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Mechanism of initiation of yeast mitochondrial DNA replication: Role of RNA polymerase

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Abstract. Yeast mitochondrial RNA polymerase was purified and resolved into 2 distinct fractions. Peak A was found to be nonspecific and exhibited characteristics of the core polymerase, whereas peak B exhibited characteristics of the holoenzyme. *In vitro* replication assays were carried out, using the peak B enzyme, the cloned *ori* sequences and other DNA templates. It was found that *ori* 2 was the most efficient template for RNA polymerase primed DNA synthesis, as compared to all the other templates studied.

Keywords. RNA polymerase; yeast; mitochondria.

1. Introduction

Mechanism of mammalian mitochondrial (Mt) DNA replication has been well elucidated by Clayton and co-workers (Fischer and Clayton 1985; Wong and Clayton 1986; Chang and Clayton 1987; Chang *et al* 1987; Fischer *et al* 1987; ME Schmitt and DA Clayton, personal communication). The extremely large size (78 kb) and the complexity of yeast Mt DNA makes the research on the exact mode of its replication a little difficult but challenging. In yeast seven origins of replication were first identified by Bernardi *et al* (1983) using genetics of hyper suppressive petite mutants. Initiation of Mt DNA replication was postulated by Baldacci *et al* (1984) to be a RNA primed bi-directional process similar to mammalian Mt DNA. The strand which is transcribed was suggested to be primed by RNA polymerase and the other by DNA primase. We have earlier reported the presence of a DNA primase from yeast mitochondria, characterized it and demonstrated its preferential priming ability using a variety of templates (Desai *et al* 1989; Murthy and Pasupathy 1994). Sequence analysis of the *ori* regions showed several structural features of certain *ori* sequences held in common with mammalian origin of replica tion—shown to be primed by RNA polymerase—including the presence of conserved sequence boxes 1 and 2 (de Zamaroczy *et al* 1984). Yet there have been no investigations on *in vitro* replication of Mt DNA, using purified RNA poly merase. We report here our results on the role of RNA polymerase, which was purified by a procedure developed by Desai *et al* (1990), in promoting Mt DNA replication through the synthesis of RNA primers on a variety of templates including cloned *ori* sequences.

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2. Materials and methods

2.1 *Materials*

Fine chemicals and biochemicals were from Sigma Chemical Co., USA and molecular biology products (restriction nucleases, Klenow fragment, etc.) were from Boeringer, Mannheim. [α^3H] UTP (sp. act. 40-60 Ci/mmol) and [α^{32}P]dATP (sp. act. 5000 Ci/mmol) were from BRIT of Department of Atomic Energy, New Delhi. GF/B filters were from Whatman, England. All other chemicals used were of Analar grade.

2.2 *Cell growth*

Saccharomyces cerevisiae D 27310 B wild haploid strain was used in the present studies. Cells were grown in YEPD (Pasupathy and Pradhan 1978) medium for 18 h. *Escherichia coli* JM103 was maintained on yeast extract, tryptone and sodium chloride agar slants.

2.3 *Purification of Mt*

Mt were purified as reported earlier from aerobically grown logarthmic yeast cells (Desai *et al* 1989).

2.4 *Purification of Mt RNA polymerase*

Mt RNA polymerase was purified by the method developed in our laboratory (Desai *et al* 1990) which was a modification of the procedure described by Scragg (1976). The lysate of mitochondria was passed through the DEAE column. Column was washed with buffer A [0·02 M Tris-HCL pH 7·5; 0·01 M EDTA; 0·001 M dithiothretol (DTT) and 6 mg/ml of PMSF] containing 0·2M NaCl [DNA polymerase/primase were then eluted by pumping a gradient of 0·2-0·4 M NaCl through the column as described by Desai *et al* (1989)].

Column washing of 0·2 M NaCl which showed the presence of RNA polymerase activity was again loaded on a DEAE-cellulose column (20 × 2·5 cm), pre-equilibrated with buffer B (Tris-HCl pH 7·8, 0.002 M $MgCl_2$, 0·005 M EDTA, 0·005 M DTT and 10% glycerol).

The column was washed with 100 ml buffer B containing 0·2 M KCl and 2 ml fractions were collected. The fractions contaning active enzyme were polled and dialyzed overnight at 4°C against buffer B. The dialyzed fractions were then concen trated and stored at — 20°C.

2.5 *Assay of RNA polymerase*

The assay was carried out essentially as described by Levens *et al* (1981). The assay mixture contained in a total volume of 100 µl: 0·1 M Tris-HCl pH 7·9,0.01 M $MgCl_2$, 1 mg/ml BS A, 5% glycerol, 150 µM concentration of ATP, CTP, GTP and [α^3H] UTP (1 µCi/assay, sp.act. 40-50 Ci/mmol) and 1 µg of template DNA. The

reaction was initiated by the addition of varying of RNA polymarase preparation. The reaction mixtures were incubated at 30°C for 20 min. Incubations were 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 sodium pyrophosphate and filtered through Whatman GF/C filters presoaked in 1 M HCl and 0·1 M sodium pyrophosphate. The filters were washed three times with 5 ml of 0·1 M HCl and 0·01 M sodium pyrophosphate and once with 95% ethanol, dried and counted in a toluene based scintillation fluid. One unit of the enzyme is defined as that amount which catalyzes the incorporation of 1 nmol of UMP into RNA in 20 min at 30°C.

2.6 *Purification of Mt DNA*

Mt were purified and DNA prepared from it by the method standardized in our laboratory (Murthy and Pasupathy 1994) which was a modified method of Querol and Bario (1990).

2.7 *Amplification of ori sequences using PCR*

The *ori* sequences from the mitochondrial genome were amplified by PCR with the reagents supplied in the gene AmpTM DNA amplification kit supplied by Perkin Elmer Cetus and the N801-0150 DNA thermal cycler from Perkin Elmer Cetus. The

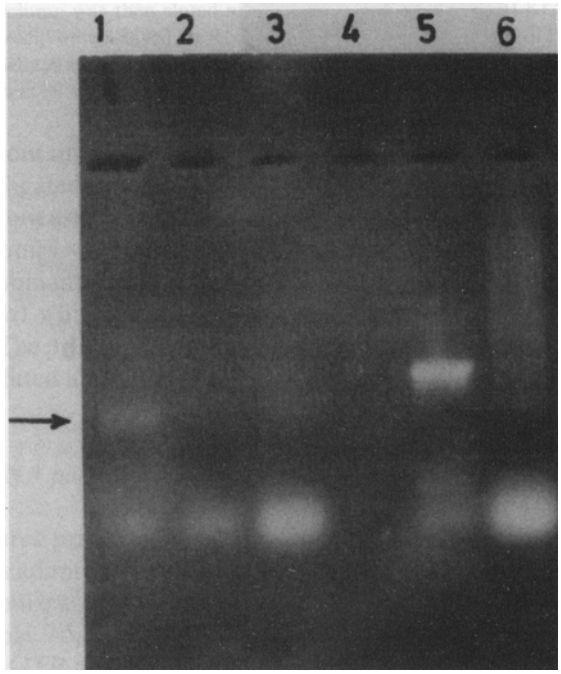

Figure 1. Agarose gel electrophoresis of the PCR product. The PCR reaction was carried out using the Mt DNA as the template as described in §2. The products of the reaction were run on 1·2% low melting agarose gel. Lane 1 shows the 400 bp PCR product. Lane 5 shows the 500 bp region from λ DNA used as the control in the PCR reaction.

primer sequences chosen for the amplification reaction were from regions which were conserved in all the seven origins of replication of the yeast Mt genome. The sequences of the two oligonucleotides were:

Primer 1: 5′ GGAAGCTTGGGGGTCCCAATTA 3′ and primer 2: 5′CCGAAT TCAAATATATACGTAATAGG 3′

The template used for the PCR was Mt DNA. The reaction was carried out as described by Sambrook *et al* (1989). The amplified PCR product was electrophoresed on a 1·2% low melting agar, eluted from it, re-extracted, dried and dissolved in water and was used directly for the *in vitro* replication assays. Figure 1 shows the products of the PCR run on a low melting agarose gel.

2.8 *Cloning of ori 2 and ori 7*

Five µg of purified Mt DNA was restriction digested with 5 units of *Eco* RI in a total reaction volume of 20 µl and the product of the reaction were then run on a 0·8% agarose gel. Nine fragments were obtained with a size range from 0·15 kb to 32 kb. The

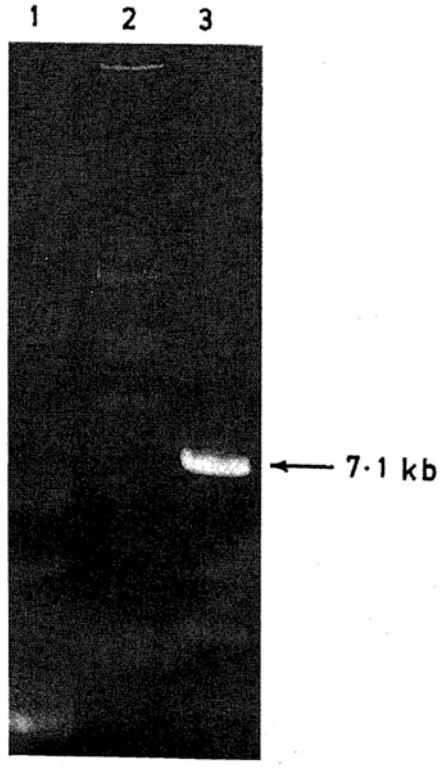

Figure 2. Gel electrophoresis of the recombinant plasmid DNA. The 7·l kb fragment obtained after digesting Mt DNA with *Eco*RI was cloned into the plasmid vector as described in §2. The recombinant plasmid obtained after transformation was run on a 1% low melting agarose gel. Lane 1, pUC18 DNA; lane 2 recombinant pUC18 DNA containing the 7·1 kb fragment; lane 3, *Eco*RI digested recombinant pUC18 DNA showing the 7· 1 kb fragment.

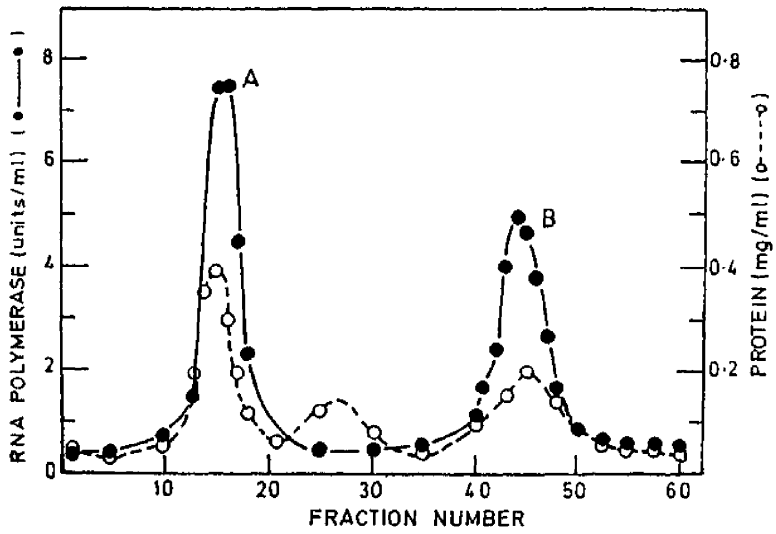

Figure 3. Purification of yeast Mt RNA polymerase by DEAE cellulose chromatography. Mt homogenate was passed through a DEAE cellulose column and eluted with buffer A containing 0·2 M NaCl. This was the washings obtained during purification of Mt DNA polymerase. The eluted fraction was then applied to another DEAE cellulose column (20 × 2·5 cm). The contaminating proteins were washed out by passage of buffer B through the column. The column was then eluted with buffer B containing 0 · 2 M KCl and the eluted fractions (2 ml each) were assayed for RNA polymerase activity (Desai *et al* 1990). One unit of the enzyme is defined as the amount which catalyzes the incorporation of 1 nmol of UM P into RNA in 20 min at 30°C.

7.1kb fragment containing the *ori* sequences 2 and 7 was then eluted from the agar, purified and then ligated to the pUC 18 plasmid DNA digested with EcoRI. Transformation was carried out as described by Sambrook *et al* (1989) in *E.coli* JM103. The white recombinant colonies were analysed for the presence of the 7·1 kb insert. Figure 2 shows the recombinant plasmid DNA digested with EcoRI. This insert was then digested with *Hha*I which yielded two fragments, one containing *ori 2* and the other containing *ori* 7. The products of the reaction were run on a 1 % agarose gel and the two fragments were eluted and used directly for the *in vitro* replication assays.

2.9 *Assay for RNA polymerase primed DNA replication*

The assay measures primer synthesis by RNA polymerase and extension by DNA polymerase and is adapted from Wong and Clayton (1986) and Murthy and Pasupathy (1994). The assay mixture contained 1 µg DNA template, 7 µg klenow fragment, 0·05 M Tris-HCl buffer pH 7·5, 0·001 M 2-mercaptoethanol, 0·010 M $MgCl_2$, 0·002 M ATP, 200 µM of CTP, GTP and UTP, 100 µm of dGTP, dCTP, dTTP, 20 µM dATP and 0·25 µCi of [α^{32}P] dATP (sp. act. 3000 Ci/mmol). The reaction was initiated by the addition of RNA polymerase (7 µg protein), and the incubation was terminated by the addition of cold 5% TCA containing the pyrophosphate, filtered and processed as described earlier.

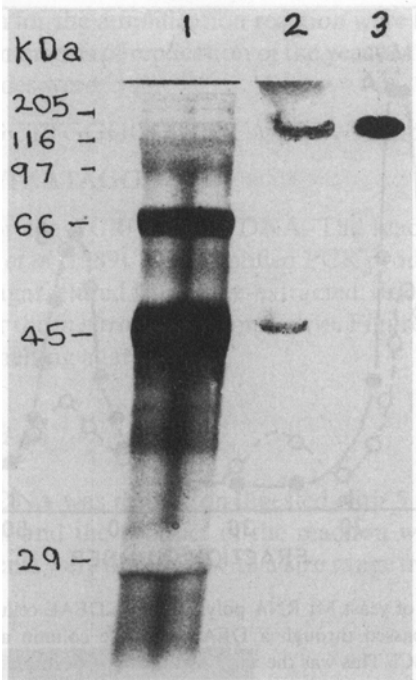

Figure 4. Electrophoresis of RNA polymerase on a 7·5% Polyacrylamide gel with SDS. Lane a, Molecular weight markers; lane b, RNA polymerase peak B enzyme; lane 3, RNA polymerase peak A enzyme.

2.10 *Estimations*

Protein was estimated by the method of Lowry *et al* (1951) and DNA was estimated by the method of Cesarone *et al* (1979).

3. Results

3.1 *Purification of RNA polymerase*

The procedure used here for isolation and purification of Mt RNA polymerase differs from other reported procedures. Levens *et al* (1981) used successive chromatography on Sepharose 4B, Heparin Sepharose 4B, phosphocellulose and DEAE Sephadex columns. One major advantage of our method was that using the same Mt lysate, it was possible to efectively fractionate the three important enzymes of Mt DNA replication, viz., RNA polymerase, DNA polymerase and DNA primase. This is essentially an adaptation of the method of Scragg (1976). The 0·2 M NaCl flow-through of the DEAE cellulose column (the first chromatographic step used for the preparation of DNA polymerase and DNA primase by Desai *et al* 1989; Murthy and Pasupathy 1994) was found to contain the Mt RNA polymerase enzyme.

The 0·2 M NaCl flow through was loaded onto a second DEAE cellulose column and the RNA polymerase was eluted from the column using 0·2 M KCl. As seen in figure 3 two widely separated peaks were obtained. Table 1 shows the results on the recovery and degree of purity of Mt RNA polymerase, both peak A and peak B fractions.

Table 1. Purification of Mt RNA polymerase.

Fraction	Total protein (mg)	Total activity (units)	Specific activity units/mg	Yield (100%)
Mt lysate	200	380	1·9	100
DEAE cellulose (I)	40	165	4·12	43·42
DEAE cellulose (II)				
Peak A	3·14	53·6	17·07	14·1
Peak B	2·98	57	19·12	15·0

RNA polymerase assay was carried out in the presence of M 13 single stranded DNA. The assay mixture details are as in §2. 1 μg of M 13 DNA and 5 μg of RNA polymerase were used. One unit of the enzyme is defined as that amount which catalyzes the incorporation of 1 nmol of UMP into RNA in 20 min at 30°C.

Table 2. Activity of mitochondrial RNA polymerase (peak A and peak B) on different templates.

Enzyme	Template	Activity pmol	Sp. activity pmol/mg
Peak A	M13 DNA	21	17·5
	Mt DNA	18	15
Peak B	M13 DNA	24	20
	Mt DNA	62	51

The RNA polymerase assay was carried out as described in §2. The concentrations of DNA templates were 1·2 μg per 100 μl reaction mixture.Activity given is pmol of NMP incorporated in 20 min.

3.2 *Priming abilities of peak A and peak B enzymes*

Levens *et al* (1981) and Desai *et al* (1990) had pooled these two distinct fractions and then further purified through glycerol gradients. In the present study, it was sought to examine the distinguishing characteristics, if any, of these two peaks: to ascertain whether there can be any differences in the template specificity. Table 2 incorporates results on the template specificities of peak A and peak B enzymes. The two enzyme fractions appeared to behave like core and holo enzymes. It is possible that peak A core enzyme could be non specific.

3.3 *Molecular size determination*

The molecular sizes as determined by the method of Laemmli (1970) on SDS-PAGE indicate that the peak A enzyme to be 140 kDa while that of peak B to be a heterodimer of 140 and 45 kDa (figure 4). Schinkel *et al* (1988), Greenleaf *et al* (1986) and Lisowsky and Michaelis (1988,1989) have established that Mt RNA polymerase is a heterodimer consisting of 145 kDa and 43 kDa polypeptide. The core polymerase has characteristics similar to those of other known RNA polymerases and the 43 kDa specificity factor is

Table 3. *In vitro* replication assay using Mt RNA poly merase (peak A and peak B enzyme).

Experimental conditions	DNA synthesis pmol/h
Mt DNA + Klenow – RNA polymerase	Not detectable
Mt DNA + RNA polymerase – Klenow	Not detectable
Mt DNA + RNA polymerase + Klenow	0·65
Mt DNA + RNA polymerase + Klenow – ribonucleotides	Not detectable

The *in vitro* replication assay was carried out in a reaction mixture of total volume of 100 µl.. The assay mixture contained 5 µg peak B enzyme, 1 µg template DNA, 7 µg Klenow fragment, 50 mM Tris-HCl pH 7.5, 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$,1 mg/ml BSA, 5 % glycerol, 150 µM each of ATP, GTP, UTP, CTP, 100 µM each of dATP, dGTP, dcTP, dTTP and [α^{32}P]dATP (specific activity 3000 Ci/mmol). The reaction mixture was incubated at 30°C for 1 h.

suggested to behave as a σ-factor. There are some differences of opinion regarding the nomenclature of 45 kDa polypeptide. Schmidt and Clayton (1994) reported that yeast Mt RNA polymerase is a single subunit enzyme (140 kDa) and transcription is dependent on a 40 kDa specificity factor. In our preparation 40 kDa polypeptide was found to be a subunit of the heterodimer of peak B fraction. Hence peak B enzyme was used in further experiments.

3.4 *RNA polymerase primed DNA synthesis in vitro*

As is evident from table 3, yeast RNA polymerase peak B enzyme is very essential for DNA polymerase catalyzed Mt replication (primer synthesis by RNA polymerase and extension by DNA polymerase). The fact that omission of ribonucleotide triphosphate precursors totally inhibited *in vitro* DNA replication lends support to the postulate that initiation of yeast Mt DNA replication requires synthesis of an RNA primer, either by DNA primase as reported earlier, Murthy and Pasupathy (1994) or by RNA polymerase (the present report).

3.5 *Template preference of RNA polymerase*

Seven putative *ori* sequences have been identified on the mitochondrial genome of *S. cerevisae* by Bernardi *et al* (1983). A mixture of population of *ori* sequences obtained by PCR using primers selected from the conserved regions present in all the seven *ori* sequences, *ori* sequences 2 and 7 cloned from the Mt DNA as described in §2 were tested for their efficacy to initiate DNA replication. As seen in table 4, *ori* 2 was the most efficient in promoting RNA polymerase primed DNA replication, *ori* 7 exhibited a much lower level of DNA synthesis.

Table 4. Activity of Mt RNA polymerase (peak B) on different DNA templates.

Template	DNA synthesis pmol/h
Mt DNA	0·70
ori 2	2·0
ori 7	0·1
ori (PCR)	1·4
pUC 18	0·07
M13	0·26

The *in vitro* assay was carried out as described in table 3.

4. Discussion

In the present experiments, the two RNA polymerase peaks on DEAE cellulose column were found to be distinctly different in their functions. Peak A fraction behaved very much like the core enzyme (140 kDa) and peak B fraction as the hole enzyme (140 ± 45 kDa). The holoenzyme had all the characteristics reported for yeast Mt RNA polymerase. Most importantly, it exhibited a distinct preference for Mt DNA among other natural templates. Baldacci *et al* (1984) have studied the potential of *ori* sequences, as templates for transcription. These sequences are highly homologous to each other and are formed by four regions. The strand containing the *'r'* sequence, a 16 bp sequence, is called the *'r* strand' and the complimentary strand is called the 'non-*r* strand'. This 16 bp AT sequence called *'r* sequence' is also found in the promoters for genes coded for by the mitochondrial genome and is shown by Baldacci *et al* (1984) to be essential for transcription initiation, *ori* 1,2,3 and 5 which have the 16 bp *'r* sequence'—essential for transcription initiation — can be efficiently transcribed whereas, *ori* 4, 6 and 7 in which *'r* sequence' is inactivated by the insertion of an extra GC cluster, Γ, are poorly transcribed. It was postulated that DNA replication could be initiated on the strand that contains the *'r'* sequence by RNA polymerase i.e. both initiation of replication and transcription could lie within the same sequences. In the present studies, a mixed population of *ori* sequences obtained by PCR and two cloned *ori* sequences, *ori* 2 (which had the V sequence'), and *ori* 7 (which had the extra GC cluster within the *'r* sequence') were used to determine the priming ability. Interestingly, *ori 2* showed much higher level of DNA synthesis (primed by RNA polymerase and elongated by Klenow), as compared to *ori* 7 in the *in vitro* replication assay. RNA polymerase is thus probably required to initiate replication on the *'r* strand' whereas primase was shown to catalyze RNA priming on the 'non-*r* strand' as demonstrated earlier (Murthy and Pasupathy 1994).

The present studies have served to highlight the feasibility as well as the usefulness of the *in vitro* studies, and should be highly beneficial. These should not only serve as the conformatory proof for the role of RNA polymerase in the initiation of DNA replication but also provide useful insights into the problem of whether one or all the putative *ori* sequences present in the mitochondrial genome, take part in the normal DNA replication (during the normal cell cycle) or whether most of these are suppressed and become active only in such situations like respiratory adaptation wherein Mt DNA copies per cell has been found to be increased from 5 to 30 (D'Souza *et al* 1992). The *in*

vitro system can also be used to study some regulatory aspects, for instance: the mechanisms that are responsible for initiation of DNA replication and suppression of further initiations unless the DNA replication cycle already initiated completes its round; whether the levels of specific transcription factors or mitochondrial processing enzyme as proposed in mammalian mitochondrial system by Chang and Clayton (1987), Chang *et al* (1987), or both, are responsible for specific initiation at specific time-points.

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