to be related in the presence of AT spacers and GC clusters[18]. Ultraviolet radiation-induced thymine dimers preferentially at the pyrimidine-rich clusters as compared to the other DNA regions even though they were potential sites for thymine dimer formation. A related aspect that was examined is the repair of mitochondrial DNA. DNA damage in fully matured mitochondria was found to be unrepaired upon incubation in dark[1]. By corollary, it may be inferred that promitochondrial structures in anaerobic cells may also be deficient in this excision repair system. A very sensitive assay was used in which an endonuclease isolated from *Micrococcus luteus* specifically cleaves UV-induced pyrimidine dimers in the DNA and the resultant gap is seen as a strand break in the subsequent alkaline-sucrose density-gradient analysis.

The results on excision repair of promitochondrial DNA of wild type strain (Table V) revealed that at low doses (0.5 J/m^2), UV radiation-induced damage was completely repaired upon incubation in dark and at higher doses (10 J/m^2) (which impair respiratory adaptation) the dark repair is partial, but nevertheless functional. Understandably no such repair takes place in the UV radiation-sensitive strains *rad* mutants which lack excision repair. This would mean that any repair of promitochondrial DNA would have been brought about by nuclear DNA repair machinery alone.

The results on partial excision of promitochondrial DNA damage at doses above 10 J/m^2 need comment. When the dark-held cells were subsequently exposed to light, all the dimers were not removed by photoreactivation. Possibly, the excision-repair enzyme could have excised the dimers but the subsequent repair leading to sealing of gap may not have been completed.

Excision repair of nuclear DNA damage was quite efficient at all the doses studied whereas no such repair, even to a limited extent, of mitochondrial DNA was observed. It may be inferred that while nuclear DNA repair machinery influences repair of promitochondrial DNA, it does not presumably act on the DNA of fully matured mitochondria of aerobic cells. It is possible that fully mature mitochondria, but not promitochondrial structures, form a membrane barrier to the nuclear DNA repair enzymes.

It is noteworthy that UV radiation-induced damages of both nuclear and mitochondrial DNA can be efficiently repaired by photoactivation brought about by post-irradiation

Table V
Fate of endonuclease sensitive sites in the promitochondrial, mitochondrial and nuclear DNAs of wild type cells of *S. cerevisiae*

Treatment	Enzyme-sensitive sites		
	Nuclear DNA	Mitochondrial DNA	Promitochondrial DNA
	Sites/10^8 daltons	Sites/10^7 daltons	
1. Unirradiated	0	0	0
2. UV-irradiation 0.5 J/m^2	0.26	1.56	1.57
3. UV-irradiation 0.5 J/m^2 and incubation in dark for 2 hr	0	1.56	0
4. Unirradiation 0.5 J/m^2 and incubation in dark for 2 hr followed by incubation in light for 1 hr	0	0	0
5. UV-irradiation 0.5 J/m^2 and incubaion in light for 2 hr	0	0	0
6. UV-irradiation 10 J/m^2	15	26	24
7. UV-irradiation 10 J/m^2 and incubation in dark for 2 hr.	0	25	12.1
8. UV-irradiation 10 J/m^2 and incubation in dark for 2 hr followed by incubation in light for 1 hr	0	0	12
9. UV-irradiation 10 J/m^2 and incubation in light for 2 hr	0	0	0

(^{3}H)-uridine labelled organellar DNA was isolated after different treatments. One set of sample was treated with the pyrimidine dimer-specific endonuclease isolated from *Micrococcus luteus*[23]. Both the enzyme-treated and untreated samples of DNA were spun on 5–20% linear alkaline sucrose density gradients. From the distribution of radioactivity, the weight average molecular weights of DNA were calculated. Number of strand breaks seen as enzyme sensitive sites were calculated using the following formula. Number of sites per 10^7 daltons = $\left(\frac{2}{(M_w)_{+enz}}\right) - \left(\frac{2}{(M_w)_{-enz}}\right) 10^7$, where $(M_w)_{+enz}$ and $(M_w)_{-enz}$ are the weight average molecular weights for endonuclease-treated UV irradiated DNA and 'minus enzyme' control, respectively.

exposure of cells to visible light. In *S. cerevisiae* it has been shown that a common enzyme (a nuclease gene product) can accomplish repair of both nuclear and mitochondrial DNA[19]. Either this enzyme (as against the complex enzyme machinery of excision repair) is easily accessible to mitochondrial DNA, or during mitochondrial biogenesis, promitochondrial structures are in the close vicinity of the nucleus. A finely tuned coordination among nuclear transcription, replication, repair machinery and cytoplasmic protein synthesizing machinery in maturing mitochondria is expected in view of the fact that mitochondrial biogenesis is not autonomous but largely dependent on nuclear functions.

References

1. PASUPATHY, K. AND PRADHAN, D. S. Extreme ultraviolet radiation sensitivity of respiratory adaptation in *Saccharomyces cerevisiae* during transition from anaerobic to aerobic state, *Biochem. Biophys. Res. Commun.*, 1978, **83**, 365–372.

2. PASUPATHY, K. AND PRADHAN, D. S. — Extreme chemical mutagen sensitivity of respiratory adaptation in yeast, *Mut. Res.*, 1981, **80**, 65–74.

3. PASUPATHY, K. AND PRADHAN, D. S. — Susceptibility of respiratory adaptation of *Saccharomyces cerevisiae* to low doses of radiations and chemicals, *Proceedings of Indo-FRG Seminar on methodological approaches for estimation of low dose effects of radiations and chemicals in the environment*, 1983, 230–233.

4. CLAYTON, D. A. — Replication of animal mitochondrial DNA, *Cell*, 1982 **28**, 693–705.

5. CHANG, L. M. S., RAFTER, E., AUZI, C. AND BOLLUM, F. J. — Purification of DNA polymerase—DNA primase complex from calf thymus glands, *J. Biol. Chem.*, 1984, **259**, 14679–14687.

6. TAPPER, D. P. AND CLAYTON, D. A. — Precise nucleotide location of the 5′ ends of RNA-primed nascent light strands of mouse mitochondrial DNA, *J. Mol. Biol.*, 1982, **162**, 1–16.

7. BORST, P. — Mitochondrial nucleic acids, *Annu. Rev. Biochem.*, 1972, **41**, 333–369.

8. LINNANE, A. W., HASLAM, J. M., LUKINS, H. B. AND NAGLEY, P. — The biogenesis of mitochondria in microorganisms, *Annu. Rev. Microbiol*, 1972, **28**, 163–198.

9. BORST, P. — Structure and function of mitochondrial DNA, *TIBS*, 1977, **2**, 31–34.

10. CHANG, D. D. AND CLAYTON, D. A. — Priming of human mitochondrial DNA replication occurs at the light strand promoter, *Proc. Natn. Acad. Sci. USA.*, 1985, **82**, 351–355.

11. WONG, T. W. AND CLAYTON, D. A. — *In vitro* replication of human mitochondrial DNA: accurate initiation of light strand synthesis, *Cell*, 1985, **42**, 951–958.

12. WONG, T. W. AND CLAYTON, D. A. — Isolation and characterisation of a DNA primase from human mitochondria, *J. Biol. Chem.*, 1985, **260**, 11530–11535.

13. WONG, T. W. AND CLAYTON, D. A. — DNA primase of human mitochondria is associated with structural RNA that is essential for enzymatic activity, *Cell*, 1986, **45**, 817–825.

14. DE ZAMAROCZY, M., MAROTTA, R., FAUGERON-FONTY, G., GOURSOT, R., MANGIN, M., BALDACCI, G. AND BERNARDI, G. — The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity, *Nature*, 1981, **292**, 75–78.

15. DE ZAMAROCZY, M., BALDACCI, G. AND BERNARDI, G. — Putative origins of replication in the mitochondrial genome of yeast, *FEBS Lett.*, 1979, **108**, 429–432.

16. BALDACCI, G. AND BERNARDI, G. — Replication origins are associated with transcription initiation sequences in the mitochondrial genome of yeast, *EMBO J.*, 1982, **1**, 1987–1994.

17. GRONOSTAJSKI, R. M., FIELD, J. AND HURWITZ, J. — Purification of a primase activity associated with DNA polymerase from the He La Cells, *J. Biol. Chem.*, 1984, **259**, 9479–9486.

18. PRUNELL, A. AND BERNARDI, G. .. — The mitochondrial genome of wild-type yeast cell. VI. Genome organisation, *J. Mol. Biol.*, 1977, **110**, 53–74.

19. DAILY, O. P., CUTTITTA, JR., F. F. AND MACQUILLAN, A. M. — The absence of photoreactivation enzyme in yeast mitochondria, *Biochim. Biophys. Acta*, 1976, **453**, 375–381.

20. CHANCE, B. AND WILLIAMS, G. R. — A simple and rapid assay of oxidative phosphorylation, *Nature* (Lond.) 1955, **175**, 1120–1121.

21. WINTERSBERGER, U. AND BLUTSCH, H. — DNA-dependent DNA polymerase from yeast mitochondria, *Eur. J. Biochem.*, 1976, **68**, 199–207.

22. LEVENS, D., LUSTIG, A. AND RABINOWITZ, M. — Purification of mitochondrial RNA polymerase from *Sachharomyces cerevisiae*, *J. Biol. Chem.*, 1981, **256**, 1474–1481.

23. REYNOLDS, R. J. Removal of pyrimidine dimers from *Sachharomyces cerevisiae* nuclear DNA under non-growth conditions as detected by a sensitive, enzymatic assay, *Mut. Res.*, 1978, **50**, 43–56.