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QUARTERLY

Characterization of the mitochondrial DNA polymerase from *Saccharomyces cerevisiae**

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The mitochondrial DNA (mtDNA) polymerase was isolated from a protease-deficient yeast strain (PY2), and purified about 3000 fold by a column chromatography on phosphocellulose, heparin-agarose, and single-stranded DNA cellulose. The purified polymerase was characterized with respect to optimal nucleotide concentration, template-primer specificity and sensitivity to some inhibitors. These results were compared with the nuclear DNA polymerase I activity. Both polymerases showed similar requirement of deoxynucleotide concentrations (K_m < 1 μM), and highest activity with poly(dA-dT) template. However, the mtDNA polymerase was more sensitive to ddTTP, EtBr and Mn^{2+} inhibition in comparison to the nuclear DNA polymerase I. The mtDNA polymerase did not need ATP as an energy source for *in vitro* DNA synthesis. This mtDNA polymerase preparation also showed 3′ → 5′ exonuclease activity.

It has been known for many years that the mitochondrion has its own genetic material distinct from the nuclear genomes. The mitochondrial genome from lower eukaryotes, such as yeast, to higher eukaryotes, such as human, carries a conserved set of genes that code for two ribosomal RNAs, a full set of tRNAs, and seven to thirteen proteins, mostly the subunits of the respiratory chain enzyme complexes [1, 2]. The rest of proteins necessary for mitochondrial biogenesis are encoded by the nuclear genome, and imported into mitochondria. Since mitochondria have a small genome (e.g., 15 kb in human to 76 kb in yeast), and their function is dependent on the expression of two separate genomes (nuclear genome and its own), they have been used as a good model system for the study of eukaryotic gene regulation.

We are interested in studying mitochondrial deoxyribonucleic acid (mtDNA)[1] synthesis in yeast, *Saccharomyces cerevisiae*. It is known that mtDNA synthesis is independent from the nuclear DNA synthesis, and continues throughout the cell cycle [3, 4]. The mechanism and control of mtDNA replication in mammals are well understood [5]. The mammalian mtDNA has two origins of replication (O_H and O_L) from which heavy strand and light strand DNA synthesis begin. First, an RNA-primed H-strand synthesis starts at O_H and then continues over the L-strand DNA. The synthesis of daughter H-strand DNA displaces the parental H-strand on which the L-strand synthesis starts from the origin of L-strand replication (O_L).

In contrast, very little is known about mtDNA synthesis in *Saccharomyces cerevisiae*. The

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[1]Abbreviations used: dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; mtDNA, mitochondrial DNA; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

genetic analysis of deleted yeast mitochondrial genome formed *in vivo* suggests that four primary origins of replication (300 bp conserved GC-rich sequences) are present in the wild type mitochondrial genome. However, in the absence of these primary origins in the petite mtDNA, other sequences are used as an alternative origin of replication [6]. Since mtDNA is maintained even in the absence of mitochondrial protein synthesis, all trans-acting gene products involved in mtDNA replication must be encoded by the nuclear genomes. Among them only the mtDNA polymerase has been isolated [7]. The fidelity of mtDNA polymerase might be lower than that of other polymerases because mitochondrial mutation is ten thousand fold higher than in the nuclear and prokaryotic systems (i.e., $> 10^{-2}$ mitochondrial mutations compared to $< 10^{-7}$ nuclear mutations/cell per generation). Presumably the mtDNA polymerase is not efficient either in initial incorporation of the complementary nucleotide, exonucleolytic proof-reading of the newly incorporated nucleotides, or postreplicative scanning for mismatched base pairs. To address this problem, we have purified and partially characterized the mtDNA polymerase from *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Chemicals and enzymes. Deoxynucleoside triphosphates were from P-L Biochemicals; [^{3}H]dTTP was from ICN Biochemicals, and [^{32}P]nucleotides were from New England Nuclear. Salmon sperm DNA, poly[dA-dT], poly[dA-dC]:poly[dG-dT], poly[dA]:poly[dT], poly[A]:[dT]$_{10}$ were purchased from Sigma. DNase I, Klenow fragment of *E. coli* DNA polymerase I, T_4 polynucleotide kinase, and restriction endonucleases were obtained from Bethesda Research Laboratories.

Polymerase assays. The mtDNA polymerase activity was measured in a 50 µl reaction mixture containing 20 mM Tris/HCl, pH 7.9, 20 mM $MgCl_2$, 0.5 mg/ml rabbit serum albumin, 1 mM DTT, 5% glycerol, 50 µM each dGTP, dATP, dCTP and 5 µM dTTP, 1 µCi of [^{3}H]dTTP, 150 µg/ml activated salmon sperm DNA, and the enzyme [7]. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 1 ml of cold trichloroacetic acid containing 1% sodium pyrophosphate. The acid precipitable radioactivity was collected on GF/C glass filter, and measured by a scintillation counter. The nuclear DNA polymerase I was assayed according to the method of Burgers & Bauer [8].

In vitro run-off DNA synthesis. A run-off DNA synthesis was performed using an M13 primer and an M13 DNA template. A double-stranded or a single-stranded M13 DNA template was hybridized with the primer by heating at 90°C for 5 min followed by cooling to room temperature. The reaction conditions were same as described above except that 5 µM dATP and 5–10 µCi of [α-^{32}P]dATP were used in a 25 µl reaction mixture. The reaction was carried out at 37°C for 20 min, and then stopped by the addition of 25 µl stop solution (0.3% SDS, 200 µg/ml tRNA) followed by phenol extraction. After centrifugation, DNA in the aqueous layer was precipitated by ammonium acetate/ethanol. The precipitated DNA was collected by centrifugation, and electrophoresed through a 5% polyacrylamide-urea (8 M) gel. The primer-extended DNA products were visualized by autoradiography.

3′ → 5′ exonuclease assay. The 3′ → 5′ exonuclease activity was measured by incubating the mtDNA polymerase with a 3′ end-labeled DNA. The 3′ ends of a 2.7 kb linear plasmid DNA were labeled by enzymatic (Klenow) filling of the 3′ recessed ends of the plasmid DNA in the presence of [α-^{32}P]dATP. The 3′ end-labeled DNA (30 fmol) was incubated with 4 µl of enzyme in a 25 µl reaction mixture containing 20 mM Tris/HCl, pH 7.9, 20 mM $MgCl_2$, 0.5% rabbit serum albumin, 1 mM DTT, 5% glycerol at 37°C for different time periods. The reaction was terminated by the addition of 25 µl stop solution (200 µg/ml tRNA and 10 µM EDTA) followed by phenol extraction. The DNA in the supernatant was precipitated with ethanol. After centrifugation, the supernatant was collected, lyophilized and the liberated [^{32}P]AMP was measured by radioactive counter (DuPond BC 2000). The amount of released [^{32}P]AMP was plotted against the incubation times.

5′ → 3′ exonuclease assay. The 5′ → 3′ exonuclease activity was measured by incubating the mtDNA polymerase with a 5′ end-labeled DNA. The 5′ ends of a 2.7 kb linear plasmid DNA were dephosphorylated with calf-intestinal alkaline phosphatase, and then radio-

labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The labeled DNA was incubated with the mtDNA polymerase at 37°C for different time periods, and the amount of released nucleotides was plotted against the incubation times.

RESULTS AND DISCUSSION

Purification of mtDNA polymerase

The enzyme was purified by phosphocellulose, heparin-agarose and single-stranded DNA cellulose column chromatography. The final enzyme preparation was purified about 3000 fold, and SDS-polyacrylamide gel electrophoresis showed 4–5 polypeptides. In brief, a protease-minus yeast strain (PY2) was grown to an early-log phase, and the yeast mitochondria were isolated by differential centrifugation of cell homogenate [9]. The isolated mitochondria were suspended in buffer A (50 mM KPO_4, pH 7.6, 1 mM EDTA, 15% glycerol and 1 mM DTT) containing protease inhibitors (soybean trypsin inhibitor, leupeptine and PMSF to a final concentration of 20 µg/ml, 5 µg/ml and 10 µg/ml, respectively), and then homogenized in a Potter-Elvehjem homogenizer. After addition of Nonidet P-40 detergent (0.5% final concentration) and KCl (0.4 M final concentration), the mitochondrial extract was further homogenized to solubilize maximum enzyme protein. The mitochondrial homogenate was centrifuged at 100000 × *g* for 1 h, and the supernatant was collected for further purification of the mtDNA polymerase.

The supernatant was dialyzed against buffer A containing protease inhibitors for 3 h to remove KCl. The dialyzed supernatant containing 220 mg of protein was loaded onto a phosphocellulose column (12 × 1.5 cm) previously equilibrated with buffer A. Protein was eluted with a 140 ml linear gradient of 50–500 mM KPO_4, and fractions of 5 ml were collected. The mtDNA polymerase was eluted at about 0.3–0.4 M KPO_4 concentration. The active fractions were pooled, dialyzed against buffer B (10 mM Tris/HCl, pH 7.6, 1 mM DTT), and then loaded onto a heparin-agarose column (8 × 1.4 cm). Protein was eluted with an 80 ml linear gradient of 50–500 mM KCl in buffer B, and 2 ml fractions were collected. The fractions containing polymerase activity were pooled, dialyzed against buffer B and then loaded onto a single-stranded DNA-cellulose column (5 × 1.2 cm). The mtDNA polymerase was eluted with a 40 ml linear gradient of 50–500 mM KCl in buffer B. The active fractions were pooled and 1 mg/ml bovine serum albumin was added as an enzyme stabilizer, and then frozen at –70°C.

For a comparative analysis, the yeast nuclear DNA polymerase I was purified according to the procedure of Burgers & Bauer [8]. Since aphidicolin inhibits nuclear DNA polymerase but not the mtDNA polymerase [10], this inhibitor was used to examine any cross contamination of the nuclear and mitochondrial DNA polymerases in these preparations.

Optimal deoxyribonucleotide concentrations for DNA synthesis

To determine the optimal concentrations of deoxynucleoside triphosphates (dNTPs) required for DNA synthesis by the mtDNA polymerase and the nuclear DNA polymerase I, an *in vitro* reaction was carried out under standard assay conditions except that 50 µM three dNTPs and different concentrations (0.1 to 25 µM) of the fourth nucleotide were used. The apparent K_m values of both DNA polymerases for four dNTPs were determined to be <1 µM (Table 1). Since there is no significant difference in nucleotide requirement between

Table 1

K_m values of the mtDNA polymerase and the nuclear DNA polymerase I for dNTPs

Nucleotide	mtDNA polymerase (µM)	Nuclear DNA polymerase I (µM)
dATP	0.26	0.56
dGTP	0.37	0.27
dCTP	0.87	0.27
dTTP	0.40	0.36

the nuclear and mitochondrial DNA polymerases, the nucleotide concentrations may not be the factor responsible for higher mutational rate of mitochondrial genome over the nuclear genome.

Template-primer utilization

A number of natural DNA and synthetic oligonucleotides were tested as templates for the mtDNA polymerase and the nuclear DNA polymerase I (Table 2). First, a time course of DNA synthesis on activated salmon sperm DNA was studied under standard assay conditions. With the particular preparations of these polymerases, DNA synthesis was linear up to 30 min (not shown). The rest of the assays were carried out at 37°C for 20 min, and the polymerase activities were expressed as percentage of activity on activated salmon sperm DNA. Among the templates tested, poly(dA-dT) was most efficiently used by both polymerases whereas the homopolymer poly[dA]:poly[dT] duplex was not used at all. Poly[rA]:poly[dT] template, which is used by reverse transcriptase and the vertebrate mtDNA polymerase [10, 11], was not a template for the yeast mtDNA polymerase and was but poorly used by the nuclear DNA polymerase I.

The primer-specific DNA synthesis by the mtDNA polymerase was carried out on two topologically different DNA templates: a double-stranded linear and a single-stranded circular M13 DNA templates. The M13 DNA template and an M13 primer were hybridized by heating at 90°C for 5 min, and then cooled to room temperature. The primer-specific DNA synthesis was carried out as described above, and the results of 20 min reaction are shown in Fig. 1. In a negative control experiment primer was not added, and no DNA product was obtained (Fig. 1, lanes 1, 5), as expected. This result suggests that neither the mtDNA polymerase has its own priming activity nor the template DNA provided a specific 3′ OH group for initiation of DNA synthesis. When an M13 primer was added, a discrete 246 nucleotide run-off product was obtained from a linear double-stranded DNA template (Fig. 1, lane 2). Since enzymes like DNA helicase [12], rec A protein [13], Rep protein [14], and ATP-driven

Table 2

Relative activities of the mtDNA polymerase and the DNA polymerase I on various DNA template.
The polymerase activity was measured under standard reaction conditions except that 100 µg/ml primer-template was used. The results were expressed as percentage of activity on activated salmon sperm DNA.

DNA template	MtDNA polymerase	Nuclear DNA polymerase I
Natural templates		
Salmon sperm DNA (native)	8	14
Salmon sperm DNA (activated)	100	100
E. coli (native)	0	5
E. coli (activated)	15	16
Yeast chromosomal DNA (native)	18	3
Yeast chromosomal DNA (activated)	51	32
Yeast mtDNA (native)	16	2
Yeast mtDNA (activated)	57	40
Double-stranded M13 DNA (native)	11	0
Double-stranded M13 DNA (activated)	20	2
Synthetic templates		
Poly[dA-dT]	186	249
Poly[dA]:poly[dT]	0	0
Poly[dA-dC]:poly[dG-dT]	5	18
$Poly[rA]_{305}:poly[dT]_{167}$	0	9

DNase [15] catalyze unwinding of the duplex DNA using ATP as an energy source, the effect of ATP on the mtDNA polymerase activity was tested. In this experiment, 1 mM ATP did not stimulate the mtDNA polymerase activity (Fig. 1, lane 3). When an unlabeled primer (random hexamer) was used as a competitor of M13 primer, the M13 primer-specific DNA synthesis was inhibited (Fig. 1, lane 4).

Interestingly, there was a remarkable difference in DNA synthesis by the mtDNA polymerase on a single-stranded DNA template. In contrast to the double-stranded DNA template, many different sizes of DNA products were made on a single-stranded DNA template. This result suggests that the mtDNA polymerase might be sensitive to the secondary structures of the single-stranded M13 template (a feature characteristic to single-stranded DNA of ϕX174 and fd) or less processive on a single-stranded DNA template. Presumably, the high A-T nucleotide contents (about 82%) of mtDNA/or the mtDNA-binding protein minimize the secondary structure of the mitochondrial single-stranded DNA template (lagging strand), and allow mtDNA replication *in vivo*.

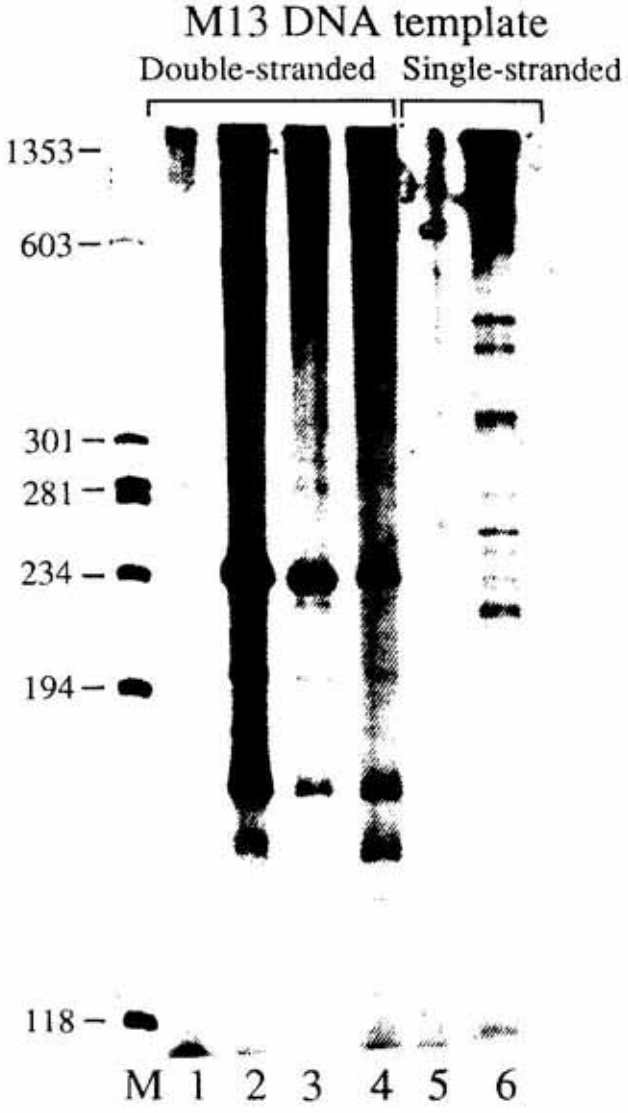

Fig. 1. In vitro primer-specific DNA synthesis on M13 DNA templates.
Reactions were carried out under standard assay conditions using an M13 primer and a double-stranded linear (lanes 1–4) or a single-stranded circular (lanes 5, 6) M13 DNA template. The results of 20 min reactions are shown. Lane "M", an end-labeled *Hae*III-digested ϕX174 DNA marker. Lane 1, no primer was added; and lane 2, primer was included. The conditions of reactions shown in lanes 3 and 4 were identical to that of lane 2, except that 1 mM ATP (lane 3) or 1 μg of random hexamer (lane 4) was added. Lane 5, no primer was added; lane 6, primer was added.

The effect of inhibitors on DNA polymerase activities

Since yeast mitochondrial mutations are induced with extremely high frequency (100 fold) by treatment with Mn^{2+} [16] or intercalating dyes like ethidium bromide [17], the sensitivity of the yeast mtDNA polymerase and the nuclear DNA polymerase I to ddTTP, EtBr and Mn^{2+} inhibition were tested.

Mn^{2+} at 3 mM or EtBr at 5 mM concentration inhibited mtDNA polymerase by about 60% whereas the nuclear polymerase I activity was stimulated by about 50% under similar conditions (Figs. 2 A, C). The effect of Mn^{2+} on primer-specific mtDNA synthesis was also tested (Fig. 2 B). The polymerase activity gradually decreased with increasing Mn^{2+} concentration. At 3 mM Mn^{2+} concentration, the polymerase activity was inhibited by 50% confirming the result of Fig. 2 A. We have also studied the effect of ddTTP (a nucleotide analog) on *in vitro* DNA synthesis by the mitochondrial and nuclear DNA polymerases (Fig. 2 D). The mtDNA polymerase was inhibited by 46% with ddTTP (dTTP/ddTTP, 5:2) whereas the nuclear polymerase was insensitive under similar conditions. The lower K_i of ddTTP for the mtDNA polymerase than for the nuclear DNA polymerase I suggests that the former has relatively lower selectivity for correct deoxynucleotides. It was found that dideoxythymidine triphosphate inhibits the mtDNA polymerase isolated from HeLa cells [18] and *Xenopus laevis*

[19], and also inhibits the *in vivo* replication of Chinese hamster mtDNA [20]. The stronger inhibition of mtDNA polymerase (in comparison to that of nuclear DNA polymerase I) could be one of the possible reasons for high mitochondrial mutational rate in the presence of these compounds in cell culture.

Exonuclease activities

The exonuclease activity, which is an important factor for high degree of fidelity of *in vivo* DNA synthesis, is associated with many DNA polymerase preparations. Therefore, we analyzed the presence of exonuclease on activity in the purified mtDNA polymerase preparation. The 3′ → 5′ and 5′ → 3′ exonuclease activities were tested by incubating the mtDNA polymerase with a 3′ or 5′ end-labeled double-stranded DNA. Interestingly, this enzyme preparation showed a 3′ → 5′ exonuclease activity, but very little 5′ → 3′ exonuclease activity (Fig. 3). The association of 3′ → 5′ exonuclease activity with the other mtDNA polymerases has been reported recently in *X. laevis* [21],

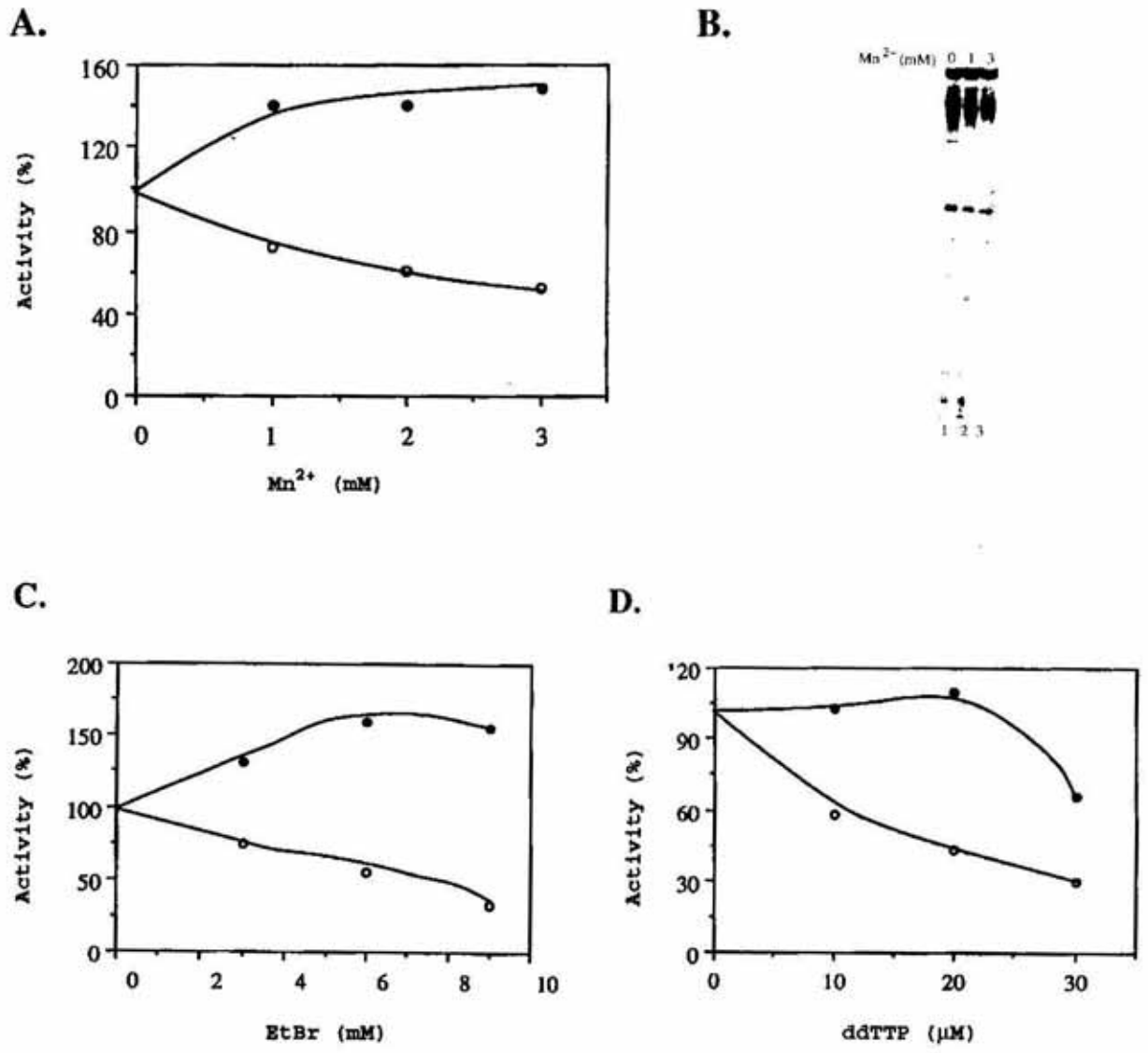

Fig. 2. Effects of Mn^{2+} (A and B), EtBr (C), and ddTTP (D) on the activities of the mtDNA polymerase and the nuclear DNA polymerase I.

Each chemical was added at the indicated concentrations to the standard reaction mixture, and the reaction was carried out for 20 min at 37°C. ○, mtDNA polymerase; ●, nuclear DNA polymerase I. B, Effect of Mn^{2+} on primer-specific DNA synthesis. The run-off reactions were performed on a linear double-stranded DNA template in the presence of different concentrations of Mn^{2+}.

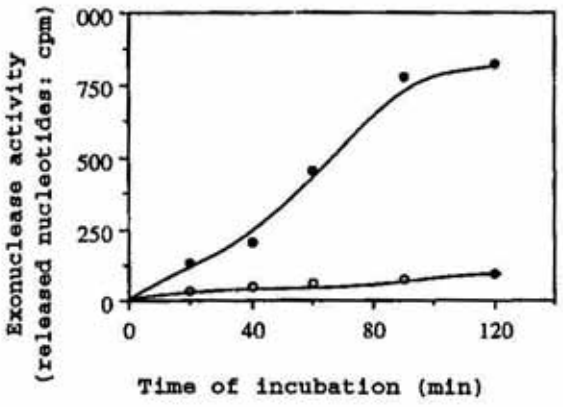

Fig. 3. The 3′ → 5′ (●) and 5′ → 3′ (○) exonuclease activities of the mtDNA polymerase preparation.

chick embryo [22], and in porcine [23]. The polymerase and exonuclease activities of both T4 DNA polymerase and *E. coli* DNA polymerase I are mediated by two separate domains of a single polypeptide [24, 25] whereas these activities in *E. coli* DNA polymerase III exist on different polypeptides. Since there were 4 - 5 polypeptides in our mtDNA polymerase preparation, it will be interesting to know whether the mitochondrial polymerase and exonuclease activities are associated with a single protein or with two different proteins.

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