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Expression and Purification of Mitochondrial RNA Polymerase and Transcription Factor A from *Drosophila melanogaster*

John P. Gajewski, Jamie J. Arnold, Tiina S. Salminen, Laurie S. Kaguni, and Craig E. Cameron

Abstract

Mitochondrial gene expression is essential in all organisms. Our understanding of mitochondrial transcription on a biochemical level has been limited by the inability to purify the individual protein components involved in mitochondrial gene expression. Recently, new systems have been identified that permit purification of these proteins from bacteria. However, the generalizability of these systems is not clear. Here, we have applied the technology from the Cameron lab to express and purify mitochondrial RNA polymerase and transcription factor A from *Drosophila melanogaster*. We show that the use of SUMO system to produce SUMO fusion proteins in bacteria is effective not only for the human and mouse proteins, but also for the fly proteins. The application of this system to produce the mitochondrial proteins from other organisms should permit detailed understanding of mitochondrial transcription from any organism.

Keywords

Mitochondria; Mitochondrial transcription; Transcription factor A; Mitochondrial RNAPolymerase; Protein expression; Protein purification

1 Introduction

It is becoming increasingly clear that the ability to understand the regulation of mitochondrial gene expression requires the ability to study transcription reconstituted from purified components *in vitro*. For example, mitochondrial transcription factor A (TFAM) is now considered to be a key regulator of mitochondrial transcription [1 – 6]. The ability to illustrate its role in mitochondrial transcriptional control required the use of transcription systems reconstituted from purified components rather than cell-free extracts [5]. Until recently, the purification of mitochondrial proteins has been achieved only using baculovirus systems, and often required co-expression of proteins such as mitochondrial RNA polymerase and transcription factor B2 [4, 7]. However, the efforts by the Cameron and Temiakov labs have simplified this situation [5, 8 – 11]. To determine the breadth of the applicability of the SUMO technology published by the Cameron laboratory [12] to the production of mitochondrial transcription proteins, we have extended it to purify mitochondrial RNA polymerase (*Dm-mtRNAP*) and transcription factor A (*Dm*-TFAM) from *Drosophila melanogaster*. The SUMO system results in the production of SUMO fusion proteins that can be purified by using an N-terminal, six-histidine tag and

subsequently cleaved by a SUMO protease (Ulp1) to produce the “authentic” untagged proteins [12]. In addition, both the expression and the yield of the SUMO fusion proteins are enhanced by employing the Overnight Express AutoInduction System that allows for the expression of proteins in bacteria at high cell density, without the need to monitor cell density and the timely addition of an exogenous inducer such as IPTG. Here we report the expression and the purification of *Dm-mtRNAP* and *Dm-TFAM*, and the surprising observation that *Dm-TFAM* alone can activate transcription on a fragment containing *Drosophila* mtDNA sequence.

2 Materials

2.1 Protein Expression by Auto-Induction

1. Equipment: 37 °C heat block, microcentrifuge, 30 °C incubator, refrigerated incubator shaker, visible spectrophotometer, 4 °C refrigerator or cold box, centrifuge, centrifuge bottles, gel electrophoresis equipment, power supply.
2. pSUMO expression plasmid (Life Sensors) containing cloned mitochondrial transcription genes for *Dm-mtRNAP* and *Dm-TFAM* from *Drosophila melanogaster*. NCBI accession numbers NM_134721.3 and NM_079691.3 for *Dm-mtRNAP* and *Dm-TFAM*, respectively. Expression plasmids resulted in the production of *Dm-mtRNAP* (amino acids 77–1369) and *Dm-TFAM* (amino acids 1–257) (see Note 1).
3. BL21(DE3) competent cells.
4. SOC media: 0.5 % yeast extract, 2 % tryptone, 10 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄, 2.5 mM KCl, autoclave sterilize.
5. NZCYM (Amresco) agar plates supplemented with 25 µg/mL kanamycin.
6. Overnight Express AutoInduction System (EMD Millipore).
7. 25 mg/mL kanamycin solution: 250 mg kanamycin in 10 mL ddH₂O, filter sterilized.
8. 2 L Erlenmeyer flasks.
9. T₁₀E₁: 10 mM Tris pH 8.0, 1 mM EDTA.
10. 2× SDS-PAGE sample buffer: 225 mM Tris pH 6.8, 50 % glycerol, 0.70 M β-mercaptoethanol, 0.05 % bromophenol blue, 5 % SDS.
11. 1× SDS-PAGE sample buffer: 2× SDS-PAGE sample buffer diluted with an equal volume of T₁₀E₁.
12. 8 and 15 % SDS-PAGE gels.
13. 1× SDS-PAGE running buffer: 25 mM Tris, 190 mM glycine, 0.1 % SDS.

¹Mitochondrial transcription genes are cloned into the pSUMO expression plasmid in frame with SUMO to produce a SUMO fusion protein containing a N-terminal histidine tag for affinity purification. The gene for mitochondrial RNA polymerase was cloned lacking the putative coding sequence for the mitochondrial targeting signals, and that for TFAM was cloned intact.

14. Coomassie blue stain: 0.1 % Coomassie brilliant blue R-250, 40 % ethanol, 10 % acetic acid.
15. Destain: 10 % ethanol, 10 % acetic acid.

2.2 Protein Purification

1. Equipment: Homogenizer, French press and pressure cell, peristaltic pump and tubing, HisPur (Thermo Scientific) cobalt resin, Kontes-flex columns (10 mm × 50 mm), centrifuge, conical tubes, dialysis tubing: 3000 and 30,000 molecular weight cut-off (MWCO), gel electrophoresis equipment, stir plate, Vivaspın concentrators: 3000 and 30,000 MWCO.
2. Lysis buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, Roche Complete protease inhibitor tablets.
3. Post-lysis supplement reagents: 100 mM phenylmethylsulfonylfluoride (PMSF) prepared in ethanol, 10 % nonidet P-40 (NP-40).
4. 1st Column wash buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF, 0.1 % NP-40, Roche Complete protease inhibitor tablets (1 tablet per 50 mL wash buffer).
5. 2nd Column wash buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF.
6. Column elution buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 150 mM imidazole, 5 mM β-mercaptoethanol, 3.0 μg/μL pepstatin A, 3.0 μg/μL leupeptin, 1 mM PMSF.
7. Ulp1 SUMO protease (Life Sensors).
8. Dialysis buffer: 100 mM potassium phosphate pH 8.0, 500 mM NaCl, 20 % glycerol, 5 mM β-mercaptoethanol.
9. Bradford reagent: 50 mg Coomassie Blue G250 dissolved in 50 mL methanol, 100 mL of 85 % H₃PO₄ was added, followed by H₂O to 1 L.

2.3 Mitochondrial Transcription Assay

1. Equipment: thermocycler, agarose gel electrophoresis equipment (gel tray, caster, comb, running apparatus), Nanodrop spectrophotometer, radioactive work space, heat blocks at 20, 32, 50, and 65 °C, large gel electrophoresis equipment (gel stand, plates, combs, and spacers), power supply, phosphorimager screen, and Typhoon variable mode imager (GE).
2. Plasmid containing cloned mitochondrial DNA promoter sequences. NCBI accession number NC_001709.1 for *Drosophila melanogaster* mitochondrion, complete genome. Two sequences that correspond to the central and rightward

regions of the A + T-rich non-coding region of *Drosophila melanogaster* mtDNA (Oregon R strain) anticipated to contain promoters for both DNA strands and spanning regions 16972–17597 (designated OR-Ori) and 19108 to 19517/1–225 (designated OR-Rend) were cloned into pCR4-TOPO plasmid DNA.

3. 10× Thermopol buffer (NEB).
4. 100 mM MgSO₄.
5. 10× dNTPs (4:1): 4 mM dATP, 4 mM dTTP, 1 mM dCTP, 1 mM dGTP, dilute each dNTP in ddH₂O from 100 mM Ultrapure dNTPs solution.
6. DNA oligonucleotide primers: M13 forward and M13 reverse.
7. Deep Vent DNA polymerase (NEB).
8. 5× Xylene cyanol (XC) solution: 50 % glycerol, 2.5× TBE, 0.05 % xylene cyanol.
9. 1.0 % Agarose gel in 0.5× TBE with 0.25 µg/mL ethidium bromide.
10. 0.5× TBE: 44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA with 0.25 µg/mL ethidium bromide.
11. Wizard SV Gel and PCR Clean-Up System (Promega).
12. T₁₀E_{0.1}: 10 mM Tris pH 8.0, 0.1 mM EDTA.
13. 100 mM TCEP (Tris(2-carboxyethyl)-Phosphine Hydrochloride): 286 mg TCEP-HCl, 500 µL H₂O, 300 µL 10 N NaOH, check pH with pH strips and pH to 7.0 with 10 N NaOH, add H₂O to 1 mL final volume.
14. 10× Reaction buffer: 500 mM Hepes pH 7.5, 100 mM MgCl₂, 10 mM TCEP pH 7.0.
15. 10× BSA: 10 mg/mL diluted from 100× BSA.
16. Enzyme dilution buffer (EDB): 10 mM Hepes pH 7.5, 20 % glycerol, 2 mM TCEP pH 7.0.
17. 5 M NaCl.
18. 100 mM Ultrapure NTP solutions: ATP, CTP, GTP, UTP.
19. 10 µCi/µL [α -³²P]-UTP.
20. RQ1 DNase (Promega).
21. 0.2 M EDTA/4.2 M urea in 1× TBE.
22. Proteinase K.
23. Gel loading dye: 95 % formamide, 0.025 % XC, 0.025 % BPB.
24. 10 and 100 bp DNA ladder.
25. 10 µCi/µL [γ -³²P]-ATP.
26. Denaturing polyacrylamide gel: 6 % acrylamide/0.5 % bisacrylamide, 42 % formamide, 7 M urea, 1× TBE.

27. Gel running buffer/1× TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

3 Methods

3.1 Protein Expression by Auto-Induction

1. Dilute pSUMO expression plasmid to 5 ng/μL in ddH₂O and add 5 μL to the bottom of sterile 1.7 mL microcentrifuge tube (*see* Note 2).
2. Thaw BL21(DE3) competent cells at the bench-top and add 100 μL competent cells to pSUMO plasmid DNA in the 1.7 mL tube. Place tube on ice for 10 min.
3. Transfer tube to 37 °C water bath and incubate for 1 min.
4. Place tube back on ice for 2 min.
5. Add 900 μL SOC media and transfer tube to 37 °C water bath and incubate for 60 min.
6. Place tube in microcentrifuge and spin at 16,000 × *g* for 30 s.
7. Remove the majority of the supernatant without disrupting pellet and leaving behind approximately 100 μL SOC media. Use this remaining SOC media to suspend pellet.
8. Transfer 100 μL suspended pellet to NZCYM Agar plate and spread evenly over plate.
9. Place plate in 30 °C incubator overnight, approximately 16–18 h (*see* Note 3).
10. Prepare 500 mL Overnight Express AutoInduction media in 2 L Erlenmeyer flask per manufacturer's instructions.
11. Add kanamycin solution to media to a final concentration of 25 μg/mL.
12. Inoculate media with a smear of colonies from transformed cells on agar plate.
13. Place flask in 37 °C incubator shaker and shake at 250 rpm until an OD₆₀₀ = 1.0 as measured using visible spectrophotometer.
14. Save 1 mL uninduced sample to be prepared for SDS-PAGE (step 18 below).
15. Once culture reaches OD₆₀₀ = 1.0, place flask in 4 °C refrigerator or cold box until temperature reaches 25 °C. Alternatively, culture can be cooled directly in 25 °C incubator or at the bench-top.
16. Place flask in 25 °C incubator shaker and shake at 250 rpm for 16–20 h. The OD₆₀₀ should reach between 10 and 20 (*see* Notes 4 and ⁵).
17. Remove 0.2 mL induced culture and prepare sample for SDS-PAGE (step 18 below).

²Use autoclaved sterile tips and tubes during transformation.

³Incubate plates at 30 °C overnight, if 37 °C is used the colonies can grow too large and complicate protein expression.

⁴Expression is typically induced once culture reaches an OD₆₀₀ greater than 7.0.

⁵The temperature for expression can be adjusted to maintain solubility. Typical temperatures for expression are 25 °C or 15 °C. Growth at 25 °C is performed for 16–20 h, whereas growth at 15 °C is performed for 36–40 h.

18. Spin uninduced and induced culture in microcentrifuge at $12,000 \times g$ for 1 min and remove media.
19. Suspend pellet to an OD₆₀₀ of 20 OD/mL in 1× SDS-PAGE sample buffer. Heat sample to 100 °C for 10 min.
20. Run 10 µL of uninduced and induced SDS-PAGE samples on the appropriate percentage of SDS-PAGE gels to confirm expression.
21. Once expression is confirmed, harvest cells by centrifugation at $6000 \times g$ for 10 min at 4 °C, pour off media.
22. Suspend pellet with ice-cold T₁₀E₁ (use 50 mL TE per 500 mL culture).
23. Spin cells at $6000 \times g$ for 10 min at 4 °C and remove TE wash.
24. Weigh cell pellet and freeze at −80 °C until use.

3.2 Protein Purification

1. Pre-equilibrate 1.0–2.0 mL cobalt resin with 10 column volumes of lysis buffer in the Kontes column by gravity or using peristaltic pump at 1 mL/min.
2. Suspend frozen cell pellets on ice with 5 mL lysis buffer per gram of cell mass.
3. Dounce homogenize cells on ice to break clumps and attain a thick suspension of bacteria cells.
4. Use ice-cold French pressure cell and lyse cells three times at 20,000 psi (internal) in the appropriate volume pressure cell (*see* Note 6).
5. Clarify lysate by centrifugation at $75,000 \times g$ for 30 min at 4 °C. Decant and combine clarified lysate into a larger centrifuge tube.
6. Add pre-equilibrated cobalt resin to clarified lysate in centrifuge tube and swirl immediately to bind His-SUMO tagged protein to resin via batch binding. Rotate capped tube for 30 min to bind His-tagged protein to resin (*see* Note 7).
7. Gently sediment loose cobalt resin by centrifugation at $200\text{--}300 \times g$ for 2 min at 4 °C. Rotate the centrifuge tube to 180° and repeat process until all resin is at the bottom of tube. Decant supernatant, which is the “flow through” or “pass” to a new beaker. Suspend resin in several mL of 1st column wash buffer, transfer resin to chromatography column and gravity pack, collecting the remaining flow through (*see* Note 8).
8. Wash resin with 10 column volumes of 1st column wash buffer containing the full complement of protease inhibitors and NP-40 (*see* Note 9).

⁶Lysis by French press is necessary as to not shear chromosomal DNA. Other methods may be used but could complicate purification with contaminating nucleic acid.

⁷Batch binding allows for more efficient interaction with the cobalt resin for affinity purification.

⁸Directly adding the resin to the Kontes column after batch binding can be performed without centrifuging sample to pellet resin.

⁹It is crucial to wash the resin substantially to remove contaminants.

9. Wash resin with 10 column volumes of 2nd column wash buffer containing only pepstatin A, leupeptin, and PMSF (no Roche tablets or NP-40). The column should be washed to near baseline as measured by using the Bradford assay.
10. Elute protein with elution buffer, collect 1-column volume fractions. Use the Bradford assay to determine location of protein followed by SDS-PAGE to determine purity. Mix 5µl-10µl of each fraction with an equal volume of 2× SDS-PAGE sample buffer, heat to 100 °C for 10 min and load SDS-PAGE gel.
11. Pool elution fractions based upon purity. Measure protein concentration by using the Bradford assay.
12. Add 1 µg Ulp1 per 1 mg purified SUMO fusion protein to cleave SUMO from protein of interest (*see* Note 10). Mix well and transfer protein solution to dialysis tubing. Use 3000 MWCO for TFAM, and 30,000 MWCO for *Dm-mtRNAP*. Dialyze overnight for 16–18 h with stirring.
13. Next morning break dialysis and transfer protein to a fresh conical tube. Check sample via SDS-PAGE for completion of cleavage reaction (Fig. 1).
14. Pre-equilibrate 0.5 mL cobalt resin in the Kontes column with 10 column volumes of dialysis buffer.
15. Pass entire volume of dialyzed, cleaved protein over cobalt resin to bind SUMO, Ulp1, and any uncleaved target protein (*see* Note 11). A final bed volume or column volume of buffer can be used to clear resin of protein. Save pass-through as it contains final purified protein. Confirm by Bradford assay and SDS-PAGE (Fig. 2).
16. Concentrate sample as necessary using Vivaspin concentrator as per manufacturer's instructions at 4 °C. 3000 MWCO for TFAM and 30,000 MWCO for *Dm-mtRNAP*. Calculate final concentration by Bradford assay. Aliquot proteins, flash freeze on dry ice and store at –80 °C.

3.3 Mitochondrial Transcription Assay

1. Set-up 500 µL PCR reaction with 1× Thermopol buffer, 1× dNTPs (4:1 ratio), 0.5 µM DNA oligonucleotide forward and reverse primers, 0.5 ng/µL plasmid DNA containing cloned mitochondrial DNA sequences (*see* Note 12), 0.02 units/µL Deep Vent DNA polymerase (final reaction concentrations). Assemble in PCR tubes (*see* Note 13).

¹⁰A ratio greater than 1 µg Ulp1 per mg of purified SUMO-fusion protein may be necessary to cleave completely the fusion protein to remove the SUMO tag. This can result from the nature of the terminal amino acid residue next to the cleavage site.

¹¹Passing the cleaved fusion protein over a second cobalt resin is necessary to remove the histidine tagged SUMO, Ulp1 and any uncleaved SUMO-fusion protein.

¹²Mitochondrial DNA promoter sequences should be cloned into plasmids such as pCR4-TOPO or pUC18 such that promoter sequences can be PCR-amplified using M13 forward and reverse primers.

¹³PCR reactions and cycling conditions to amplify mitochondrial DNA sequences are optimized based upon high AT-rich DNA sequences of the *Drosophila melanogaster* mtDNA genome. If mitochondrial DNA sequences to be used contain a lower A + T content, then 4 mM of each dNTP can be used in the 10× dNTP stock, and PCR cycling conditions can be varied for annealing and extension times and/or temperature. Titration of MgSO₄ may be required to optimize PCR reaction conditions.

2. Place PCR reactions in thermocycler with following settings: 95 °C 4 min for 1 cycle, 95 °C 15 s, 50 °C 30 s, 60 °C 3 min per 1 kb for 30 cycles, 60 °C 20 min for 1 cycle.
3. After PCR reaction finishes, mix 10 µL PCR reaction with 2 µL 5× BPB and run on 1.0 % agarose gel to verify PCR product.
4. Once PCR reaction is confirmed, purify PCR product using Wizard SV Gel and PCR Clean-Up System (follow manufacturer's instructions).
5. Quantitate PCR product using Nanodrop spectrophotometer.
6. Assemble mitochondrial transcription assay reagents at room temperature (final concentrations, 50 µL volume per each reaction): 1× reaction buffer, 1× BSA, 100 nM PCR product, 100 mM NaCl, 500 µM ATP, 500 µM CTP, 500 µM GTP, 100 µM UTP, 0.2 µCi/µL [α -³²P]-UTP.
7. Thaw purified mitochondrial transcription proteins on ice. Dilute *Dm*-mtRNAP to 10 µM final using ice-cold EDB. Dilute *Dm*-TFAM to 5, 10, and 25 µM final using ice-cold EDB.
8. Place mitochondrial transcription reaction mix at 20 °C for 5 min, follow subsequent steps (schematic of assay illustrated in Fig. 3a).
9. Add 1/10th volume of each *Dm*-TFAM dilution to individual transcription reactions. Wait 1 min (*see* Note 14).
10. Add *Dm*-mtRNAP to 1 µM final to transcription reaction to initiate reaction. Let reaction proceed for 30 min (*see* Note 15).
11. After 30 min, add 1 unit RQ1 DNase to each reaction. Transfer reactions to 32 °C and incubate for 10 min (*see* Note 16).
12. Add 1/4th volume of 0.2 M EDTA/4.2 M urea in 1× TBE to each reaction.
13. Add Proteinase K to 0.05 mg/mL final and incubate at 50 °C for 15 min.
14. Add an equal volume of gel loading dye solution to reaction, vortex, quick spin in microcentrifuge (*see* Note 17).
15. Label DNA ladder with [γ -³²P]-ATP as per manufacturer's instructions.
16. Heat reactions at 65 °C for 5 min and load 5 µL on 6 % denaturing PAGE. Run gel at 90 W for 2 h.
17. Disassemble gel and expose gel to phosphorimager screen for 10–60 min (*see* Note 18).

¹⁴TFAM titrations in mitochondrial transcription assays are required to determine the optimal concentration for promoter-dependent transcriptional activity.

¹⁵Reaction times for transcription assays can vary and time points can be processed and quenched accordingly.

¹⁶RQ1 DNase treatment is required to prevent RNA transcripts from reannealing to DNA templates during gel electrophoresis.

Proteinase K treatment prevents proteins from associating with RNA transcript products in case denaturation is not complete.

¹⁷Transcription assays contain significant amounts of radioactivity so proper care should be taken to shield user from exposure, and the user should check for contamination regularly.

18. Scan screen using Typhoon variable mode imager (Fig. 3b).

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¹⁸Denaturing PAGE gel can be placed onto a suitable plastic backing and covered in plastic wrap to prevent contamination to Phosphorimager screen.

Fig. 1. Coomassie-stained SDS-PAGE gels of *Drosophila melanogaster* mitochondrial transcription proteins cleaved from the SUMO tag by the Ulp1 SUMO protease. Shown are the pre- and post-Ulp1 SUMO-fusion proteins for *Dm-TFAM* and *Dm-mtRNAP*. The ** indicates SUMO-fusion protein and * indicates cleaved target protein

Fig. 2. Final fractions of purified mitochondrial transcription proteins. Shown are 1.0 and 5.0 µg of the purified *Dm-TFAM* and *Dm-mtRNAP* on a Coomassie-stained SDS-PAGE gel, 15 % for *Dm-TFAM* and 8 % for *Dm-mtRNAP*

Fig. 3. Mitochondrial transcription assay. **(a)** Schematic of transcription assay. **(b)** Denaturing PAGE gel of *in vitro* mitochondrial transcription products using OR-Ori and OR-Rend as DNA templates. Ori and Rend correspond to the central and rightward regions of the A + T-rich non-coding region of *Drosophila melanogaster* mtDNA (Oregon R strain), and encompass nucleotides 16972–17597 and 19108–19517/1–225. The *arrows* indicate specific transcription products produced as a result of internal initiation using the indicated DNA fragment