J. Biochem. **141**, 201–211 (2007)
doi:10.1093/jb/mvm020

The C-terminal Tail of Mitochondrial Transcription Factor A Markedly Strengthens its General Binding to DNA

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Received November 27, 2006; accepted November 29, 2006; published online December 13, 2006

Mitochondrial transcription factor A (TFAM) contains a basic C-terminal tail which is essential for the promoter-specific transcription. TFAM is also a major component of a protein–mitochondrial DNA (mtDNA) complex, called nucleoid, as a non-specific DNA-binding protein. However, little is known about a role of the C-tail in the nucleoid. Overexpression of full-length TFAM decreased the amount of a D-loop form of mtDNA in cells, while overexpression of TFAM lacking its C-tail (TFAM-ΔC) did not, suggesting that the C-tail is involved in destabilization or formation of the D-loop. An mRNA for mtDNA-derived ND1 was hardly decreased in the former but rather decreased in the latter. Given that the D-loop formation is coupled with the transcription, the decrease in the D-loop is likely due to its destabilization. The recombinant full-length TFAM much strongly unwound DNA than TFAM-ΔC, which is consistent with the above idea because D-loop is resolved by unwinding of the supercoiling state. Notably, truncation of the C-tail decreased DNA-binding activity of TFAM by three orders of magnitude. Thus, the C-terminal tail of TFAM is important for the strong general binding to mtDNA. This strong DNA-binding conferred by the C-tail may play an important role in the nucleoid structure.

Key words: D-loop, mitochondrial DNA, nucleoid, TFAM, transcription.

INTRODUCTION

Mitochondria play a central role in generation of ATP through the respiratory chain. Mitochondrial DNA (mtDNA) encodes 13 proteins essential for the oxidative phosphorylation and RNAs (22 tRNAs and 2 rRNAs) required for translation. In contrast to nuclear DNA that winds around histone proteins and takes on a nucleosome structure, human mtDNA had long been considered to be devoid of such a higher structure. However, recently, human mtDNA has been recognized to take on a higher structure called nucleoid. We have reported that the amount of human mitochondrial transcription factor A (TFAM) is sufficient to cover the entire region of mtDNA (*1*), that most of TFAM is indeed bound to mtDNA in human cells (*2*) and that the mtDNA amount is correlated with the amount of TFAM but not with the transcription level (*3*). TFAM has an ability to bind DNA without sequence specificity (*4, 5*) like many other HMG family proteins (*6–8*). These results suggest that TFAM architecturally packages mtDNA. To form a higher structure throughout mtDNA, TFAM should distribute evenly over mtDNA. However, it is not shown so far that TFAM is indeed evenly associated with the entire region of mtDNA *in vivo*.

TFAM is well characterized as a transcription factor of mtDNA. *In vitro* transcription assays reveal that TFAM enhances the mtDNA transcription in a promoter-specific fashion in concert with mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor B (TFBM) (*9*). TFAM is composed of two high-mobility group (HMG)-boxes and a basic carboxyl terminal region (C-terminal tail). TFBM binds to the C-terminal tail of TFAM (*10*) and TFAM lacking the C-terminal tail does not initiate the transcription (*11*), indicating an essential role of the C-terminal tail in the initiation of the promoter-specific transcription. However, a role of the C-terminal tail in the nucleoid has not been examined thus far.

In this study, we show that the C-terminal tail of TFAM markedly strengthens the general binding of TFAM to DNA. This strong binding conferred by the C-terminal tail may affect mitochondrial transcription and/or replication via the change of mtDNA conformation.

MATERIALS AND METHODS

Antibodies—Antibodies against human TFAM were produced by immunizing rabbits with recombinant glutathione S-transferase-tagged human TFAM (*12*). The antibodies were affinity-purified using resins on which histidine-tagged TFAM protein was immobilized. Anti-P32 and anti-cytochrome *b* antibodies were produced as described previously (*3, 13*). Anti-calnexin antibody was obtained from Stressgen (Ann Arbor, MI, USA)

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Expression and Purification of Recombinant TFAM-Full and TFAM-ΔC—To express N-terminally histidine-tagged full-length TFAM (TFAM-full) and TFAM lacking the C-terminal 25 amino acids (TFAM-ΔC), DNA fragments encoding mature TFAM-full (43-246 amino acids residues) and TFAM-ΔC (43-221 amino acids residues) were inserted between BamHI and EcoRI sites of the pPRO-EX-HTb (Invitrogen, Carlsbad, CA, USA). The recombinant TFAM-full was expressed in *Escherichia coli* BL21 cells. The recombinant TFAM-full was recovered from a soluble fraction after disruption of the cells by sonication. Then the recombinant protein was purified sequentially with three columns: Ni^{+}-bound chelating Sepharose resins, Heparin Sepharose and SP Sepharose (Amersham Biosciences, Piscataway, NJ, USA). TFAM-ΔC was purified as was TFAM-full (Supplementary Fig. 1). The recombinant proteins were dialyzed in phosphate-buffered saline (PBS) containing 20% glycerol and stored at –80°C.

Tetracycline-Regulated TFAM-Overexpressing Cell Lines—Tetracycline-regulated TFAM-overexpressing cell lines were produced as previously reported (*3*). Briefly, the DNA fragments for human TFAM-full (1-246 amino acids residues) and human TFAM-ΔC (1-221 amino acids residues) were inserted between BamHI and NheI sites of pTRE2hyg (Clontech, Mountain View, CA, USA). A HeLa Tet-off cell line was obtained from Clontech. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA) and 100 μg/ml G418 (Sigma, St. Louis, MO, USA). We transfected the HeLa Tet-off cells with the plasmids using a Fugene 6 reagent (Roche, Indianapolis, IN, USA) and selected cells bearing the transgenes in the presence of 400 μg/ml G418, 200 μg/ml hygromycin B (Wako, Osaka, Japan), and 1 μg/ml doxycycline (a tetracycline derivative) (ICN Biomedicals, Aurora, OH, USA). The hygromycin-resistant clones were isolated, cultured in DMEM with 10% fetal bovine serum (the Tet system approved, Clontech) and with or without doxycycline for 10 days, and then examined for the expression of the exogenous proteins by western blotting. Finally, we selected three clones (full-11.1, full-11.4 and full-2) and two clones (ΔC-4 and ΔC-8) for TFAM-full and TFAM-ΔC, respectively.

Isolation of Crude and Purified Mitochondria from HeLa Cells—Mitochondria were isolated from HeLa cells as described previously (*3*). All procedures were done at 4°C or on ice. Briefly, HeLa cells were harvested by scraping, pelleted by centrifugation and resuspended in homogenization buffer (10 mM HEPES-KOH, pH 7.4 and 0.25 M sucrose). The cells were homogenized with a Potter-Elvehjem homogenizer. The homogenized sample was centrifuged at 900*g* for 10 min to remove unbroken cells and nuclei. After the first supernatant was centrifuged at 900*g* for 10 min again, the second supernatant was centrifuged at 10,000*g* for 6 min. The pellet was used as a crude mitochondria fraction. For the isolation of further purified mitochondria, the first supernatant (~2 ml) was diluted with 2 ml of 20% Percoll buffer [20 mM HEPES-KOH, pH 7.4, 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 20% Percoll (Amersham Bioscience)] and overlaid onto a discontinuous Percoll density gradient (4 ml of 40% and 4 ml of 20% Percoll buffer) in a 12-ml centrifugation tube. After centrifugation at 70,000 *g* for 1 h using an SW41Ti rotor (Beckman, Fullerton, CA, USA), a band located in a middle of the tube was taken and centrifuged at 10,000*g* for 6 min. The pellet was used as purified mitochondria.

Separation of Mitochondrial NP-40-Soluble and Insoluble Fractions—The crude mitochondria were resuspended in TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) containing 0.5% NP-40 (1.0 mg protein/ml) and incubated for 30 min on ice water with intermittent mixing. The mitochondria were centrifuged at 20,000*g* for 30 min at 4°C and separated into a pellet (P) and a supernatant (S). mtDNA in P and S fractions was detected by PCR as described previously (*2, 3*). Briefly, total mitochondrial, P and S fractions (from 10 μg protein of mitochondria) were incubated at 55°C for 30 min in the digestion buffer (50 mM Tris-HCl, pH 8.0, 200 μg/ml proteinase K, 1% sodium dodecyl sulphate (SDS), 1 mM EDTA). After diluted with 10 volumes of distilled water, DNA samples were incubated at 100°C for 10 min to inactivate proteinase K and then diluted with additional 20 volumes of distilled water. Using one microlitre each of the diluted samples, a fragment of mtDNA was PCR-amplified with primers 5′-TTGCCACA ACTAACCTCCTC-3′ (nucleotide position; nps 8762-8781) and 5′-TGTGGTAAGAAGTGGGCTAG-3′ (nps 8918–8899)]. The number of amplification cycle was 25. The PCR products were separated on a 1% agarose gel.

Western Blotting—HeLa cells were scraped into cold PBS, and solubilized with SDS denaturing buffer [PBS containing 0.5% SDS and 1 × Complete Mini^{TM} (mixture of proteinase inhibitor, Roche)]. After briefly sonicated, the sample was boiled at 95°C for 3 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with indicated specific antibodies. The signals were visualized with horseradish peroxidase-labelled anti-rabbit immunoglobulin G and an ECL reagent (Amersham Biosciences). The chemiluminescence was recorded and quantified with a chilled charge-coupled device camera, LAS1000plus (Fuji Photo Film, Tokyo, Japan).

Southern Blotting—Total DNA was extracted from HeLa cells with a DNeasy tissue kit (Qiagen, Hilden, Germany). The DNA concentration was determined by measuring the absorbance at 260 nm. Three micrograms of total DNA was digested by 24 U BamHI (Toyobo, Osaka, Japan) in 120 μl of 1 × universal High buffer (Toyobo) at 37°C for overnight. Then the mixture was boiled at 90°C for 5 min to release a displacement loop (D-loop) strands. The DNA was precipitated with ethanol and solubilized in 15 μl of TE buffer. The DNA was quantified with a PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Eugene, OR, USA). Five hundred nanograms of the DNA was applied in each lane and resolved electrophoretically on a 1% agarose gel. DNA corresponding to the D-loop region (nps 16070-671) was used as a probe. This probe was labelled with alkaline phosphatase using an AlkPhos Direct kit (Amersham Biosciences) according to the manufacture's instruction. The signal was visualized

using a chemiluminescent regent (CDP-Star, Amersham Biosciences), recorded and quantified with a chilled charge-coupled device camera, LAS1000plus.

Quantification of mRNAs—Total RNA was extracted from HeLa cells with an SV Total RNA Isolation System (Promega, Madison, WI, USA). The concentration of the total RNA was determined by measuring the absorbance at 260 nm. The reverse transcription of 300 ng of the total RNA was performed with a SYBR RT-PCR Kit (Takara, Otsu, Japan) according to manufacturer's instructions. Random 6-mer primers were used in the reverse transcription reaction. The expression of mitochondrial genes, NADH-ubiquinone reductase subunit 1 (ND1), cytochrome c oxidase subunit II (COXII), cytochrome c oxidase subunit III (COXIII) and cytochrome *b* (CYTB) was detected by quantitative PCR with a LightCycler (Roche). The PCR primers were: 5′-TCGCC CTATTCTTCATAGCC-3′′ (nps 3965–3984) and 5′-AGAA GTAGGGTCTTGGTGAC-3′ (nps 4103–4084) for ND1, 5′-TCTGCTTCCTAGTCCTGTATG-3′ (nps 7686–7706) and 5′-ATGAGGACTAGGATGATGGC-3′ (nps 7812–7793) for COXII, 5′-TAACGCTCCTCATACTAGGC-3′ (nps 9325–9344) and 5′-GGATTATCCCGTATCGAAGG-3′ (nps 9459–9440) for COXIII, 5′-CTATCCATCCTCATCCT AGC-3′ (nps 15632–15651) and 5′-TGGTTGTCCTCCGA TTCAGG-3′ (nps 15772–15753) for CYTB. For a nuclear-encoded gene, β-actin, 5′-CAGGATGCAGAAGGAGATCA-3′ and 5′-GTCATACTCCTGCTTGCTGA-3′ were used likewise. The amounts of four mRNAs of mitochondrial genome were corrected by the amount of β-actin mRNA. The amount of each mRNA is described as a relative amount to that in the cells without the induction of TFAM.

Quantification of mtDNA—Quantification of mtDNA was carried out as described previously (*3*), with minor modifications. Briefly, the DNA extracted from HeLa cells was quantified with a PicoGreen double-stranded DNA quantitation kit. The relative amounts of mtDNA between the cell lines were determined by real-time PCR with a LightCycler. mtDNA was amplified with SYBR Premix Ex Taq (Takara) and primers (nps 8762–8781 and nps 8918–8899). To estimate the amount of genomic DNA as an internal standard, a nuclear anti-thrombin III gene was amplified with primers (5′-TCAGAA CAGAAGATCCCGGA-3′ and 5′-CAAAGGTGCTCCTAA CAAGG-3′). The amount of mtDNA was adjusted by the amount of the nuclear DNA.

MtChIP Assay—Each step was done at 4°C or on ice unless especially mentioned. The purified mitochondria isolated from HeLa cells were incubated with 1% formaldehyde for 30 min in crosslinking buffer (20 mM HEPES-KOH, pH 7.4, 0.25 M sucrose, 2 mM EDTA and 25 mM NaCl). The crosslinking reaction was stopped by addition of 125 mM glycine. After centrifugation at 10,000g, the pellets were suspended in 500 μl of PBS containing 1× Complete Mini. We sonicated 200 μl of the 500 μl sample (the remaining sample was stored at −80°C) and fragmented mtDNA (Supplementary Fig. 2). After centrifugation at 10,000g, 80 μl of the supernatant was taken into two tubes (40 μl each). The supernatants were diluted with 2 ml of IP buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% bovine serum albumin (BSA) and 0.5% NP-40). Each sample was incubated with magnet beads (tosylactivated Dynabeads M-280, Dynal, Oslo, Norway) conjugated with affinity-purified anti-TFAM antibody or control rabbit IgG. After 1 h rotation, the beads were washed three times with IP buffer without BSA and two times with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% SDS). Then, the beads were incubated in 10 μl of elution buffer [0.1 M sodium bicarbonate, 1% SDS and 10 mM dithiothreitol (DTT)] at room temperature for 15 min. After the first elution sample was collected, the beads were incubated with another 10 μl of elution buffer at room temperature for 15 min. The mixtures of the first and second elution samples (total volume 20 μl) were mixed with 0.8 μl of 5 M NaCl and incubated at 65°C for 6 h to reverse the crosslinking. Then, the samples were mixed with digestion buffer (finally 25 mM EDTA, 10 mM Tris-HCl, pH 6.8 and 300 μg/ml proteinase K) and incubated at 45°C for 60 min. DNA was extracted from the samples with a PCR purification kit (Qiagen). Finally, the DNA was eluted with 50 μl of buffer (10 mM Tris-HCl, pH 8.5). The DNA was analysed by quantitative PCR with a LightCycler. In order to detect the mtDNA regions bound by TFAM, we designed 32 pairs of specific primers at about 500-bp regular intervals over mtDNA. The reaction mixture for PCR contained 4 μl of the DNA solution, 10 pmol each of primers and 10 μl of SYBR Premix Ex-Taq in 20 μl. As quantification standards, 1 ng, 10 pg and 100 fg of human mtDNA were amplified for all the 32 regions under the same reaction conditions. We determined the amount of mtDNA immunoprecipitated with anti-TFAM antibody for each region based on the standard human mtDNA. Similarly, we determined the mtDNA amount immunoprecipitated with control IgG. The amounts of mtDNA immunoprecipitated with control IgG was reproducibly below 3% of those with anti-TFAM antibody in any regions. We subtracted the latter from the former to estimate the TFAM-specific immunoprecipitation.

Unwinding Assay—The DNA-unwinding capacity of recombinant TFAM-full and TFAM-ΔC was analysed using a DNA Unwinding Kit (TopoGEN, Port Orange, FL, USA) according to the manufacturer's instructions. Briefly, 0.25 μg plasmids (pBR32) were treated with 2 U of topoisomerase I (Takara) in 20 μl buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 30 min at 37°C (step I). The relaxed plasmids were incubated in the presence of 0.125, 0.25, 0.5, 1.0 and 2.0 μM recombinant TFAM-full or TFAM-ΔC (Supplementary Fig. 1) at 37°C for another 30 min (step II). Ethidium bromide (0.5 μg/ml) was used as a positive control for the unwinding activity at this step. Then 1% SDS and 60 μg/ml proteinase K were added to the reaction mixture. After the reaction mixture was incubated for another 20 min at 56°C, the mixture was mixed with an equal volume of chloroform–isoamylalcohol (24/1, v/v) and agitated vigorously. The resultant aqueous phase was analysed by agarose gel electrophoresis (1% agarose gel) in buffer consisting of 45 mM Tris-phosphate, pH 7.5, 1 mM EDTA. During electrophoresis, a constant voltage of 2.5 V/cm was applied for 16 h at room temperature. The gel was stained with

0.5 μg/ml ethidium bromide for 1 h and destained in water for 30 min before photodocumentation.

Kinetic Analysis of TFAM-Binding to Double-Stranded Oligonucleotides—Biacore 1000 (Biacore AB, Uppsala, Sweden) was used to assess the interaction of TFAM with DNA. As ligands, three different 30-mer double-stranded oligonucleotides with mtDNA sequences were prepared: light-strand promoter (LSP: nps 420–449), termination-associated sequence (TAS: nps 16151–16180) and cytochrome *c* oxidase subunit I (COXI: nps 6991–7020). For LSP, oligonucleotides biotin-5′-CACTTT TAACAGTCACCCCCCAACTAACAC-3′ and 5′-GTGTT AGTTGGGGGGTGACTGTTAAAAGTG-3′ were annealed each other. For TAS, oligonucleotides biotin-5′-CCTGTA GTACATAAAAACCCAACCCACATC-3′ and 5′-GATGTG GGTTGGGTTTTTATGTACTACAGG-3′ were annealed. For COXI, oligonucleotides biotin-5′-CTAGACATCGTAC TACACGACACGTACTAC-3′ and 5′-GTAGTACGTGTCG TGTAGTACGATGTCTAG-3′ were annealed. The double-stranded oligonucleotides were immobilized on an SA sensor chip (Biacore AB). Recombinant TFAM-full and TFAM-ΔC were dialyzed in reaction buffer (PBS with 0.005% Tween-20). The association reaction was started by passing the reaction buffer containing the protein over the surface of the sensor chip at 30 μl/min and then the dissociation reaction was started by replacing the solution by the reaction buffer containing no protein. In parallel, the association/dissociation reaction was done using an SA sensor chip on which oligonucleotides were not immobilized. The association and dissociation on the empty sensor chip was subtracted as a background for the calculation of kinetic parameters. The association rate constant (K_a) and the dissociation rate constant (K_d) were calculated according to BIAevaluation version 4.1 (Biacore AB) by global fitting using a program named 1:1 (Langmuir) binding model. The dissociation constant (K_D) is K_d/K_a.

RESULTS

Overexpression of TFAM-Full Induces Conformational Change of mtDNA—A part of human mtDNA molecules form a D-loop structure that contains a third 700-b strand called a D-loop strand or 7S DNA (*14*). Because the proportion of the D-loop form in mtDNA molecules varies depending on cell types and growing conditions (*15–17*), the D-loop is supposed to be somehow involved in metabolisms of mtDNA though the biological function of this structure remains enigmatic. Since TFAM has an ability to resolve the D-loop structure *in vitro* (*1*), we observed the time-shift of the amount of the D-loop strand in TFAM-overexpressing cells. The expression of

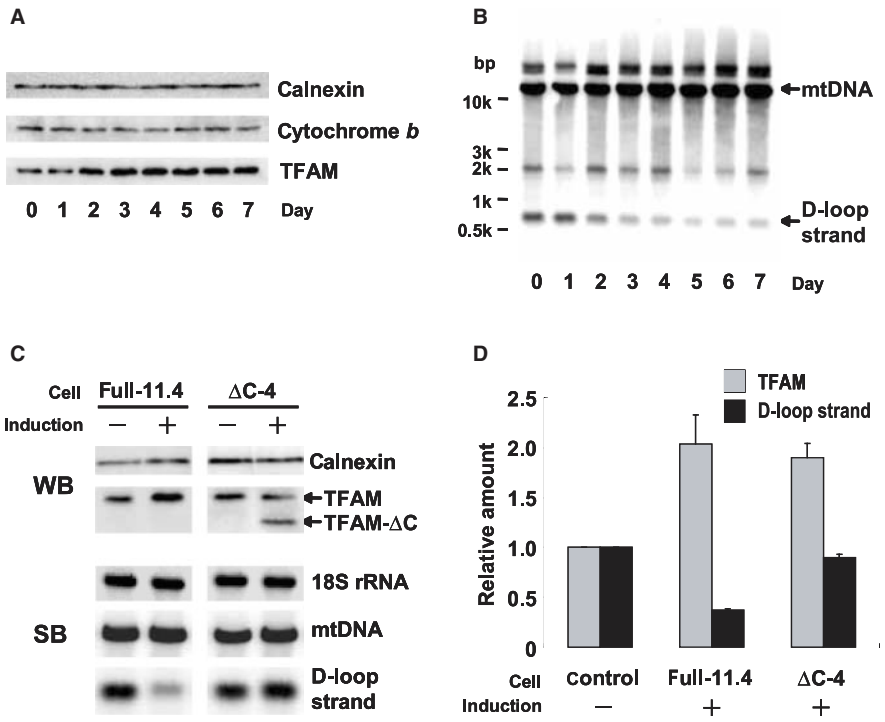

Fig. 1. Time-shift of the D-loop strand in TFAM-overexpressing cells. The time-shift of the amounts of TFAM protein (A) and the D-loop strand (B) was examined after induction of TFAM-full expression. TFAM-full-overexpressing cells, full-11.1, were cultured with doxycycline (day 0). The cells were collected every day after induction of TFAM-full expression by removal of doxycycline. Total cell lysates were used for western blotting. Calnexin, a microsomal protein; cytochrome *b*, a mitochondrial protein encoded by mtDNA. Total DNA extracted from HeLa cells was used for detection of the D-loop strand by Southern blotting. (C) TFAM-full-overexpressing cells, full-11.4 and TFAM-ΔC-overexpressing cells, ΔC-4, were cultured with doxycycline [induction (−)] or without doxycycline [induction (+)] for 10 days. WB, western blotting; SB, southern blotting. 18S rRNA is shown as an internal standard. (D). The relative amounts of TFAM (gray bar) and D-loop strand (black bar). An error bar indicates SD of three independent experiments..

TFAM was induced by removal of doxycycline from the culture medium (Fig. 1A). As previously reported (*3*), the TFAM-overexpression hardly affected the amount of cytochrome *b*, a mitochondrial protein encoded by mtDNA, during this time course. The overexpression of full-length TFAM (TFAM-full) decreased the amount of the D-loop strand to one-third (Fig. 1B and D). The decrease in the D-loop strand means the decrease in the D-loop form of mtDNA. Since the synthesis of the D-loop strand is considered to be coupled with the transcription from the LSP (*18–20*) and the C-terminal tail of TFAM is essential for the promoter-specific transcription (*11*), we overexpressed TFAM-ΔC, which is devoid of the C-terminal tail and little initiates the LSP-dependent transcription. We then examined the amount of the D-loop strand. The TFAM-ΔC overexpression hardly affected the amount of the D-loop strand although its expression level was almost equal to that of TFAM-full (Fig. 1C and D), suggesting that the decrease in the D-loop by TFAM-full is not due to the decrease in the D-loop strand synthesis by the promoter-dependent transcription. In consistent with this idea, the mRNA amount of ND1 was not changed (Fig. 4B). Therefore, it is suggested that TFAM-full inhibits the formation of the D-loop or resolves the D-loop *in vivo* and the C-terminal tail of TFAM is required for the decrease in the D-loop.

The C-terminal Tail of TFAM Strengthens General DNA Binding—The D-loop is known to be resolved by unwinding of a supercoiled state of DNA (*21, 22*), and TFAM can unwind DNA (*4*). Hence, we compared the DNA-unwinding by purified recombinant TFAM-full to that by purified recombinant TFAM-ΔC (Fig. 2; see also Supplementary Fig. 1). TFAM-full induced supercoiling of relaxed pBR322 plasmids in the presence of topoisomerase I in a dose-dependent manner (Fig. 2, lanes 4–8). Eight-fold more TFAM-ΔC was required for unwinding the DNA to the same extent (Fig. 2, lanes 9–13). This stronger unwinding activity of TFAM-full further supports the idea that the D-loop is destabilized by TFAM-full.

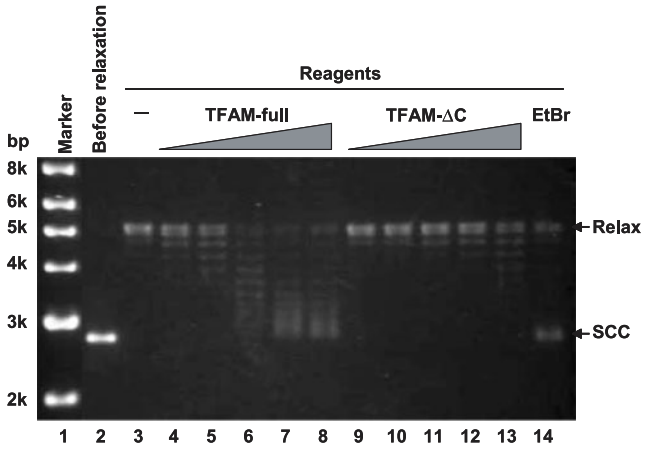

Fig. 2. DNA unwinding by TFAM-full and TFAM-ΔC. The DNA unwinding activity of TFAM-full and TFAM-ΔC was analysed. Plasmids, pBR322, were relaxed with topoisomerase I (lanes 3–14). The relaxed plasmids were incubated with nothing (lane 3), increasing concentrations (0.125, 0.25, 0.5, 1.0, 2.0 μM) of recombinant TFAM-full (lanes 4–8) or TFAM-ΔC (lanes 9–13) or 0.5 μg/ml ethidium bromide (lane 14) in the presence of topoisomerase I. Then the plasmids were electrophoresed on a 1% agarose gel. Lane 1, molecular weight marker; lane 2, plasmids before relaxation. Relax, relaxed form of pBR322; SCC, supercoiled circular pBR322.

Next, we measured the DNA-binding ability of recombinant TFAM-full and TFAM-ΔC using three double-stranded oligonucleotides with different mtDNA sequences, LSP, TAS and COXI (Fig. 3). The association rate constant (K_a), the dissociation rate constant (K_d) and the dissociation constant (K_D) of TFAM-full and TFAM-ΔC to each sequence are shown in Table 1. The K_Ds of TFAM-full and TFAM-ΔC were ~0.5 nM and 0.5 μM, respectively, irrespective of the DNA sequences. It is notable that TFAM-full has three orders of magnitude lower K_D than TFAM-ΔC. Particularly, the absence of the C-terminal tail markedly elevated the dissociation rate (Table 1), suggesting that the C-terminal tail is important for the stable association. A fact that TFAM-full has very low K_D for all DNA sequences is well consistent with the architectural function of TFAM in the nucleoid formation.

Overexpression of TFAM Decreases mRNAs—The transcription of the heavy-strand of mtDNA is initiated from the heavy-strand promoter (HSP) by POLRMT. POLRMT synthesizes a long polycistronic premature RNA. This premature RNA is cut into the separated mRNAs, and mature mRNAs are produced by the addition of poly(A) tails to their 3′ ends as shown schematically in Fig. 4A. To see if the TFAM overexpression affects the transcription of mtDNA, we measured the amounts of mRNAs in TFAM-overexpressing cells. Four genes, ND1, COXII, COXIII and CYTB, encoded by the heavy-strand, were selected. The TFAM protein levels (the combined amount of endogenous and exogenous TFAM) of full-11.4 and ΔC-4 clones were almost 2-fold of the control level and those of full-2 and ΔC-8 were almost 3-fold (Fig. 4B, lowest column). Interestingly, the transcript of ND1 hardly decreased but those of COXII, COXIII and CYTB descended in this order in the TFAM-full-overexpressing cells (Fig. 4B, full-11.4 and full-2), indicating that mRNA of a gene more distant from HSP decreases more strongly (Fig. 4A). Considering the manner of the mRNA maturation (Fig. 4A), these results suggest that TFAM-full does not inhibit the transcription initiation but inhibits the elongation of the RNA synthesis probably by obstructing the processivity of POLRMT. In contrast, the transcripts of the four genes hardly changed in the ΔC-4 cells while the four transcripts decreased almost equally in the ΔC-8 cells (Fig. 4B), suggesting that the excessive overexpression of TFAM-ΔC inhibits the transcription initiation but not the elongation.

TFAM/mtDNA Ratio in TFAM-Overexpressing Cell Lines—In order to know if the exogenous TFAMs are bound to mtDNA, we solubilized mitochondria with 0.5% NP-40 and separated the soluble and insoluble fractions. We have previously shown that the NP-40 insoluble TFAM is mostly associated with mtDNA by using a sucrose-density gradient assay (*23*). As shown in Fig. 5, most of the exogenous TFAMs as well as endogenous

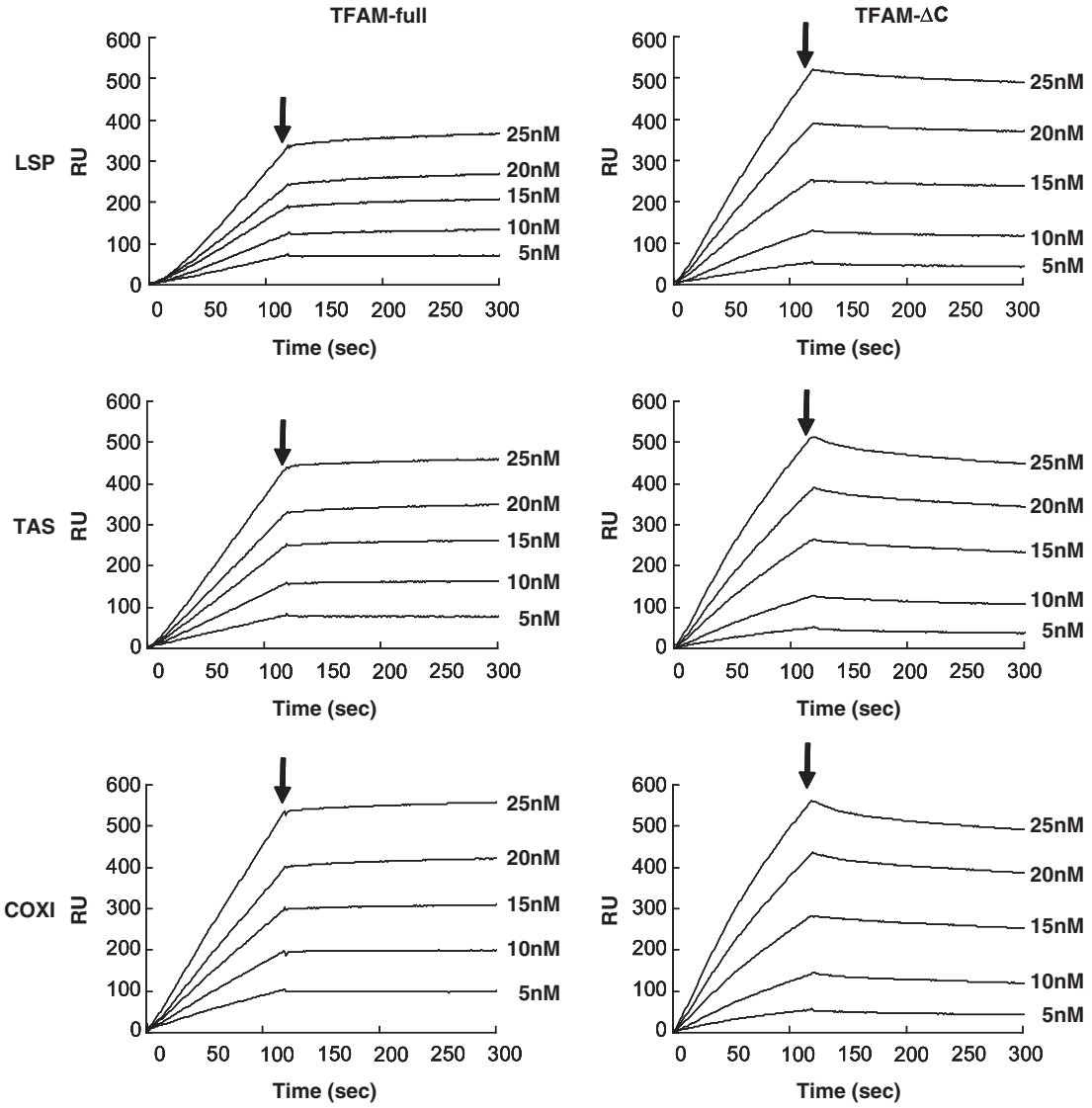

Fig. 3. Binding of TFAM to oligonucleotides. The binding of TFAM to immobilized 30-mer oligonucleotides was started by flowing the buffer containing the indicated recombinant proteins (5, 10, 15, 20 and 25 nM) in PBS with 0.005% Tween-20. After about 2 min, the dissociation reaction was started by flowing the buffer without the recombinant protein. An arrow indicates the buffer change. LSP, light strand promoter; TAS, termination-associated sequence; COXI, cytochrome *c* oxidase I.

TFAM were detected in the particulate fraction while p32, a mitochondrial matrix protein, was recovered from the soluble fraction. Given that the TFAM in the particulate fraction is in the nucleoid, TFAM associated with mtDNA increased in all of the four TFAM-over-expressing cell lines (Table 2), raising the possibility that TFAM-full, as well as TFAM-ΔC, is increasingly associated with mtDNA in those cells.

Distribution of TFAM on mtDNA In Vivo—TFAM should distribute throughout mtDNA if it forms the nucleoid structure all over mtDNA. To examine whether TFAM binds entirely to mtDNA *in vivo*, we used an mtChIP assay. The fragmented TFAM–DNA complexes were immunoprecipitated with anti-TFAM antibody or the same amount of control IgG and then the immunoprecipitated mtDNA was quantified by quantitative PCR using 32 pairs of mtDNA-specific primers (Table 3). The amounts of mtDNA immunoprecipitated with control IgG was reproducibly below 3% of those with anti-TFAM antibody in any regions. The difference between the former and latter in each primer set was determined as anti-TFAM-specific immunoprecipitation in each area. The primer pairs are distributed over mtDNA at about 500-bp regular intervals. Since standard DNA was not properly PCR-amplified with a primer set No. 1 or 4, the two regions were excluded in Fig. 6. The amount of the immunoprecipitated mtDNA by anti-TFAM was markedly lower in a No. 28 region than in any other regions in all three independent experiments. The amount of the immunoprecipitated mtDNA by control IgG in a No. 28 region was essentially the same as in the other regions. Hence, the relative amount to the No. 28 region was

Table 1. **Binding of TFAM to oligonucleotides.**

	TFAM-full	TFAM-ΔC	ΔC/full
LSP			
K_a	1.33×10^{3}	5.86×10^{2}	0.44
K_d	6.72×10^{-7}	3.88×10^{-4}	577
K_D	5.07×10^{-10}	6.61×10^{-7}	1304
TAS			
K_a	7.77×10^{3}	2.23×10^{3}	0.29
K_d	4.05×10^{-6}	9.58×10^{-4}	237
K_D	5.21×10^{-10}	4.30×10^{-7}	825
COXI			
K_a	4.81×10^{3}	1.43×10^{3}	0.30
K_d	3.74×10^{-6}	7.25×10^{-4}	194
K_D	7.78×10^{-10}	5.08×10^{-7}	653

The kinetic constants were calculated from the experiments shown in Fig. 3. K_a, association rate constant ($M^{-1}S^{-1}$); K_d, dissociation rate constant (S^{-1}); K_D, dissociation constant (M).

calculated in every experiment and then averaged. As a whole, any mtDNA regions were well immunoprecipitated except for the No. 28 region, suggesting that TFAM distributes all over mtDNA except for the No. 28 region (Fig. 6). The presence of an area that reproducibly much less recovered may become an internal control for that the anti-TFAM did not non-specifically precipitate mtDNA.

DISCUSSION

The C-terminal Tail Markedly Strengthens the General DNA-Binding of TFAM—The most important finding in this study is that the C-terminal tail of human TFAM markedly increases the general DNA-binding activity. *Physarum polycephalum* has a protein called Glom, which is required for the higher structure formation of mtDNA (*24*). This protein is consisted of a lysine-rich basic domain at an N-terminal and a TFAM-like domain containing two HMG-boxes at a C-terminal. This TFAM-like domain, however, does not harbour the region corresponding to the C-terminal tail of TFAM. Instead, the lysine-rich basic domain of Glom is essential for tight packaging of mtDNA in *P. polycephalum* (*24*). Likewise, the basic C-terminal tail of human TFAM can be important for the tight packaging of mtDNA. The C-terminal tail contains eight basic amino acids, four arginines and four lysines, in 25 amino acids. So, it is possible that the C-terminal tail contributes to the stabilization of the TFAM/mtDNA complex through electrostatic interaction.

In the ΔC-8 cells that expressed TFAM (endogenous and exogenous) about 3-fold, the four kinds of transcripts were evenly decreased (Fig. 4B), suggesting that the transcription initiation is suppressed but its elongation is not. TFAM-ΔC may competitively inhibit the transcription initiation because TFAM-ΔC is not able to elicit the promoter-dependent transcription. Here, a question is raised that the K_D of TFAM-ΔC for DNA seems too higher than that of TFAM-full (about 1000-fold) (Table 1)

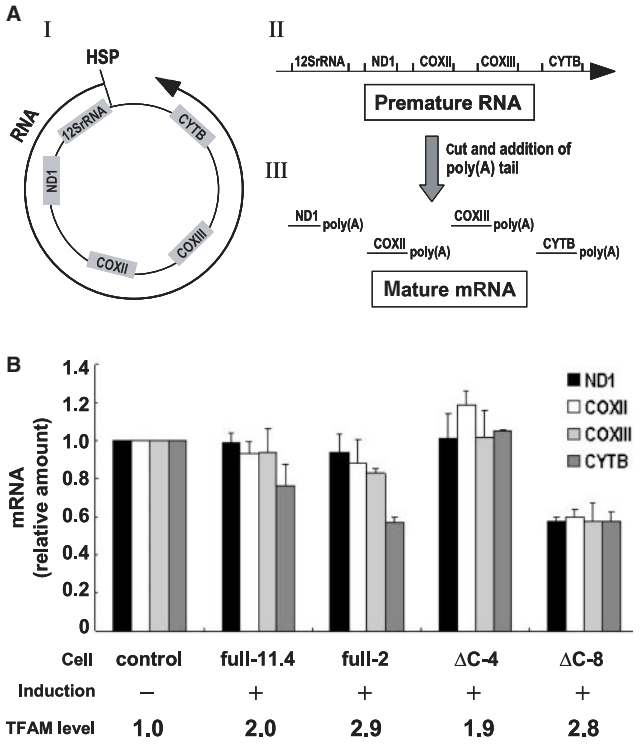

Fig. 4. mtDNA transcripts in TFAM-overexpressing cells. (A). A scheme for the transcription of mtDNA from HSP. (i). Transcription of the heavy-strand of mtDNA is initiated from HSP by POLRMT. (ii). POLRMT synthesizes a polycystronic premature RNA including transcripts of all genes encoded by the heavy-strand. (iii). Then, the premature RNA was cut and poly(A) was added to each mRNA. In this scheme, the five genes, 12SrRNA, ND1, COXII, COXIII and CYTB, were shown as examples. (B). TFAM-full-overexpressing cell lines, full-11.4 and full-2, and TFAM-ΔC-overexpressing cell lines, ΔC-4 and ΔC-8, were examined. The cells of each cell line were divided to two dishes and cultured with doxycycline [control: induction (−)] or without doxycycline [induction (−)] for 10 days. The mRNAs of the mitochondrial genes and that of the nuclear encoded gene, β-actin, were measured by RT-PCR. The amounts of four mRNAs derived from mitochondrial genome were adjusted by the amount of β-actin mRNA. The amount of each mRNA is shown as a relative amount to the control. The relative amount of TFAM protein level (the combined amount of endogenous and exogenous TFAM) in each cell was described in the lowest column. An error bar indicates SD of three independent experiments. ND1, black; COXII, white; COXIII, light gray; CYTB, dark gray.

to competitively bind to the promoter. However, the truncation of the C-terminal tail mostly affected the dissociation rate and hence TFAM-ΔC may fairly well bind to DNA but dissociate rapidly. We have previously hypothesized that once-bound TFAM on the promoter would rapidly dissociate by an unknown mechanism *in vivo* for the regulation of the transcription while it would be rather stably associated with mtDNA in the other regions (*3*), which may explain an observation that a small amount of exogenous TFAM can upregulate the transcription (*25*). If so, the increase in the dissociation rate of TFAM-ΔC may not much affect the competition

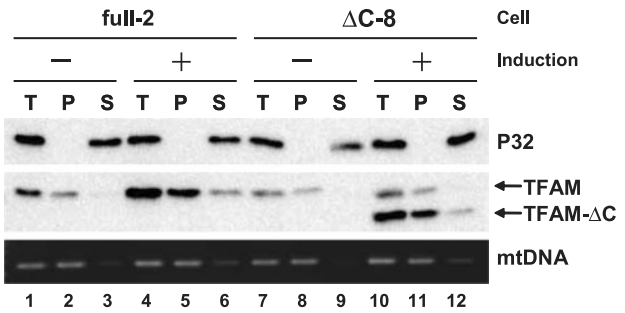

Fig. 5. Exogenous/endogenous TFAM bound to mtDNA. The TFAM-full cell line (full-2) and TFAM-ΔC cell line (ΔC-8) were cultured with doxycycline [induction (−)] or without doxycycline [induction (+)] for 10 days. Crude mitochondria were prepared from these cells and solubilized with 0.5% NP40 (T). The solubilized mitochondria were separated into pellet (P) and supernatant (S) by centrifugation. Each sample was analysed by western blotting with anti-human TFAM and anti-P32 antibodies. P32 is shown as a standard protein existing in mitochondrial matrix. mtDNA in each fraction was detected by PCR.

Table 2. **The ratio of mtDNA-bound TFAM to mtDNA (TFAM/mtDNA).**

Cell line	TFAM/mtDNA
full-11.4	1.6 ± 0.3
full-2	2.1 ± 0.2
ΔC-4	1.3 ± 0.2
ΔC-8	2.1 ± 0.3

TFAM (endogenous and exogenous) in the particulate fraction was quantified as TFAM bound to mtDNA (Fig. 6). The TFAM/mtDNA ratio in the control cells was set as 1.0. The relative values to the control were determined in each independent experiment and then averaged. The values are shown as a mean ± SD (N=3).

against endogenous TFAM. Because TFAM-ΔC rather maintains its association rate (about one-third of TFAM-full) (Table 1), TFAM-ΔC would be able to competitively inhibit the transcription initiation. On the other hand, the easy dissociation of TFAM-ΔC well explains why TFAM-ΔC does not affect the elongation of the RNA synthesis.

The dissociation constants were measured using the short linear oligonucleotides (Fig. 3). This experimental condition might not necessarily reflect the *in vivo* situation where mtDNA is closed circular. TFAM recognizes kinked structures of DNA, e.g. oxidized bases (*26*), cross-linked DNA (*27*) and branched structure (*12*). In fact, the difference between TFAM-full and TFAM-ΔC was at most 10-fold in the unwinding activity (Fig. 2), where closed circular DNA was used. Thus, the dissociation constants of the two TFAMs could depend on the DNA conformation and so could be different between for the closed circular mtDNA and for the short oligonucleotides. Furthermore, TFAM unwinds and bends DNA, which can affect the binding of TFAM to adjacent regions. Therefore, we do not exclude the possibility that difference of the dissociation constants between TFAM-full and TFAM-ΔC is not so large *in vivo* as that obtained by using the short oligonucleotides and so, TFAM-ΔC can be more competitive *in vivo* than expected from the *in vitro* results. Such possible stronger binging of TFAM-ΔC *in vivo* might explain why TFAM-ΔC is increasingly associated with mtDNA as is TFAM-full (Fig. 5). Taken together, DNA-binding of TFAM-ΔC *in vivo* may be sufficient to stably associate with mtDNA but insufficient to inhibit the transcription elongation.

Modulation of Mitochondrial Transcription by Overexpression of TFAM—In the TFAM-full-overexpressing cells, we have noticed two phenomena. First, mtDNA in a D-loop form was decreased (Fig. 1). In other words, the overexpression of TFAM induces a conformational change of mtDNA. It suggests that TFAM is involved in the regulation of D-loop formation *in vivo*. Second, the transcripts were decreased depending on the distance of their genes from the promoter (Fig. 4B), suggesting that the transcription initiation is not inhibited but its elongation is repressed. These phenomena may be explained by the general tight binding of TFAM to mtDNA because of the following two reasons. Firstly, most of endogenous and exogenous TFAM is likely to be associated with mtDNA nucleoids in the TFAM-full-overexpressing cells (Fig. 5), i.e. TFAM molecules could be increasingly associated with one mtDNA molecule (Table 2). Secondly, there may be little alteration of the transcription initiation by overexpression of TFAM-full (Fig. 4B). mtDNA would have a more tight structure in the TFAM-full-overexpressing cells than in normal cells. This probable strong packaging may obstruct the processivity of POLRMT.

TFAM/mtDNA Ratio—Since most of TFAM and mtDNA bind to each other *in vivo* (*2*) and forced decrease in TFAM by RNA interference reduces the amount of mtDNA (*3*), we have supposed that a ratio of TFAM and mtDNA is constant in mitochondria. The current work suggests that the number of TFAM bound to one mtDNA molecule is not necessarily under a strict control but is able to increase (Fig. 5; Table 2), possibly by forming oligomers on mtDNA. The TFAM/mtDNA ratio may be regulated to be constant in cells under a normal state. However, under special conditions, the overpackaged state seems to happen. In the course of maturation of *Xenopus* oocytes, the inactivation of mtDNA transcription is accompanied by a high TFAM/mtDNA ratio (*28*). The high TFAM/mtDNA ratio may result in inhibition of mtDNA transcription probably through overpackaging of mtDNA and/or regulation of the D-loop. In another report, H_2O_2 causes a loss of mtDNA in C2C12 myoblastic cells while TFAM is less decreased, resulting in an increase in TFAM per mtDNA (*29*). Since TFAM has a stronger affinity to DNA with kinked structures, e.g. oxidized bases, cross-linked DNA and branched structure than to normal DNA, it is possible that the overpackaging by TFAM locally happens when mtDNA are injured by some DNA-damaging agents.

TFAM Binds to the Entire Region of mtDNA in Human Cells—Fisher *et al*., (*4*) have reported that TFAM can bind specifically to the upstream control regions of LSP and HSP *in vitro* although TFAM binds to multiple sites within the control region at saturating concentrations. Furthermore, Ghivizzani *et al*., (*5*) have shown by *in organello* footprinting analysis that TFAM binding is not restricted to the LSP and HSP regions but occurs at

Table 3. **Primer sets for the mtChIP assay.**

Primer set	Forward primer	Reverse primer	(nps)	(nps)
1	5′-TAGAGGCGACAAACCTACCG-3′	5′-TCCTAGTGTCCAAAGAGCTG -3′	(1983-2002)	(2130-2111)
2	5′-ATCACCTCTAGCATCACCAG-3′	5′-AAGAGACAGCTGAACCCTCG-3′	(2509-2528)	(2661-2652)
3	5′-CGATGTTGGATCAGGACTATC-3′	5′-AAGGCGCTTTGTGAAGTAGG-3′	(2988-3007)	(3167-3148)
4	5′-TCTCACCATCGCTCTTCTAC-3′	5′-AGTTTGATGCTCACCCTGATC-3′	(3540-3559)	(3679-3659)
5	5′-TCGCCCTATTCTTCATAGCC-3′	5′-AGAAGTAGGGTCTTGGTGAC-3′	(3965-3984)	(4103-4084)
6	5′-ACACTCATCACAGCGCTAAG-3′	5′-CGTGAGGAAATACTTGATGGC-3′	(4518-4537)	(4655-4635)
7	5′-AGCAGTTCTACCGTACAACC-3′	5′-GTTAGCTTGTTTCAGGTGCG-3′	(5042-5061)	(5182-5163)
8	5′-ACTCTGCATCAACTGAACGC-3′	5′-AAGCCAGTTGATTAGGGTGC-3′	(5605-5624)	(5722-5703)
9	5′-AGGCTTTGGCAACTGACTAG-3′	5′-ACTGTTCAACCTGTTCCTGC-3′	(6131-6150)	(6286-6267)
10	5′-AGGCTTCGGAATAATCTCCC-3′	5′-TGATTATGGTAGCGGAGGTG-3′	(6650-6669)	(6837-6818)
11	5′-AACACTTTCTCGGCCTATCC-3′	5′-AGGACTTTTCGCTTCGAAGC-3′	(7186-7205)	(7351-7332)
12	5′-TCTGCTTCCTAGTCCTGTATG-3′	5′-ATGAGGACTAGGATGATGGC-3′	(7686-7706)	(7812-7793)
13	5′-ACAGTTTCATGCCCATCGTC-3′	5′-CGGTAGTATTTAGTTGGGGC-3′	(8196-8215)	(8387-8368)
14	5′-TTGCCACAACTAACCTCCTC-3′	5′-TGTGGTAAGAAGTGGGCTAG-3′	(8762-8781)	(8918-8899)
15	5′-TAACGCTCCTCATACTAGGC-3′	5′-GGATTATCCCGTATCGAAGG-3′	(9325-9344)	(9459-9440)
16	5′-TCACTATCTGCTTCATCCGC-3′	5′-GACCCTCATCAATAGATGAG-3′	(9850-9869)	(9988-9968)
17	5′-TCTGGCCTATGAGTGACTAC-3′	5′-GAGGTGTGAGCGATATACTAG-3′	(10361-10380)	(10547-10527)
18	5′-CAACCTATTTAGCTGTTCCCC-3′	5′-TCGTGATAGTGGTTCACTGG-3′	(10900-10920)	(11032-11013)
19	5′-GTCAATAGTACTTGCCGCAG-3′	5′-TAGGTCTGTTTGTCGTAGGC-3′	(11440-11459)	(11605-11586)
20	5′-TAGTCACAGCCCTATACTCC-3′	5′-AAACCCGGTAATGATGTCGG-3′	(11961-11980)	(12130-12111)
21	5′-TCATGTGCCTAGACCAAGAAG-3′	5′-ACGAACAATGCTACAGGGATG-3′	(12497-12517)	(12623-12603)
22	5′-GGCAAATCAGCCCAATTAGG-3′	5′-GATAGCGCCTAAGCATAGTG-3′	(13000-13019)	(13185-13166)
23	5′-AGACCACATCATCGAAACCG-3′	5′-AAGCGAGGTTGACCTGTTAG-3′	(13512-13531)	(13646-13627)
24	5′-TCCTAGACCTAACCTGACTAG-3′	5′-GTAATTGAGATTGCTCGGGG-3′	(13991-14011)	(14175-14156)
25	5′-AATAACACACCCGACCACAC-3′	5′-GTAGTCCGTGCGAGAATAATG-3′	(14548-14567)	(14691-14671)
26	5′-CTCCTGCTTGCAACTATAGC-3′	5′-TGAGTAGCCTCCTCAGATTC-3′	(15101-15120)	(15253-15234)
27	5′-CTATCCATCCTCATCCTAGC-3′	5′-TGGTTGTCCTCCGATTCAGG-3′	(15632-15651)	(15772-15753)
28	5′-ACCCATCAACAACCGCTATG-3′	5′-TGTTGGTATCCTAGTGGGTG-3′	(16070-16089)	(16283-16264)
29	5′-TATTAACCACTCACGGGAGC-3′	5′-ACAGATACTGCGACATAGGG-3′	(20-39)	(131-112)
30	5′-CACCAGCCTAACCAGATTTC-3′	5′-AGAAAGGCTAGGACCAAACC-3′	(375-394)	(671-652)
31	5′-ATAGAAGCCGGCGTAAAGAG-3′	5′-ATCCCAGTTTGGGTCTTAGC-3′	(924-943)	(1076-1057)
32	5′-GTCGAAGGTGGATTTAGCAG-3′	5′-TTCGTCCAAGTGCACTTTCC-3′	(1412-1431)	(1600-1581)

Thirty-two pairs of specific primers, for the mtChIP assay, designed at about 500-bp regular intervals over mtDNA.
nps, nucleotide position.

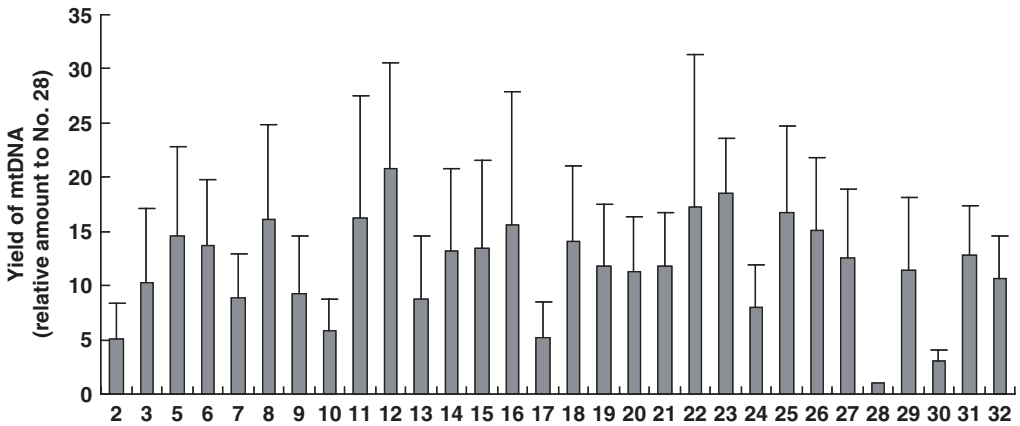

Fig. 6. MtChIP assay. After fragmentation of mtDNA by sonication (Supplementary Fig. 2), the TFAM-DNA complexes were immunoprecipitated with anti-TFAM antibody or control rabbit IgG. The immunoprecipitated DNA was quantified by quantitative PCR using 32 pairs of mtDNA-specific primers. Since the standard DNA was not properly amplified with a primer set No. 1 or 4, we excluded the two regions from the results. Based on the PCR using the standard mtDNA as a template, the absolute amounts of mtDNA immunoprecipitated with anti-TFAM antibody and with control IgG were determined. We subtracted the latter from the former and obtained the amount of DNA specifically immunoprecipitated with anti-TFAM antibody. The relative amount to the No. 28 region was determined in each experiment and then the values in three independent experiments were averaged. An error bar indicates a standard deviation (SD) from the mean of three independent experiments.

regular intervals in the control region. We have recently reported that the amount of TFAM is sufficient to cover the entire region of mtDNA (*1*) and most TFAM is bound to mtDNA *in vivo* (*2*), indicating the possibility that TFAM covers the entire region of mtDNA. However, it has not yet been known if TFAM is indeed evenly bound to the entire region of mtDNA *in vivo*. Thus far, the analysis of TFAM-binding has been limited to the D-loop control region. The mtChIP assay here shows the first direct and *in vivo* evidence that TFAM is bound to the entire region of mtDNA almost equally (Fig. 6).

The No. 28 region was reproducibly much less immunoprecipitated than any other mtDNA regions. This No. 28 region largely covers the vicinity of the D-loop termination, in which a TAS is included. TAS locates at about 50 bases upstream of a 3′ end of the D-loop strand. The region around the D-loop termination may have an unknown structure that decreases TFAM-binding. A 48-kD protein is crosslinked to the TAS region in bovine mitochondria (*30*). Given that the 48-kDa-like protein is present also in human mitochondria, it can be responsible in part for the less binding of TFAM to this No. 28 region.

Previously, we have proposed that human TFAM possesses an architectural function in addition to the transcription initiation function. In this study, we show that (i) the C-terminal tail of TFAM markedly strengthens the general DNA-binding, (ii) the conformation of mtDNA is changed by TFAM-full as evidenced by the decrease in the D-loop, (iii) TFAM-full and TFAM-ΔC differently modulate the transcription and (iv) TFAM is bound to the entire region of mtDNA *in vivo*. These results suggest that the transcription (and possibly replication also) of mtDNA would be affected by the higher structure of mtDNA.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports, and Culture of Japan. S.H.K. was supported in part by the Daegu University Research Grant, 20050052.

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