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Characterization of mitochondrial DNA primase from *Saccharomyces cerevisiae*

V MURTHY and K PASUPATHY*
Radiation Biology and Biochemistry Division, Bhabha Atomic Research Centre, Bombay 400 085, India

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Abstract. DNA primase from yeast mitochondria was shown to have a molecular weight of 67 kDa by SDS-PAGE and an S value of 5·5. It was shown to have preference for SS mitochondrial DNA especially fragments containing origins of replication, as a template to initiate DNA replication. Further examination of the enzyme showed its possible association with a ribonucleotide moiety essential for enzyme activity.

Keywords. Primase; yeast; mitochondria.

1. Introduction

Replication of duplex DNA is a highly complex and coordinated process involving the action of several enzymes in unison as explained by Kornberg and Baker (1992). In recent years particular attention has been focussed on replication of DNA in subcellular organelles of eukaryotic cells. Much of the work related to mitochondrial DNA replication is confined to the mammalian mitochondrial system. The entire process of replication and transcription in mammalian mitochondria has been very well deduced by Clayton (1991).

The biosynthesis of mitochondrial DNA (Mt DNA) in mammalian cells has been shown by Clayton (1982) to be initiated at two distinct origins on the mitochondrial genome. Heavy strand synthesis begins at the displacement loop region and appears to be primed by transcripts synthesized from a major promoter presumably catalyzed by Mt RNA polymerase. When 67% of the H strand synthesis is completed, light strand (L strand) DNA synthesis is initiated at distinct site on the separated parental H strand and a DNA primase is identified as the enzyme responsible for initiation of L strand DNA replication through formation of RNA primers. Information about the exact mode of Mt DNA replication in yeast *Saccharomyces cerevisiae* is scanty. The extremely large size (78 kb) and the complexity of yeast Mt DNA makes the research work a little difficult but challenging. Yeast Mt DNA replication was suggested by Baldacci *et al* (1984) to be an RNA primed bidirectional process similar to mammalian Mt DNA replication. However, recent reports by Schinkel and Tabak (1989) suggest the possibility of Mt DNA replication being similar to that of T3 and T7 bacteriophages, due to the striking homology in the replicative enzymes. Electron micrographic studies and pulsed-field gel electrophoretic work by Clarke-Walker *et al* (1991) suggest that yeast Mt DNA could possibly replicate

*Corresponding author.

by the rolling circle mechanism. Irrespective of the mode of replication, synthesis of an RNA primer could be carried out by RNA polymerase/primase. The presence of an yeast Mt DNA primase capable of synthesising RNA primers on single-stranded DNA templates which can promote synthesis of DNA in the presence of Klenow enzyme was reported by Desai *et al* (1989) from this laboratory. We report in this communication, further characterization of this enzyme.

2. Materials and methods

Deoxy and ribo nucleoside triphosphates, poly dT, pancreatic RNAase inhibitor and calf intestinal phosphatase were obtained from Sigma Chemical Company, USA. *Eco*RI, SS M13 mpl9 DNA and polynucleotide kinase were from Bangalore Genei Private Ltd., Bangalore. Zymolyase was from Kirin Brewery, Japan, [α^{32}P] ATP (sp. act. 3000 Ci/mmol) and [α^{32}P] dATP (sp. act. 3000 Ci/mmol) were from BRIT of this Research Centre. GF/B filters were from Whatman, England. All other chemicals were of Analar grade.

2.1 *Cell growth*

S. cerevisiae D 273 l0B wild type haploid strain was used in the present studies. Cells were grown in YEPD (Pasupathy and Pradhan 1978) medium for 18 h.

2.2 *Purification of yeast Mt DNA primase*

Mitochondria were prepared by Standard method from *S. cerevisiae* wild type haploid strain D273 l0B. The purification of DNA primase was carrying to the procedure of Desai *et al* (1989).

2.3 *Assay of enzymes*

The standard assay for DNA polymerase was carried out according to Wintersberger and Wintersberger (1970). Activated calf thymus DNA was prepared to assay DNA polymerase according to Aposhian and Kornberg (1962).

DNA primase assay was performed according to Clayton and Wong (1985) using poly dT, SS M13mp l9 DNA, SS Mt DNA and DS Mt DNA and restriction cut fragments of Mt DNA.

2.4 *Mt DNA preparation*

Mt DNA was prepared from *S. cerevisiae* as described by Querol and Barrio (1990). It was made single stranded by heat denaturation. Mt DNA was cut with *Eco*RI and individual fragments isolated according to S.ambrook *et al* (1989).

2.5 *Electrophoresis*

Electrophoresis (SDS-PAGE) was done according to Laemmli (1970).

2.6 *Sucrose density gradient centrifugation*

Molecular weight of DNA primase was determined using sucrose density gradients. Standard proteins thyroglobulin, catalase, alcohol dehydrogenase and bovine serum albumin were used to calibrate the gradient. Enzyme (20 µg) was loaded on a 5–20% sucrose gradient in buffer A (0.02M Tris HCl, pH 7·5, 0·01 M EDTA, 0·001 M DTE, 6 mg/1 PMSF and 10% glycerol) with 0·1 M NaCl and centrifuged for 25 h at 1,10,000 g in an SW28 rotor in L8 Beckman ultracentrifuge.

2.7 *Radiolabelling of RNA*

Five end labelling of RNA moiety was carried out as described by Sambrook *et al* (1989).

2.8 *Preparation of cytoplasmic and mitochondrial RNA*

Mt RNA was prepared as described by Rickwood *et al* (1988). Cytoplasmic RNA was prepared as described by Sambrook *et al* (1989).

2.9 *Estimation of protein*

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a Standard.

3. Results and discussion

DNA primases are generally found to be associated with DNA polymerases in a number of systems as reviewed by Roth (1987). Recent studies from this laboratory (Desai *et al* 1989) and by Wilson and Sugino (1985) have shown that it is possible to purify primase completely free from DNA polymerase.

3.1 *Initiation of DNA synthesis by primase*

In the present experiments, DNA primase was isolated from purified yeast mitochondria using DEAE-cellulose ion exchange chromatography followed by DNA-cellulose affinity chromatography. Initiation of DNA synthesis by primase was examined using SS M13 DNA, poly dT, SS Mt DNA and DS Mt DNA as templates. The results incorporated in table 1 show that Klenow in the absence of primase failed to synthesize DNA, as studied by incorporation of labelled dNMP in the acid-precipitable fraction even after 60 min incubation. However, addition of primase could initiate DNA synthesis which was extended by Klenow.

3.2 *Template preference of primase*

Results presented in table 1 also show that primase was most efficient on SS Mt DNA as compared with other DNA templates tested. It was observed that natural DNA served as better template than synthetic DNA. The enzyme exhibited no activity with DS Mt DNA. The order of efficiency on various templates was as follows; SS Mt DNA > SS M13 DNA > poly dT > DS Mt DNA. The preference

Table 1. Initiation of DNA syhnthesis by primase.

Template	Enzyme	[α^{32}P] dNMP incorporated (pmol/mg protein/h)
SS M13 mp19DNA	Klenow[a]	Not detectable
	Klenow + primase[b]	3·2
Poly dT	Klenow	Not detectable
	Klenow + primase	0·393
SS Mt DNA	Klenow	Not detectable
	Klenow + primase	7·48
DS Mt DNA	Klenow	Not detectable
	Klenow + primase	Not detectable

Assay conditions in a total of 25 µ1: For SS Ml3 and Mt DNA; 25 µg BSA, 50 mM Tris HCl, pH 7·4,·1 mM 2 mercaptoethanol, 10 mM $MgCl_2$, 2mM ATP, 200 µM each of CTP, UTP and GTP, 100 µM dGTP, dCTP, dTTP, 20 µM dATP, [α^{32}P] dATP (1 µCi/assay), 1 µg template and 0·6 µg primase enzyme.
For Poly dT the assay components remain the same except the template is poly dT, only ATP as ribonucleotide, [α^{32}P] dATP (µCi/assay), Klenow 7 µg.
[a]Reaction mixturte incubated with Klenow for 15 min or 60 min.
[b]Reaction mixture incubated with Klenow for 15 min preceded by incubation with primase for 45 min.

for SS Mt DNA as a better template indicated that Mt DNA might have specific sites that primase could recognise. Baldacci *et al* (1984) have reported that amongst 7 *ori* sequences present in wild type yeast mitochondrial genome, some are known to be initiated by RNA polymerase and some by primase. This possibility was further checked using restriction fragments of Mt DNA. Yeast Mt DNA was cut with *Eco*RI and electrophoresed on agarose to separate the 9 different fragments. The fragments of size 32·5, 17, 7·1, 3·5 and 2·4 kb were isolated, denatured and used for primase assay. Results shown in table 2 suggest that primase showed a better priming ability with fragments of size 32·5, 17, 7·1 kb which contained the *ori* sequences, as compared with 3·5 and 2·4 kb fragments, which lack *ori* sequences. This to a large extent proves that Mt DNA primase not only shows a preference for Mt DNA but the priming ability is more pronounced in regions where priming sequences (RNA start sites) have been mapped by Baldacci *et al* (1984). This could also be due to conformational changes introduced in the template primer enzyme complex.

Table 2. Preference of primase for Mt DNA fragments carrying *ori*sequences.

Template Mt DNA	Size (kb)	*Ori* sequences	[α^{32}P] dAMP incorporated (pmol/mg protein/h)
1	32·5	6,3,4	4·0
2	17	5,1	3·6
3	7·1	2,7	2·5
4	5·1	–	0·53
5	3·5	–	0·49

Assay was carried out as described in table 1. Templates used were different fragments of *Eco*RI digested Mt DNA.

3.3 *Molecular size determination*

Further studies were directed towards determining the molecular size of the enzyme. The enzyme was analyzed on a 7·5% PAGE containing SDS which showed a major band at the position of 67 kDa (figure 1). Further analysis on sucrose density

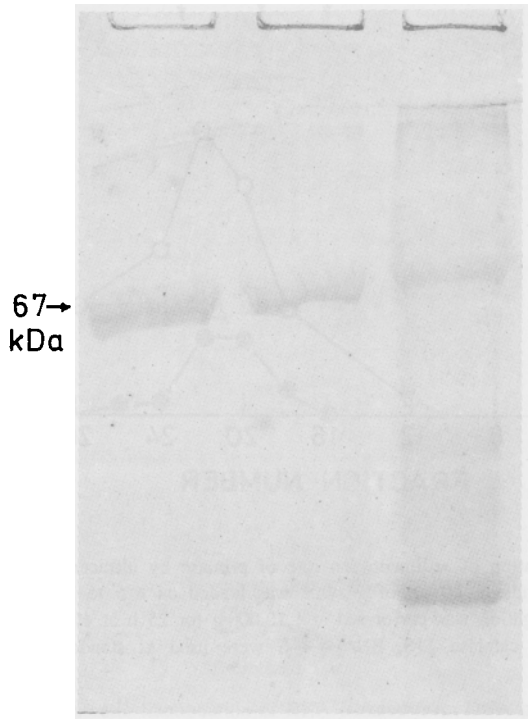

Figure 1. SDS PAGE of primase on 7·5% PAGE containing SDS. Lane 1, DNA primase before digestion of RNA; lane 2, DNA primase after digestion of RNA; lane 3, molecular weight markers.

gradient revealed the native enzyme to have an S value of 5·5 and molecular size of 76 kDa as seen in figure 2. Since there does not seem to be any subunit, it was thought that the enzyme could be associated with some other moiety other than protein. The absorption of the enzyme at 280 and 260 nm suggested the association of nucleic acid moiety up to a concentration of 4% as indicated by a monograph. To identify this moiety associated with primase the enzyme was treated with proteinase K and the nucleic acids were radiolabeled and electrophoresed on a 6% PAGE with 7 M urea. From the autoradiogram in figure 3 it is clear that a major species of RNA of about 100 nucleotides is associated with the enzyme. Attempts to determine the size of the enzyme on SDS PAGE before and after digestion of RNA moiety always resulted in a protein band of 67 kDa. Possibly alkaline elecrtophoretic conditions resulted in the dissociation of the RNA moiety. Gel filtration studies (results not shown) have confirmed the size of the enzyme with RNA moiety to be 76 kDa and after digestion of RNA to be 67 kDa.

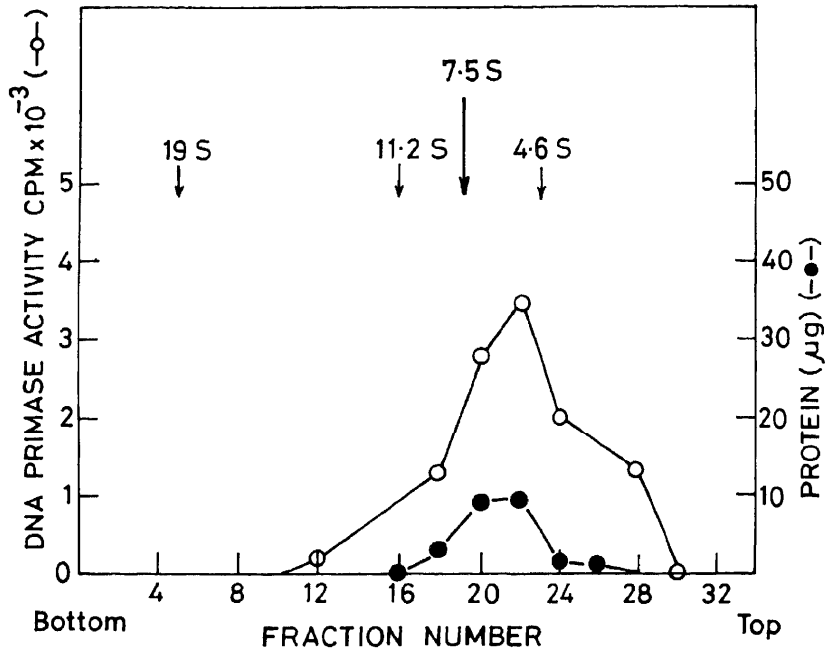

Figure 2. Determination of sedimentation rate of primase by ultracentrifugation through a 5 to 20% sucrose gradient 20 µg of enzyme was loaded on a 5 to 20% sucrose gradient in buffer A. Centrifugation was performed at 1,10,000 g for 25 h at 4°C on an SW28 rotor. Thyroglobulin 19·25, catalase 11S, BSA 4·4 S were used as standard molecular weight markers.

3.4 *Requirement of RNA moiety for primase activity*

The role of the RNA moiety on the primase activity was further studied. RNAase treatment of the enzyme resulted in removal of the RNA moiety and a decrease in the priming ability of the enzyme (table 3). Thus this moiety seems to be important for the activity of the enzyme. Substituting an exogenous RNA to the enzyme previously treated with RNAase restored its activity partially. Wong and Clayton (1986) while studying the mammalian Mt DNA primase reported the presence of an RNA moiety with primase which was found to be essential for the enzyme activity as evidenced by the loss of activity upon RNAase treatment. They observed that substituting the moiety by other exogenous RNA (cytoplasmic, mitochondrial), brought back its activity partially. This suggested that any RNA moiety, and not a specific species of RNA, could bring back the activity of primase enzyme which was earlier treated with RNAase. They have subsequently purified and sequenced the RNA moiety associated with primase and shown it to be similar in sequence to 5·8 S ribosomal RNA. The actual role of this RNA is not known. It may be required for proper interaction between the enzyme complex and the DNA template. The reason for partial restoration of primase activity by exogenous RNA may be reflection of the efficiency of the reconstitution procedure.

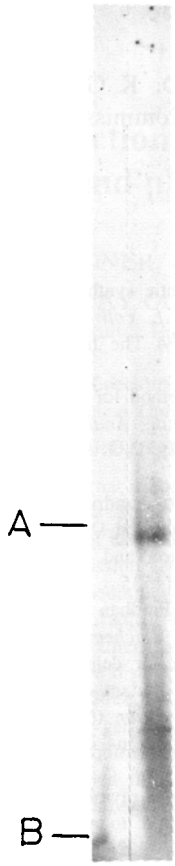

Figure 3. Electrophoretic fractionation of RNA isolated from DNA primase of a 6% PAGE with 7 M urea. Lane 1, after RNAase treatment; lane 2, without RNAase treatment. (A) Position of Xylene cyanol (106 bp). (B) Position of bromophenol blue (26 bp).

Table 3. Effect of RNAase treatment on primase activity.

Treatment	[α^{32}P] NMP incorporated (pmol/mg protein/h)
Primase	0·626 ± 0·04
Primase treated with RNAase*	0·041 ± 0·003
RNAase treated Primase + Mt RNA	0·103 ± 0·007[b]
RNAase treated Primase + Cyt RNA	0·426 ± 0·003[a]

*Primase (20 μg) was preincubated with RNAase (2 μg) at 30°C for 30 min. The RNAase activity was inhibited by the addition of 50 units of RNAase inhibitor. Control untreated primase was also treated with RNAase inhibitor. Primase assay was then carried out as detailed in table 1 using poly dT as the template and [α^{32}P] ATP (1 μCi/ assay) as the label. [a]$P < 0·05$ when compared to primase alone; [b]$P < 0·01$ when compared to primase treated with RNAase.

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