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3D model of RNA polymerase and bidirectional transcription

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Abstract

In the *in vitro* mitochondrial (mt) transcription initiation system with mt RNA polymerase fraction and mt lysate, the transcription initiation products were shown to be synthesized bidirectionally from the only H-strand-promoter (HSP)/L-strand-promoter region (LSP) of the mitochondrial D-loop genome segment. These transcription products ranged between >100 and >800 bp with the purified mitochondrial RNA polymerase fraction, but were larger (>2030–4000 bp) in size with the mitochondrial lysate in both human and mouse. In this brief report, an *in vitro* reconstituted mitochondrial transcription system purified by affinity chromatography (heparin–Sepharose) from mouse hypotetraploid letter Ehrlich ascites tumor cell mitochondria was shown to initiate transcription bidirectionally from the mitochondrial D-loop region (HSP/LSP), as evidenced by *in vitro* generated transcription products. The *in vitro* generated transcription products were separated by sequencing gel. But this *in vitro* reconstituted transcription system was not studied beyond the D-loop region. A 3D model of the enzyme RNA polymerase was docked with both ATP and CTP.

Keywords: Mitochondrial D-loop region; *In vitro* transcription; mt RNA polymerase; Gel-mobility shift assay; 3D model

Both human and mouse mitochondrial (mt) *in vitro* reconstituted transcription system and mt lysate were shown to transcribe bidirectionally from D-loop region containing H-strand-promoter/L-strand-promoter (*HSP*/*LSP*) of mitochondrial genome [1–23]. These transcripts ranged between >100 and >3000 bp in both human and mouse [1–20]. The D-loop distal genes were also transcribed in this system [16]. The nuclear coded RNA polymerase, RNase P and RNase MRP as well as several transcription factors have now been proposed to function in a co-ordinate fashion for the synthesis of the polycistronic RNA and its processing into individual RNA molecules [1–12]. The ideal human transcription complex [13] was described to constitute RNA polymerase, transcription factor TFB1M (or TFB2M having weaker activity than TFB2M), *LSP*/*HSP* template (1–741 bp) and mt general transcription factor TFAM. The mouse homologs (or orthologs) mTfb1m and mTfb2m were identified [13]. The transcription termination was reported to occur by 34 kDa mt-*TERM*/mt*TERF* DNA binding protein [14,15], although 24 kDa Tf1 transcription factor, 48 kDa TAS-A protein (which binds to termination associated sequence of mt D-loop region) as well as other putative binding proteins (TAS-B, -C, -D, -E, -F, -G; A/B and F/G which bind to partially overlapping sites) in the mt D-loop segment were reported [15,16]. Two-to-three putative mitochondrial transcription termination sites were stipulated, e.g., 3230 bp (701 bp downstream of RNA–tRNA^{leu} gene boundary) 5′-AACAAGGGTTT#GTTAAGATGG CAGAGCCC-3′ (human), 16274–16295 mouse D-TERM DNA 5′-TTACGCAATAAACATTAACAA-3′ [14]. In the *in vitro* reconstituted transcription system (isolated by the heparin–Sepharose chromatography), the *HSP*/*LSP* region (where both major and minor promoters lie within close proximity) of the mitochondrial D-loop segment in both human and mouse were reported previously to initiate

Abbreviations: CCC, closed circular; dd, double distilled; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; mt, mitochondrial; NP40, non-iodet P40; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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transcription products which ranged between >100 and 700 bp runoff transcripts [13–17,19,20,29,30], although the mitochondrial lysate transcribed >2050 to >3000 bp [16] in an *in vitro* assay system. The mitochondrial lysate produced comparatively larger transcripts (>4–5 kb) from HSP/LSP fused with larger human mitochondrial DNA segment [16]. The *in vitro* transcription with the mt lysate with the human mt template $tRNA^{leu}$–16SrRNA–$tRNA^{val}$–12SrRNA–$tRNA^{phe}$-D-loop-7SrRNA initiated bidirectional initiation products ranging between 200 and 2050 bp [16]. Both RNA polymerase and RNase MRP are involved for mt DNA replication from a RNA–D-loop DNA hybrid [1–10], whereas these two enzymes in addition to RNase P are involved in transcription as well as RNA (mRNA, rRNA and tRNA) processing of human and mouse mt RNA [1–12,23]. RNase MRP cleaves mRNA substrate at an adjacent conserved decamer sequence 5′-CGACCCCUCC-3′ (relative to *CSB2* in D-loop), whereas RNase P is involved in 5′ and 3′ end-processing of mt tRNA [1–16]. This conserved decamer sequence 5′-CGACCCCUCC-3′ is also present in MRP RNA [3,4,7,11–13].

In this brief report, an *in vitro* reconstituted mitochondrial transcription system purified by affinity chromatography (heparin–Sepharose) from the mouse hypotetraploid letter Ehrlich ascites tumor cell mitochondria was shown to initiate transcription bidirectionally from the mt D-loop region (*HSP*/*LSP*), as evidenced by the *in vitro* generated transcription products. But this *in vitro* reconstituted transcription system was not studied further beyond the D-loop region. A 3D model of mouse mitochondrial RNA polymerase was constructed. The active site of the enzyme was predicted by docking experiment. Furthermore, the physico-chemical parameters of the enzyme were determined from web-based programs and discussed here.

Materials and methods

Cells and tissues. The cell types used in this study included letter Ehrlich hypotetraploid ascites tumor cell (LES) as well as mouse and rat liver, and mouse embryonic liver. The maintenance and growth of LES cells in the peritoneal cavity of Swiss mice [25–30 g] and male Sprague–Dawley rats (150–200 g) were used as the source of mitochondria [18–23].

Isolation of mitochondria. Freshly harvested LES cells from mouse bearing 7-day-old tumors and rat livers washed free of blood clots were used for the isolation of mitochondria [18,21–23]. The mitochondria were purified using differential sucrose density gradient centrifugation [18,21,23]. The mitoplasts were prepared by the digitonin (75 μg/mg protein) method as described before [18,21,23] to strip off the outer membrane. The mitolplasts were washed once with mitochondrial isolation buffer and once with a buffer containing 0.25 M sucrose, 30 mM Tris–HCl (pH 7.4), 100 mM KCl, 10 mM Mg–acetate, 7 mM of 2-mercaptoethanol and 5 mM potassium phosphate, and used for protein or mRNA transcription from the template DNA.

Isolation of mitochondrial in vitro reconstituted transcription activity. The purification procedure was adapted from human KB cell mitochondrial RNA polymerase activity purification with little modification [20]. The elaborate procedure of purification of single band (a major doublet of 45 kDa pattern) was described before [14] from this laboratory.

In vitro mitochondrial transcription assay. The *in vitro* mitochondrial transcription assay procedure was adapted from human KB cell mitochondrial RNA polymerase activity purification with little modification [20]. The mt RNA polymerase activity was measured in 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 μg/mL of bovine serum albumin, 1 mM dithiothreitol, 150 μM each ATP, GTP, and CTP, RNAsin (1/10th volume) and 150 μg/mL of heat-denatured calf-thymus DNA. UTP (7.5 μM) was included either as [5′-^{3}H]UTP, (10.9 Ci/mmol, Amersham Corp.), or [α-^{32}P]UTP, (200–400 Ci/mmol, Amersham Corp.). The assay reaction volumes were either 25, 50 or 100 μL. 1/10th volume of enzyme was added. For the transcription run-off experiment from the mouse mitochondrial D-loop DNA segment, the same conditions were used except that the calf-thymus DNA was replaced by the restriction endonuclease-cut linearized and gel-eluted DNAs as specified in the figure legends and Tables, at concentrations of 5–25 μg/mL. The reactions were done at 37 °C for 30 min. Except for the runoff assays, which were analyzed by gel electrophoresis, the transcription activity was determined by measuring the radioactivity in tricarboxylic acid-precipitable material in nitrocellulose membrane (or nitrocellulose filter, Fisher Company) in a LS-230 scintillation counter (Beckman). Alternatively, the run-off transcripts were precipitated with buffer c (1 mL of 1 M HCl, 0.1 M sodium pyrophosphate) at 0 °C, collected on Whatman GF/A, washed with 15 mL of ice-cold 1 M HCl followed by 5 mL of ice-cold 95% ethanol, and assayed in Omni flour (New England Nuclear) in an LS-230 scintillation counter. Finally, the run-off transcripts were extracted with phenol, chloroform, isoamyl alcohol (or isopropanol) and ether followed by ethanol precipitation with addition of commercially available tRNA at a final concentration of 0.3 M sodium acetate (or 0.2 M sodium chloride). These RNAs were then denatured with glyoxalation and resolved on 3.5% or 8% urea–polyacrylamide gels with glycerol–phosphate buffer [14,18,21].

Protein concentration. Protein concentration was measured by the method of Bradford [24].

Preparation of mitochondrial lysate. Mitoplasts (about 25 mg NP40/mg protein) were suspended in a buffer d (10 mM Hepes (pH 7.5), 8 mM magnesium acetate, 40 mM KCl, 1 mM DTT) at a protein concentration of 10 mg/mL and lysed with non-ionic detergent, e.g., NP40 with gentle shaking on ice [14,18,21,23].

Defined DNA templates. The plasmid P407 contained *Xb*1/*Hae*III (15973–16380 bp) of the mouse mitochondrial DNA fragment cloned in *Xba*1/*Sal*1 sites of pUR250 plasmid [18,21]. p622 contains *Bal*II/*Xba*1 (15330–15973 bp) mouse mitochondrial DNA segment cloned in *Bam*H1/*Xba*1 sites of pUR250 plasmid [18,21]. The plasmid p10 contained *Sac*1 (16216 bp)–Alu1 (16294-O-through 85 bp) mouse mitochondrial DNA segment cloned in pUR250 [18,21]. All these three mitochondrial DNA segments lie within the D-loop region [18,21]. The sequence of mouse mitochondrial DNA from which defined DNA templates (Fig. 1) were generated was described before [25]. The two other plasmids pT71-3369 and pT72-3370 containing cytb-ND6-ND5 region (11970–15330 bp) cloned in T7 polymerase promoter-directed pT71 and pT72 vectors in both orientations were described before [22]. The plasmids were transformed into *Escherichia coli* JM107, and purified by alkaline lysis procedure followed by Biogel-A150 chromatography, and finally extracted with the standard phenol–chloroform–isoamyl alcohol (or isopropanol) protocol followed by chloroform and ether separately [22]. The restriction fragments eluted from the gel of the plasmids were linearized with restriction enzymes, and were used for template in the *in vitro* reconstituted transcription complex system [16,17].

Isolation of other mitochondrial enzymes for control. The mitochondrial RNA processing RNase P activity was purified as described [19,22]. The purification of mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) was described before [22,27,28]. Subunits (I–IV) of cytochrome *c* oxidase were gel purified for raising antiserum/antibody in rabbit as described before [1].

Gel electrophoresis. Protein samples were electrophoresed on 8–15% gradient polyacrylamide gels using Laemmli's procedure [14,18,21–23]. DNA were electrophoresed through 1–1.2% agarose gel using Tris–acetate buffer [26]. RNA were electrophoresed through 1–1.2% agarose–formaldehyde gel and 3.5–8% sequencing gel (urea–polyacrylamide) using buffers

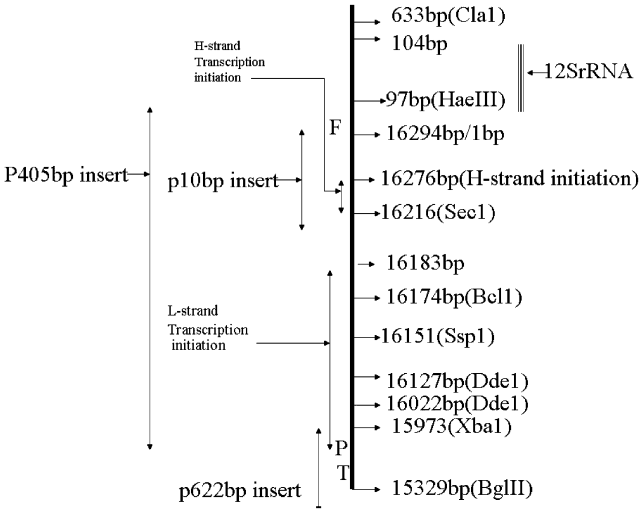

Fig. 1. Mitochondrial D-loop region of mouse. The line drawings of the mouse mt D-loop region reveal all inserts of plasmids used in the transcription initiation reaction as well as putative HSP and LSP regions, which are not on the scale. P, T and F represent three tRNAs i.e., $tRNA^{pro}$, $tRNA^{thr}$ and $tRNA^{phe}$, respectively.

as described before [14,18,21–23]. The gels were flurographed using En-^{3}hance (New England Nuclear Corp.) and exposed to intensifying X-ray film (Kodak) at −70 °C.

Gel mobility shift assay. The gel mobility shift assay was performed on 1–1.2% agarose gel using 10 mM Tris–acetate buffer [26] In brief, 3.36 kb $tRNA^{thr}$cytb–$tRNA^{glu}$–ND6–ND5 mt DNA were excised from both L- and H-strand specific PT71-3369 and pT72-3370 plasmid DNA with restriction endonucleases, gel-purified, and end-labeled with [γ-^{32}P]dATP or [γ-^{32}P]dCTP [14,26]. The binding reaction was performed in mt RNA polymerase buffer (mentioned above). 0.1–0.2 ng [γ-^{32}P]-labeled gel-purified double-stranded DNA probe (30,000 cpm) was incubated at 30–35 °C for 10–30 min with the *in vitro* reconstituted transcription complex (1–2 μg) or mitochondrial lysate (2–5 μg) as described before [14,26]. The DNA/protein complex were loaded onto the 1.2% agarose gel or 3.5–4% polyacrylamide gel in Tris/acetate/EDTA buffer as described [14,26] and dried by gel drier on filter paper and exposed to X-ray film (fast film, Kodak). MW markers were labeled with nick-translation kit (Bethesda Research Laboratory) including [α-^{32}P]dATP or [α-^{32}P]dCTP (Amersham Corp.) [26].

3D modeling. 3D modeling was performed with EsyPred 3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) which utilized modeler program.

Calculation of free energy of unfolding. The FOLD-X (http://fold-x.embl.de/) energy function includes terms that have been found to be important for protein stability. The free energy of unfolding (ΔG) of a target protein is calculated using Eq. (1):

$$\Delta G = Wvdw \cdot \Delta Gvdw + WsolvH \cdot \Delta GsolvH + WsolvP \cdot \Delta GsolvP + \Delta Gwb + \Delta Ghbond + \Delta Gel + Wmc \cdot T \cdot \Delta Smc + Wsc \cdot T \cdot \Delta Ssc \quad (1)$$

where $\Delta Gvdw$ is the sum of the Van der Waals contributions of all atoms with respect to the same interactions with the solvent. $\Delta GsolvH$ and $\Delta GsolvP$ is the difference in solvation energy for apolar and polar groups, respectively, when going from the unfolded to the folded state. $\Delta Ghbond$ is the free energy difference between the formation of an intra-molecular hydrogen-bond compared to intermolecular hydrogen-bond formation (with solvent). ΔGwb, is the extra stabilising free energy provided by a water molecule making more than one hydrogen-bond to the protein (water bridges) that cannot be taken into account with non-explicit solvent approximations. ΔGel is the electrostatic contribution of charged groups, including the helix dipole. ΔSmc is the entropy cost for fixing the backbone in the folded state. This term is dependent on the intrinsic tendency of a particular amino acid to adopt certain dihedral angles. Finally ΔSsc is the entropic cost of fixing a side chain in a particular conformation. The energy values of $\Delta Gvdw$, $\Delta GsolvH$, $\Delta GsolvP$ and $\Delta Ghbond$ attributed to each atom type have been derived from a set of experimental data, and ΔSmc and ΔSmc have been taken from theoretical estimates. The terms *Wvdw*, *WsolvH*, *WsolvP*, *Wmc* and *Wsc* correspond to the weighting factors applied to the raw energy terms. They are all 1, except for the Van der Waals' contribution which is 0.33 (the Van der Waals' contributions are derived from vapor to water energy transfer, while in the protein we are going from solvent to protein) [29–31].

Results

Initiation of in vitro transcription initiation by in vitro reconstituted LES transcription complex

The LES cell mitochondrial lysate were fractionated by heparin–Sepharose chromatography. Fraction 1 (0–0.5 M KCl linear gradient fractions) and Fraction 2 (0.5 M KCl wash) were apparently a heterogeneous population of polypeptides (which ranged between 30 and 100 kDa including major 45 kDa and major 70 kDa) in contrast to Fraction 3 (1 M KCl wash containing a major doublet of 45 kDa pattern). Fraction 3 was dialyzed against elution buffer containing 50% glycerol before transcription experiments. These results were similar as discussed [22] before and shown [14]. The *in vitro* reconstituted RNA polymerase activity was tested with the mouse mitochondrial D-loop DNA segment of mainly three plasmid constructs (p405, p622 and p10) at 30 and 35 °C for transcription (Table 1). Fig. 1 illustrated the description of the plasmids used in the *in vitro* transcription initiation assay. The transcription initiation products were resolved in 8% sequencing gel (Fig. 2). The linearized mt DNAs (or plasmids) p405(containing 15973–16294 bp-O-through 95 bp LSP/HSP), p622 (containing 15329–15973 bp LSP) and p10 (containing 16200–16294 bp HSP) with restriction enzymes resulted in transcription initiation products. These transcription initiation products were viz., 210 bp (L), 164 bp (H) and 94 bp (H) from p405 construct, >210 bp (L) and >150 bp (L) from p622 construct and 94 bp (H) from p10 constructs. These results were in good agreement with

Table 1
RNA polymerase activity

DNA (μg)	Rate of transcription ([^{3}H]UTP incorporated × 10^{4} cpm/mL)	RNA synthesis (pmol/mL)
Effect of DNA concentration		
1 (Calf-thymus)	8.46	1726.5
0.3 (Calf-thymus)	21.2	436.7
3.0 (Calf-thymus)	9.8	200
Transcription of specific cloned DNA		
1 (P405)	1.4	232.6
0.3 (P405)	1.0	204.1

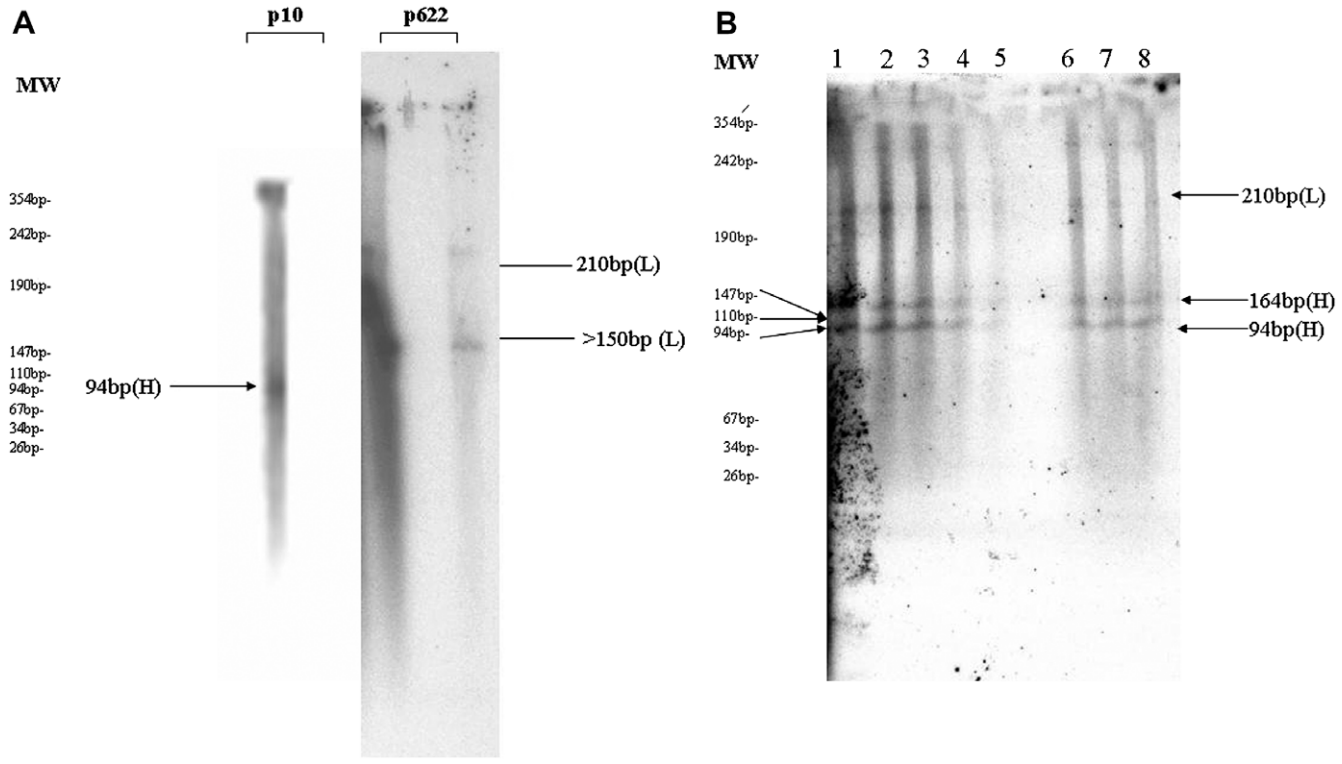

Fig. 2. *In vitro* reconstituted transcription initiation. (A) RNA transcripts from p10 and p622 mt DNA segments. (B) RNA transcripts from p405. The MW markers were 354, 242, 190, 147, 110, 94, 67, 34 and 26 bp.

the previously reported sizes (ranging between >100 and >700 bp) for both human and mouse mitochondrial D-loop segment [13,17,20,27,32,33]. The D-loop distal mitochondrial DNA segment (15330–11970 bp) cloned in both orientations in pT71 and pT72 vectors lacked the D-loop region (LSP/HSP major and minor promoters) and failed to initiate transcription *in vitro* reconstituted transcription system as evidenced both by nitrocellulose-filter-binding assay for radioactive incorporation counting in liquid-based scintillation fluid and 3.5% sequencing gel analysis of the initiation products. However, these [^{32}P]-labeled 3.36 kb 15330–11970 bp mouse mitochondrial DNA segments were shown to bind with the heparin–Sepharose-purified RNA polymerase fractions and also with the mitochondrial lysate in the gel mobility shift assay (Fig. 3). The reason for this binding of the *in vitro* reconstituted transcription system to 3.36 kb mitochondrial DNA segment (which is devoid of *HSP*/*LSP*) can presumably be attributed to a specific or non-specific DNA binding factor(s) (other than the mt RNA polymerase) which possibly copurified with this transcription complex. Even under electrophoretically driven force, the separation of these two forms (bound and unbound) were very poor because of relatively high molecular weight of this DNA molecule (3.36 kb) which bound to DNA binding protein(s) (present in the RNA polymerase column fractions) (Fig. 3). The top of almost all lanes (especially lanes 2,3.7,8) showing radioactivity (lesser extent) possibly arose from the clump or association/aggregate of the DNA/protein complex which barely migrated in the gel. Although RNA polymerase activity contained no nuclease activity in this context, albeit the affinity purified fraction might contain DNA binding protein(s), which copurified with this chromatographic separation procedure at a low concentration and escaped detection from gel staining. Whether this DNA binding protein was RNA polymerase

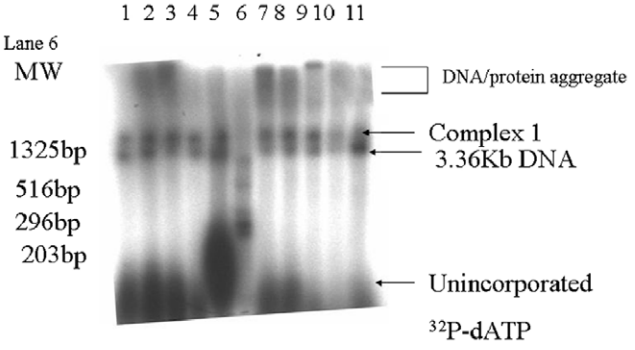

Fig. 3. Gel mobility shift assay of 3.36 kb tRNA^{thr}–cytb–tRNA^{glu}–ND6–ND5 mt DNA. Lanes (1–3), 3.36 kb DNA (pT71-3369 mt DNA fragment) and heparin–Sepharose RNA polymerase fraction; lanes 4 and 5, 3.36 kb (pT71-3369 mt DNA fragment) and mitochondrial lysate; lane 6, DNA MW markers (1325, 516, 296 and 203 bp); lanes (7–9), 3.36 kb (pT72-3370 mt DNA fragment) and heparin–Sepharose RNA polymerase fraction; lanes 10 and 11, 3.36 kb (pT72-3370 mt DNA fragment) and mitochondrial lysate. Lanes (1, 2, 4, 7, 8 and 10) were incubated for 5 min and lanes (3, 5, 9 and 11) were incubated for 10 min at 30–35 °C in the mt RNA polymerase assay buffer. The MW marker DNAs were nick-translated with [α-^{32}P]dATP or [α-^{32}P]dCTP as described before [26].

itself was not proved using antibody against RNA polymerase in this study. The only explanation is that the topoisomerase activity which caused ccc (or linear) from supercoiled form of plasmid was reported to be associated with RNA polymerase fraction [16] and may somehow bind to this 3.36 kb mt DNA.

In vitro transcription with mitochondrial lysate (incubation for 1, 2 and 30 min) was not shown. The mt lysate preparation contained a broad spectrum of proteins and enzymes including nucleases and were inefficient for nice autographs (i.e., generating nice transcription pattern). The mt RNA polymerase should not be expected to bind to the mt DNA segment other than the D-loop region and there is no such report until now. Results of transcription and purification were explained in Tables 1–3.

Mitochondrial specific RNAse P and cytochrome c oxidase

The mitochondrial RNase P and cytochrome *c* oxidase were purified from the same mitochondrial preparation as control enzymes.

3D structure of mouse mitochondrial RNA polymerase

3D structure of mouse mitochondrial RNA polymerase (Protein Sequence ID AAI10698) was made using modeler program (website) with target protein PDB ID 1msw_D (20.5% identity in Clustal W interface) and docked with ATP as well as CTP using ZDOCK program (http://www.zdock.edu/). All models (PMDB IDs are PM0074785-PM0074792) were deposited to Protein Model Data Base (PMDB), Caspur, Itally (http://a.caspur.it/PMDB/). The contact residues for ATP and CTP were found to be A799, T918, H887, Q892, I885,R763, F798, E797, E800, A792, H786, W766, L787, R763, A799, V764, F65, N785, and H769 using Rastard 3D program (Fig. 4). These contact residues can be predicted as active site binding residues, although experimental validation is required for definitive conclusion. CE (http://cl.sdsc.edu/) and EVA (http://www.nbcr.net/tools.php) exhibited align/gap 16/1,RMSD 2.2 Å and Z-score 2.3. The molecular parameters (only important characteristics) were evaluated with website programs Procheck,Gromacs, Vadar (http://redpoll.pharmacy.ualberta.ca/vadar/), Verify 3D, WhatIf, PDB2PQR 1.1.2 version(http://agave.wustl.edu/pdb2pqr/server.htmL; http://nbcr.net/pdb2pqr; http://enzyme.ucd.ie/Services/pdb2pqr/), PROPKa (http://propka.ki.ku.dk; http://propka.chem.uiowa.edu/) and Foldx (http://foldx.embl.de/), Rastard 3D, VMD 1.4, DeepView (PDB viewer), Molsoft programs. β-turn is made of 4 residues. In general, the carbonyl O of the 1st residue is H-bonded to the N of the 4th residue, which helps to stabilize the structure. Type III β-turn were found within 392–395, 399–403, 437–444, 504–507, 528–531, 539–542, 608–611, 651–658, 690–693, 772–775, 785–788, 818–821, 849–852, 855–857, 948–951, 957–960, 967–970, 973–976, 989–992, 997–1000, 1043–1046, 1126–1129, 1144–1147, 1149–1156 and 1195–1202 residues, whereas type I β-turn was found within 913–916 residues. The 3D molecule (Fig. 4A) had 50% helix, 11% β-turn, 38% coil, 15% turn, 44338.4 $Å^2$ total accessible area (ASA), 124421.5 $Å^3$ total volume (packing), 95525.83 Da molecular weight, +14.0 e (parse forcefiled) total charge, 19.0 e buried charges, −330.32 kcal/mol free energy for folding and 0.97–12.50 (surface)/−0.83–17.06 (buried) pK_a of amino acid residues. The other molecular properties was obtained by loading the PDB co-ordinate (after energy minimization) to Vadar or Foldx program. The energy minimization was performed before docking (Zdock). When the ionic strength was increased from 0.01 to 0.5 M at 35 °C in Foldx program, the conformational stability, molecular total energy, backbone H-bond energy, Van der waals energy, electrostatic energy, salvation polar energy, Van der waals clash, cis-bond energy and m-loop entropy remained unchanged, whereas sidechain H-bond energy and backbone clash increased. When the temperature was increased from 1 to 100 °C in Foldx program, the conformational stability, free energy, total energy, sidechain H-bond energy, torsional clash and backbone clash decreased, whereas solvation hydrophobic energy and van der waals clash increased. But, van der waals energy, electrostatic energy, salvation polar energy, m-loop entropy and cis-bond energy remained unchanged. The change in free energy for unfolding with temperature was shown in Fig. 5.

Table 2
Transcription efficiency with p10 DNA (12 ng/mL at 35 °C)

Incubation (min)	Transcription ([^{3}H]UTP incorporated × 10^4 cpm/mL)	RNA synthesis (pmol/mL)
10	1.1	22.5
30	1.2	24.5

Table 3
Concentration-dependent kinetics with p10 DNA

Concentrated activity of the enzyme (mL)	Incubation for 15 min		Incubation for 30 min	
	Transcription ([^{3}H]UTP incorporated × 10^4 cpm/mL)	RNA synthesis (pmol/mL)	Transcription (^{3}H-UTP incorporated × 10^4 cpm/mL)	RNA synthesis (pmol/mL)
1	1.6	326.6	1.23	251
4.5	1.7	246.9	1.23	285.7
6	1.7	351	1.03	379.5

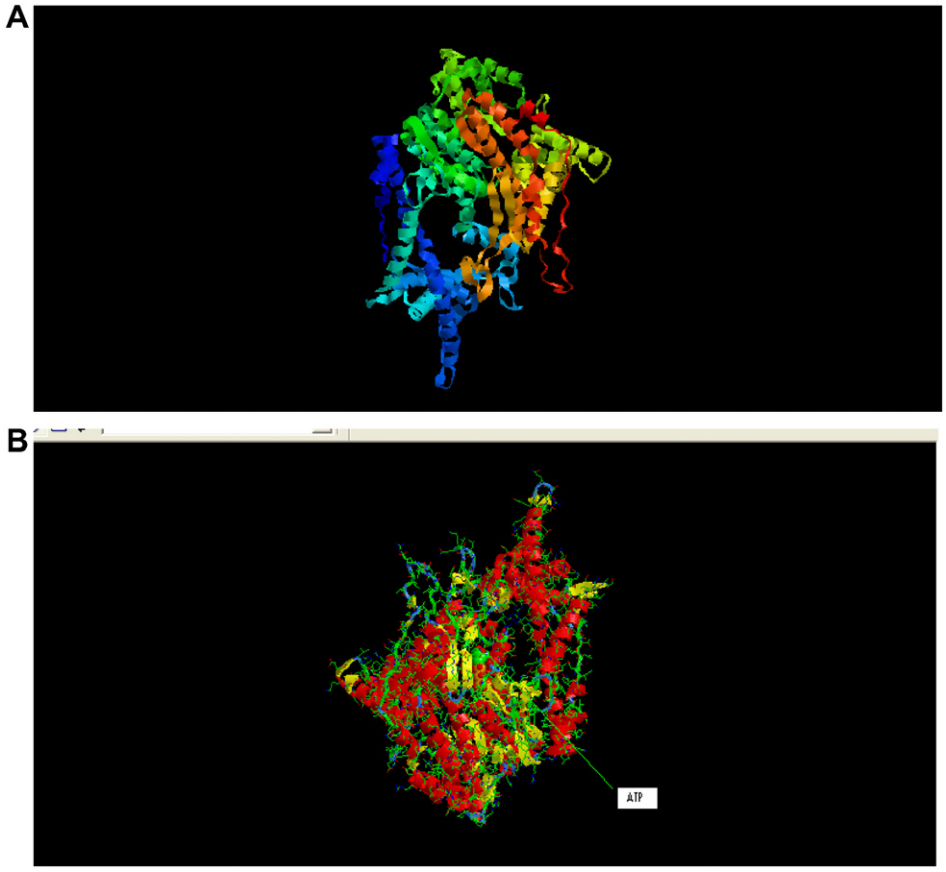

Fig. 4. Docking of ATP and CTP with mouse mitochondrial RNA polymerase (341–1207 amino acids). (A) Mitochondrial RNA polymerase; (B) mitochondrial RNA polymerase and ATP.

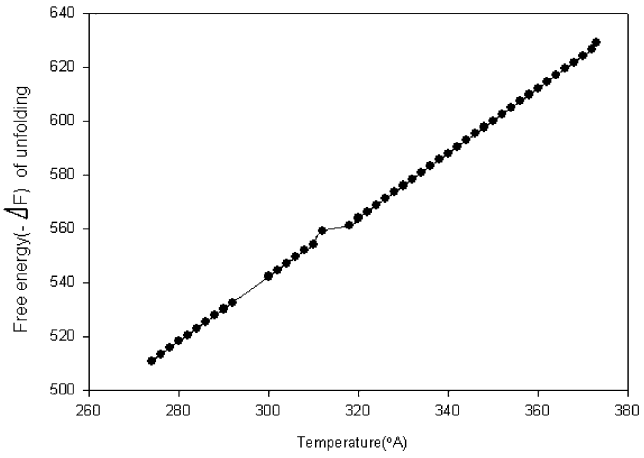

Fig. 5. Free energy of unfolding versus temperature plot of mouse mitochondrial RNA polymerase.

Discussion

By S1 nuclease protection assay, the identification of 5′capped RNA, Northern blot with the specific DNA probes, hybridization of the pulse-labeled RNA to the cellulose-linked DNA, analysis of protein synthesis of the mt specific proteins in the submitochondrial fractions or the mitochondrial lysate, the mouse mt HSP was mapped between 16216 and 16276 bp [16,33–35]. LSP was mapped between 16183 bp and inside region of the $tRNA^{pro}$ [16,33–35]. These results of the *in vitro* generated transcription products presented in this study are also in good agreement with the previous reports [13,17,20,30] of the *in vivo* transcription initiation products from the *HSP*/*LSP* containing the mt D-loop region spanning at one end of $tRNA^{leu}$ and the other end of $tRNA^{thr}$. All of these results discussed here indicate that the LES *in vitro* reconstituted transcription complex was able to transcribe bidirectionally from both H-strand–promoter (*HSP*) and light-strand-promoter (LSP) which lie within the D-loop segment. The recent report from this laboratory explained a faithful transcription termination from a human *HSP*/*LSP* fused with a mouse mt *D-TERM* DNA (16274-5′-ATTACGCAA TAAACATTACAA-3′-16295) using Hela cell *in vitro* reconstituted mt transcription complex and failure of transcription termination from mouse liver/heart *in vitro* reconstituted mt transcription complex [14,18]. This appears to be cross-species variation between human and mouse, and one species factor may not bind to other species DNA or RNA for proper function. In this context, other results of this region (but of different DNA length) of mouse mitochondrial DNA are discussed below. The mt D-loop segment (15973 bp-O-85 bp) cloned in Sp6 vector

with the *in vitro* reconstituted mouse LA9 cell mt transcription complex initiated transcription products of 550 bp (H-read-through transcript up to Sp6 plasmid Sph1 site) [29] and 210 bp (L) bidirectionally [32]. The larger mt DNA segment (15329 bp-O-85 bp) cloned in pBR322 in the same system (mouse LA9 cell) initiated bidirectional transcription products 95 bp (H) and 210 bp (L) [32]. RNA (650 bp) was transcribed from the cloned mt non-coding sequence 95–1004 bp [32]. Both human HSP (495–741 bp) and LSP (440–324 bp) were fused with human mt segment (3165–4121 bp containing 16SrRNA–$tRNA^{leu}$ gene) in association with or without a 10–16 bp linker consisting of those two genes boundary sequence (D-TERM), and were shown to initiate transcription products (that ranged between <60 and >622 bp) with human KB cell mt *in vitro* transcription system [17]. The transcription initiation products from p405, p622 and p10 plasmids were not studied further. The ideal mouse mt transcription complex containing transcription factors of mouse was explained elsewhere [13] for the better transcription initiation complex *in vitro*. The properties of 3D model of this mt RNA polymerase were discussed.

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