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Import of mitochondrial transcription factor A (TFAM) into rat liver mitochondria stimulates transcription of mitochondrial DNA

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ABSTRACT

Mitochondrial transcription factor A (TFAM) has been shown to stimulate transcription from mitochondrial DNA promoters *in vitro*. In order to determine whether changes in TFAM levels also regulate RNA synthesis *in situ*, recombinant human precursor proteins were imported into the matrix of rat liver mitochondria. After uptake of wt-TFAM, incorporation of [α-^{32}P]UTP into mitochondrial mRNAs as well as rRNAs was increased 2-fold ($P < 0.05$), whereas import of truncated TFAM lacking 25 amino acids at the C-terminus had no effect. Import of wt-TFAM into liver mitochondria from hypothyroid rats stimulated RNA synthesis up to 4-fold. We conclude that the rate of transcription is submaximal in freshly isolated rat liver mitochondria and that increasing intra-mitochondrial TFAM levels is sufficient for stimulation. The low transcription rate associated with the hypothyroid state observed *in vivo* as well as *in organello* seems to be a result of low TFAM levels, which can be recovered by treating animals with T3 *in vivo* or by importing TFAM *in organello*. Thus, this protein meets the criteria for being a key factor in regulating mitochondrial gene expression *in vivo*.

INTRODUCTION

Mitochondria are the major sites of energy production in almost all eukaryotic cells, using oxidative phosphorylation (OXPHOS) to convert redox energy of substrates into ATP. The organelles contain a small genome which, in animals, encodes 13 essential subunits of the respiratory chain complexes as well as all the rRNAs and tRNAs necessary for their translation (1,2). Mitochondrial biogenesis is regulated, which becomes most obvious during embryonic development, leading to cells with a wide range of OXPHOS capacity, but also during adaptational processes. In yeast, for example, oxygen and glucose availability regulate mitochondrial content (1,3). In mammals, on the other hand, several hormones have a profound stimulatory effect on mitochondrial biogenesis (4–6), and other examples are endurance training of muscle (7) or cold adaptation in brown fat tissue (8). Steady-state levels of mitochondrial transcripts vary over the same wide range in various rat tissues and correlate well with OXPHOS capacity (9). Early studies have already demonstrated that these levels are predominantly regulated by modulating transcriptional activity (10) and, furthermore, thyroid hormone as well as dexamethasone treatment have been shown to increase mitochondrial transcript levels by stimulating transcription rate (11–13). In summary, regulation of mtDNA transcription seems to be an essential step in the regulation of mitochondrial biogenesis.

It has long been known that *in vitro* a partially purified mitochondrial RNA polymerase preparation and a single additional protein, mitochondrial transcription factor A (TFAM), are necessary and sufficient for efficient and correct initiation of transcription from mammalian mtDNA promoters (14). Recently, two other factors necessary for mtDNA transcription have been cloned, TFB1M and TFB2M, which, together with TFAM and mitochondrial RNA polymerase (POLRMT), eventually allowed reconstitution of a faithful *in vitro* mtDNA transcription system with pure recombinant proteins (15,16). In these *in vitro* systems, elevating the concentration of the DNA-binding protein TFAM increases the yield of run-off transcripts, and there seems to exist an optimal stoichiometry between the protein and template DNA, since high concentrations of TFAM have an inhibitory effect (17,18). The TFBM proteins, on the other hand, bind with high affinity directly to the polymerase, with the maximal transcription rate being obtained at a 1:1 stoichiometry, while adding more of the TFBM proteins has no effect (15). Thus,

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in vitro the levels of TFAM regulate the rate of mtDNA transcription.

Indeed, disruption of the *tfam* gene in the mouse resulted in early death in homozygous embryos, while heterozygous mice showed reduced TFAM levels and decreased mtDNA copy number in several tissues, emphasizing its central role in mtDNA maintenance (19). However, mitochondrial transcripts were only reduced by 20% in heart and kidney of these mice and not at all in liver and muscle, and OXPHOS capacity was only marginally decreased in heart muscle. Recently, TFAM was also ablated specifically in skeletal muscle and in this case, both mtDNA as well as mitochondrial transcripts were reduced dramatically (20). These findings clearly show that levels of TFAM strongly influence copy number of mtDNA, but the actual role played by TFAM in the process of transcription regulation still remains unclear, since copy number control and transcription regulation cannot be dissected from each other *in vivo*.

In order to approach this question in an alternative way, TFAM was imported into isolated rat liver mitochondria, which faithfully synthesize RNA for several hours (21). The effect of increased intra-mitochondrial levels of TFAM on the transcription rate *in organello* was subsequently analyzed. The experiments were performed with mitochondria from normal and also from hypothyroid rats in order to test whether reduced TFAM availability in the matrix may be responsible for the reduced mitochondrial transcript levels observed in hypothyroid liver *in vivo* (22) and the low rate of mtDNA transcription in mitochondria isolated from such tissue (23).

MATERIALS AND METHODS

Cloning and subcloning of human TFAM and a truncated version

The full-length human TFAM coding sequence including the mitochondrial targeting sequence (24) was amplified by reverse transcription–PCR using total human RNA as template. The PCR product was cloned into the XbaI and ClaI sites downstream of the HCMV-IE promoter in the expression vector pKEX-2-XR (25), yielding pKEX-TFAM; restriction sites had been introduced by the forward (CCTCTAGAGCGATGGCGTTTCTCCGAA) and reverse (CCATCGATCCATTGTGAACACATCTC) primers. The correct sequence was confirmed by dideoxy sequencing of both strands. The TFAM insert was then subcloned into the XbaI and XhoI sites of pBS-II-KS (Stratagene), yielding pBS-TFAM used for *in vitro* transcription/translation analyses. A truncated version of TFAM (ΔC-25-TFAM) was produced lacking 25 amino acids at the C-terminal end. The PCR product was cloned into the XbaI and XhoI sites of pKEX-2-XR using pBS-TFAM as template and appropriate primers containg XbaI and XhoI restrictions sites. (forward, CCTCTAGAGCGATGGCGTTTCTCCGAA; ΔC-25-reverse, CCGGCTCGAGTTATTGTTCTTCCCAAGACTTCA). The insert was also subcloned into pBS-II-KS, yielding pBS-ΔC-TFAM.

Animal treatment

Female Sprague–Dawley rats (~120 g) were made hypothyroid by adding 6-propyl-2-thiouracil to the drinking water (0.05% w/v) for 5 weeks. To induce hyperthyroidism, animals received daily i.p. injections of 125 μg/kg body wt thyroid hormone (triiodothyronine, T3) for five consecutive days (22). Animals were killed after carbon dioxide anesthesia by cervical dislocation.

In vitro synthesis of TFAM and import into isolated rat liver mitochondria

Labeled TFAM proteins were synthesized in a coupled transcription/translation rabbit reticulocyte lysate system (Promega) in the presence of [α-^{35}S]methionine by addition of pBS-TFAM or pBS-ΔC-25-TFAM and T7 polymerase. Rat liver mitochondria were prepared as described in detail previously (26). Import was studied in 100 μl of the *in organello* transcription buffer system containing ATP (1 mM) in the presence of mitochondrial protein (2 mg/ml) and rabbit reticulocyte lysate (10% v/v) containing the radiolabeled proteins. After incubation at 30°C for the indicated periods of time, the incubation mixture was divided into aliquots and further incubated for 15 min at 0°C with proteinase K (0.25 mg/ml), proteinase K plus Triton X-100 (1% v/v, final concentration) or no further additions. Where indicated, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of the mitochondrial inner membrane potential, was added during the incubation at 30°C (2 μM). Subsequently, mitochondria were sedimented by centrifugation, dissolved in SDS sample buffer and analyzed by SDS–PAGE. Gels were treated for fluorography, dried and exposed to X-ray film.

In organello transcription

Incorporation of [α-^{32}P]UMP into mitochondrial transcripts was studied as previously described in detail (21). Briefly, mitochondria (2 mg of mitochondrial protein) were incubated in 500 μl of transcription buffer, containing malate (2.5 mM) and glutamate (10 mM) as substrates, ADP at 1 mM and 20 μCi [α-^{32}P]UTP (400–600 Ci/mmol), for 1 h at 37°C on a rotating wheel. Reticulocyte lysate (typically 35 μl) was added to the incubation medium, which had been preincubated for 1 h with T7 polymerase and pBS-II-KS (control), pBS-TFAM or pBS-ΔC-25-TFAM, respectively (see above). Unlabeled methionine (20 μM final concentration) was also added. Mitochondria were sedimented by centrifugation, washed twice in transcription buffer, dissolved in lysis buffer and total nucleic acids were extracted and loaded in a single gel lane (21). The labeled RNA was analyzed by vertical methylmercury hydroxide–agarose gel electrophoresis; prior to use the methylmercury hydroxide was deionized with Amberlite MB-1A (16–50 mesh; Sigma). Gels were dried and exposed to X-ray film. Autoradiograms were analyzed with a video camera-based gel analysis system (AIDA software; Raytest) and arbitrary values were obtained for some selected mitochondrial transcripts which were well separated in this gel system.

Immunoblotting

Small pieces of frozen liver were homogenized in hot (95°C) 50 mM Tris (pH 6.8), 4 M urea, 10% SDS, 15% glycerol and 20 mM dithiotreitol in a small Potter homogenizer at ~10 mg/100 μl. Equal amounts of protein, measured by the method of Bradford (27), were loaded on 12.5% polyacrylamide/SDS gels prepared according to Laemmli

(28) and run at 60 V and 15 mA at room temperature. Proteins were transferred to nitrocellulose in 154 mM glycine, 20 mM Tris (pH 8.3) and 20% methanol at 12 V and 100 mA for 75 min at room temperature. Blots were blocked in TBST (200 mM NaCl, 50 mM Tris, 0.5% Tween, pH 7.5) and 2% bovine serum albumin for 2 h. The 24 kDa mtTFA subunit was visualized with a polyclonal antibody raised against mouse mtTFA (kindly donated by Dr D. A. Clayton, Stanford University School of Medicine, Stanford, CA). The antiserum was diluted 1:5000 in TBST containing 2% bovine serum albumin. Goat anti-rabbit immunoglobulin G (diluted 1:10 000 in TBST and containing 2% bovine serum albumin) was used as the secondary antibody coupled to horseradish peroxidase and was visualized with an ECL chemoluminescence system (Amersham) by exposure to X-ray film. Lanes were scanned by densitometry.

Extraction and blotting of DNA for hybridization

To estimate the relative abundance of mtDNA, 20–50 mg tissue were thawed, minced on ice with 500 µl lysis buffer (10 mM Tris, 100 mM NaCl, 25 mM EDTA, 1% SDS and 10 mg/ml proteinase K, pH 8.0) and incubated in a gyratory water bath shaker at 48°C overnight. RNase A (50 µg/ml) was added and the incubation was continued for 30 min at 37°C. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1) and the DNA was precipitated at –20°C, centrifuged and dissolved in 10 mM Tris, 1 mM EDTA (29). To linearize the closed circular mtDNA molecule, 2.5 µg DNA were digested with BamHI and XbaI under conditions recommended by the manufacturer. DNA was denatured by acid treatment, neutralized and blotted in three amounts (1.25, 0.63 and 0.31 µg) on nitrocellulose membranes using a slot blot apparatus.

Extraction and blotting of RNA for hybridization

For estimation of mitochondrial RNA content, total RNA was extracted by the acid guanidinium isothiocyanate procedure (30) with 100–200 mg of tissue pulverized under liquid nitrogen and analyzed by slot blots, loading RNA in a serial dilution, blotting 2.5, 1.25 and 0.635 µg (29).

Hybridization of blots

Blots were prehybridized for 2 h and hybridized overnight at 42°C (prehybridization: 40% formamide, 5× SSC, 50 mmol/l phosphate buffer, pH 7.4, 10× Denhardts solution, 0.2% SDS, 500 µg/ml salmon sperm DNA; hybridization: 50% formamide, 3× SSC, 10 mmol/l phosphate buffer, pH 7.4, 2× Denhardts solution, 0.2% SDS, 170 µg/ml salmon sperm DNA). Probes were labeled to high specific radioactivity by the random priming method. DNA blots were probed with a cloned cDNA probe for cytochrome c oxidase (COX) subunit I. RNA blots were probed with cloned cDNA probes for COX subunits I and II and 12S rRNA. After hybridization, blots were washed at 42°C (2 × 15 min in 2× SSC, 0.1% SDS, followed by 2 × 15 min in 0.1× SSC, 0.1% SDS). After exposure, the previous probe was stripped from the blots (4 × 5 min incubations in boiling 0.01× SSC, 0.01% SDS) and they were hybridized to a probe for cytosolic 28S rRNA. For this probe, hybridization temperature was 44°C and the last two washing steps were performed at 50°C. Blots were exposed to X-ray films, the absorbance of the autoradiograms was measured with a scanning densitometer and arbitrary numbers for mtDNA levels were used only from the densitometric values of those dilutions which were in the linear range. For normalization, densitometric data for mtDNA were normalized to the 28S rRNA signal, also taking care that the signal was in the linear range.

Statistical analysis

Data were compared by a two-tailed Student's *t*-test. A confidence level of $P < 0.05$ was considered indicative of a statistically significant difference between groups.

RESULTS

Import of TFAM into isolated rat liver mitochondria

In order to show successful uptake into the mitochondrial matrix, rat liver mitochondria were incubated with labeled recombinant wt-TFAM and ΔC-25-TFAM proteins, sedimented, lysed and subjected to SDS–PAGE and fluorography (Fig. 1A and B). This truncated version of TFAM was chosen as a control for any non-specific effects of protein import because it was shown to have considerable DNA binding but no transactivation activity *in vitro* among a series of truncated proteins (18). The reticulocyte lysate exclusively contained the precursors, and they were produced in approximately similar amounts judged by band intensity when loaded on the same gel (not shown). By comparison of lysate and supernatant after incubation with mitochondria, it could be estimated that more than 90% of both TFAM precursors were sedimented together with the organelles. It should be noted that both TFAM proteins migrate slower than expected from their predicted molecular masses. As already shown previously (31), at 30°C only a part of the 29 kDa wt-TFAM precursor pool was converted into a 24 kDa protein within 1 h (Fig. 1A). This process was completely inhibited by the uncoupler CCCP. The 24 kDa protein was resistant to proteinase K treatment, while the 29 kDa precursor was accessible. Lysis of mitochondrial membranes by Triton X-100 made both proteins digestable (Fig. 1A). Likewise, the 26 kDa ΔC-25-TFAM was converted into a mature protein, which was resistant to proteinase K digestion (Fig. 1B). Interestingly, in this case an intermediate sized protein was also produced (~25 kDa), which was not accessible to digestion. At 37°C, the temperature of the *in organello* transcription assay (see below), all of the wt-TFAM as well as the ΔC-25-TFAM had been imported after 1 h of incubation (data not shown).

Thus, both TFAM precursor proteins were imported into the mitochondrial matrix to about the same extent and processed to the mature forms.

Effect of TFAM import on mitochondrial transcription rates *in organello*

In order to study the effect of imported TFAM on mitochondrial transcription rate, isolated rat liver mitochondria were incubated in the presence of reticulocyte lysate containing TFAM proteins and [α-^{32}P]UTP. The radiolabeled RNA was analyzed by methylmercury–agarose gel electrophoresis. Ethidium bromide staining was routinely used to

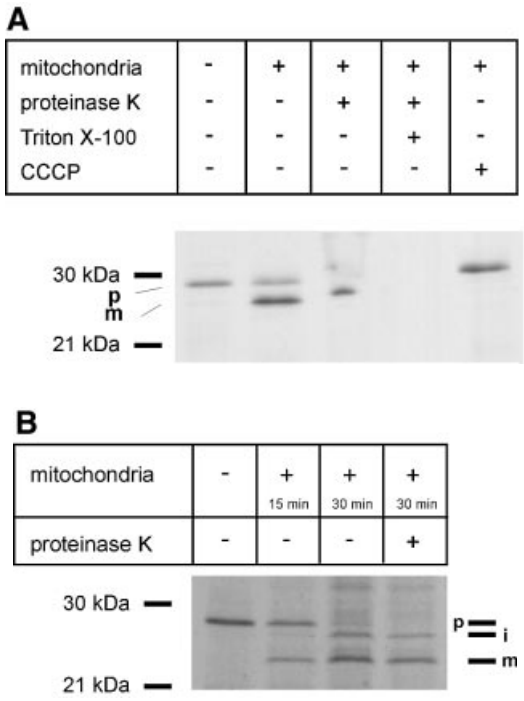

Figure 1. Import of TFAM into rat liver mitochondria. Mitochondria were incubated for 1 h (**A**) with ^{35}S-labeled wt-TFAM or (**B**) with ^{35}S-labeled ΔC-25-TFAM for the indicated times. Import was studied by SDS–PAGE and fluorography. Where indicated, CCCP was added during the incubation, or proteinase K and Triton-X-100, respectively, were added after the incubation. In the first lanes, aliquots of reticulocyte lysate are shown, while the other lanes contain protein from sedimented mitochondria. p, precursor protein; i, import intermediate protein; m, mature protein.

confirm that equivalent quantities of nucleic acids had been extracted from individual reactions and loaded onto the gel. This is best seen by similar amounts of mtDNA, present in multiple conformations, or 16S and 12S mitochondrial rRNAs, respectively (Fig. 2A). In this gel system, it is possible to identify the characteristic set of transcripts derived from the heavy strand of mtDNA, as previously described in HeLa cells (32), as well as rat liver and brain mitochondria (26). In preliminary experiments, we found that 50 µl of reticulocyte lysate decreased the rate of [α-^{32}P]UTP incorporation compared to control (Fig. 2B), probably due to the inhibition of mitochondrial transcription by high levels of ATP (21), which is generated by the creatine kinase–phosphocreatine ATP back-up system in the lysate. Consequently, different concentrations were tested, and we found that 50 µl of lysate containing TFAM stimulated incorporation of radioactivity into mitochondrial transcripts compared to 25 µl, while no further stimulation was seen when 75 µl of lysate were added (Fig. 2B). Thus, routinely, 35 µl of lysate were added to the reactions in all further experiments (Fig. 2C) as a compromise between inhibition of trancription by lysate versus stimulation by TFAM. It is evident from a first inspection that incorporation of radioactivity was higher after import of wt-TFAM and that this increase affected precursor RNAs, mRNAs and rRNAs equally. Bands that were especially well separated from others and could be reliably analyzed were selected for quantification (Table 1). Densitometric analysis showed that in this experimental series, incorporation of [α-^{32}P]UTP had increased by an average factor of two ($P < 0.05$) and that there was no preferential stimulation of transcription of any mRNA or rRNA species. After import of ΔC-25-TFAM, no significant stimulation of nucleotide incorporation into mitochondrial transcripts was observed (Fig. 2D and Table 1). A different lot of methylmercury hydroxide used for these gels resulted in some loss of resolution. However, identification of the transcripts was easily possible and quantitation was performed using a phosphorimager.

In conclusion, freshly isolated rat liver mitochondria transcribe at a submaximal rate and import of wt-TFAM stimulates transcription up to 2-fold, while the isoform lacking 25 amino acids at the C-terminus is ineffective.

TFAM expression and mtDNA levels in rat liver in different thyroid states

Thyroid hormone is a potent regulator of mitochondrial transcription. It has been shown before that TFAM mRNA is up-regulated by thyroid hormone in the liver together with mitochondrial transcripts after giving a high dose of the hormone when compared to hypothyroid rats (33,34). It was confirmed in the present experiments that mitochondrial transcripts (mRNAs for COX subunits I and II and 12S rRNA) as well as the TFAM mRNA were reduced in the hypothyroid and increased in the hyperthyroid state (data not shown). However, although we found a slight decrease in TFAM protein in hypothyroid and an increase in hyperthyroid liver on western blots (Fig. 3A), similar small changes in mtDNA content were observed (Fig. 3B). Thus, it is clear that by this approach it cannot be demonstrated that TFAM is indeed responsible for the T3-mediated regulation of mitochondrial DNA transcription observed *in vivo*.

Effect of TFAM import on mitochondrial transcription rates in mitochondria from hypothyroid livers

In organello transcription experiments were performed using liver mitochondria from hypothyroid rats. In this series of experiments, stimulation of transcription rate after import of TFAM into euthyroid mitochondria was less pronounced compared to the series described above, i.e. albeit it was consistently increased compared to control lysate, this was only by ~20–30% (data not shown). However, in experiments with hypothyroid animals, incorporation of [α-^{32}P]UTP into mitochondrial transcripts after import of TFAM increased by factors ranging from 1.5 to 4 compared to control lysate (Fig. 4). Again, synthesis of all transcript species was stimulated, irrespective of whether they were precursors RNAs, mRNAs or rRNAs.

Thus, we conclude that liver mitochondria from hypothyroid animals are subsaturated with TFAM protein *in vivo* when compared to mitochondria from euthyroid rats, resulting in greater accessibilty of binding sites to the imported protein.

DISCUSSION

In reconstituted systems, TFAM is absolutely required for efficient and correct initiation of transcription, however, it is still unclear whether it regulates mtDNA transcription *in vivo*.

These *in vitro* systems are composed of template DNA with a mtDNA promoter sequence and, initially, contained a partially purified mitochondrial polymerase. About one molecule of TFAM per template DNA was needed to stimulate transcription from the light strand promoter (LSP) above basal levels; further increases in TFAM up to a molar ratio of about 5:1 led to further stimulation, but transcription efficiency decreased and even completely ceased when TFAM concentration exceeded DNA concentration by more than 10-fold (17,18,35). Recently, such a system was even reconstituted from pure human recombinant proteins including POLRMT and the MTFB1/TFB2 proteins. In this case, measurable transcription activity from an LSP template was observed, again at a molar ratio of about 1, which increased up to a molar ratio of about 10, remained fairly constant up to a molar ratio of about 100 and then decreased (15). The heavy strand promoter (HSP) was activated only at much higher concentrations, with maximal rates reached at a stoichiometry of about 100 mol TFAM/mol template, and again, further increasing the concentration decreased transcription rate. These results suggest that the matrix concentration of TFAM may control the activity of mitochondrial promoters *in vivo*, as was proposed previously (36)

However, some properties of TFAM have always cast some doubt on its function as a physiologically relevant regulator. The yeast homolog, the 19 kDa high mobility group Abf2 protein, does not affect transcription efficiency in reconstituted systems (17). Also, this class of proteins was originally described as histone-like proteins involved in nucleosome formation, and only some members, like the UBF protein necessary for transcription of rRNA genes, were demonstrated to function as true activators (37). In addition, the low sequence specificity for DNA binding might indicate that the major function of TFAM and Abf2p is DNA packaging. This would also explain its high abundance, with initial reports of about 15 molecules per mitochondrial genome copy in human KB cells (38), 200–2500 molecules per mtDNA in *Xenopus* oocytes, depending on the maturation stage (35,39), and even enough molecules to completely cover the entire mitochondrial genome in yeast (40). Surprisingly, Kang and colleagues reported very recently that there may also be sufficient TFAM to entirely cover the mtDNA in HeLa cells as well as in human placental mitochondria (41).

In addition, although they consistently proved that TFAM is absolutely necessary for mitochondrial function, the TFAM knockout mice produced by Larsson and co-workers did not allow discrimination between TFAM function in transcription versus copy number control. When the gene encoding mouse TFAM was ubiquitously disrupted, heterozygous mice showed reduced TFAM levels and concommittently reduced

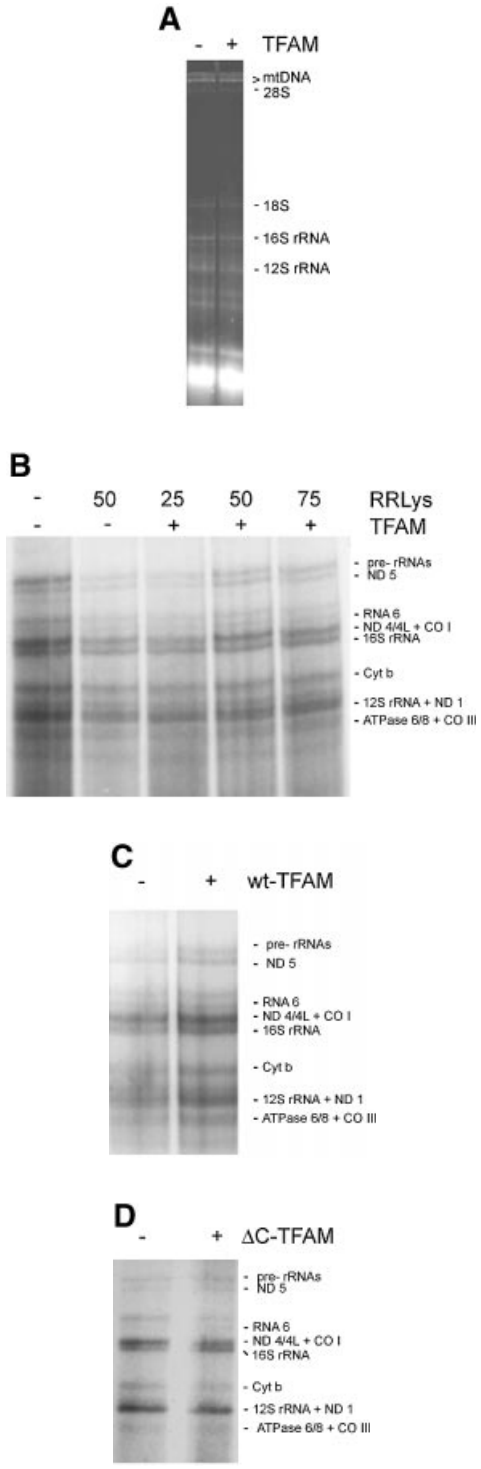

Figure 2. Effect of import of TFAM on mitochondrial transcription rate in isolated rat liver mitochondria. Radiolabeled mitochondrial transcripts were separated on vertical methylmercury–agarose gels. (**A**) Representative gel stained with ethidium bromide showing equal loading of lanes with mitochondrial nucleic acids. (**B**) Mitochondrial transcripts were labeled with [α-^{32}P]UTP *in organello* in the absence or presence of mock reticulocyte lysate without template (–) or with different amounts of lysate containing wt-TFAM (+). (**C**) In further experiments, mitochondrial transcripts were labeled with [α-^{32}P]UTP *in organello* in the presence of 35 µl of reticulocyte lysate containing wt-TFAM (+) or lysate preincubated without template (–). The autoradiogram that is shown is derived from the dried gel presented in (A). (**D**) Mitochondrial transcripts were labeled in the presence of 35 µl of reticulocyte lysate containing ΔC-25-TFAM (+) or lysate preincubated without template (–). The positions of mtDNA present in multiple conformations and a small amount of contamination with cytosolic 28S and 18S rRNA (A), as well as of several mitochondrial transcripts, are indicated (B–D). (C) and (D) are representative for five independent experiments with mitochondria from five different rats (wt-TFAM) or six independent experiments with mitochondria from three different rats (ΔC-25-TFAM). Quantitative data are given in Table 1.

Table 1. Effect of import of TFAM on incorporation of radioactivity into mitochondrial transcripts in isolated rat liver mitochondria

	Control (n = 4)	+ wt-TFAM (n = 4)	+ ΔC-25-TFAM (n = 3)
Precursor rRNAs	100	232 ± 49	97 ± 15
ND 5	100	202 ± 15	101 ± 20
RNA 6	100	209 ± 16	101 ± 25
ND 4 + ND 4L + CO I	100	187 ± 29	101 ± 24
16S	100	166 ± 39	109 ± 22
Cyt b	100	206 ± 40	99 ± 4
12S + ND 1	100	157 ± 28	97 ± 8
ATPase6/8 + CO III	100	163 ± 18	129 ± 30

Values are densitometric units obtained from autoradiograms as shown in Figure 2C (TFAM) or phosphorimager values as shown in Figure 2D (ΔC-25-TFAM). Band intensities after import of TFAM proteins were compared to the same bands in control lanes; intensities of the bands in control lanes were arbitrarily set to 100 units. Data are means ± SD.

mtDNA copy number, while homozygous mutants died very early *in utero*. These homozygous embroys did not reveal any mtDNA in Southern blot analyses, confirming that TFAM is absolutely essential for the maintenance of mtDNA *in vivo* (19). However, it remained to be proven whether TFAM acts as a transcription factor, since in heterozygous mice mitochondrial transcripts were only marginally reduced, and only in heart and kidney and not at all in liver and muscle. Even more puzzling, muscle-specific disruption of the TFAM gene led to severe myopathy with an onset at 3–4 months, however, with virtually no TFAM protein visible on western blots in 1-month-old animals. At that stage, mitochondrial transcripts were reduced to 70% and gradually decreased to 30% at 4 months, while mtDNA was reduced to 30% and stayed at that level for up to 4 months (20). Stabilization of mitochondrial transcripts and mtDNA could explain these results, although the half-life of transcripts has been shown to be in the range of several hours, and an extension to several months is hard to envisage. Another possibility would be that different populations of mtDNA molecules may be present in the mitochondrial nucleoid (42) and TFAM may serve as a packaging factor for the majority of molecules, which become degraded upon TFAM depletion, while a mtDNA subpopulation may remain transcriptionally active with stably bound TFAM which is below detectable levels. Indeed, Shadel and co-workers recently showed that recovery of mitochondrial transcription after the release of mtDNA depletion by ethidium bromide occurred before there was any significant recovery of mtDNA or TFAM protein (43). They also showed that recovery of mtDNA after removal of the chemical was faster than recovery of TFAM levels, which may again be explained by two populations of mtDNA with different TFAM occupancy or, alternatively, by TFAM binding consecutively to different binding sites in all mtDNA molecules.

Thus, we felt that more experiments and alternative approaches are necessary to help further reveal the complex function of this protein for mitochondrial DNA and RNA synthesis and maintanance. In the present paper, we have investigated whether increasing the concentration of TFAM in the matrix of rat liver mitochondria is sufficient to stimulate mitochondrial transcription from the HSP promotor *in situ*. In a preliminary study, we reported that incubation of mitochondria with reticulocyte lysate containing TFAM increased incorporation of [α-^{32}P]UTP into mitochondrial RNA, however, quantitative data and differentiation between light and heavy strand transcripts as well as mRNAs and rRNAs were not provided at that time (44). Recently, however, we have unequivocally shown that TFAM is imported into the matrix of isolated rat liver mitochondria and that it stimulates the synthesis rate of 7S DNA (31). From this result, one could anticipate that it is indeed a transcription regulator *in situ*, since this process is thought to be trancription primed from the LSP (14). In this *in organello* system it was possible to determine the stoichiometry between mtDNA and newly imported TFAM, and we calculated that each copy of mtDNA present in the assay within the matrix of the mitochondria had been challenged with between two and four additional TFAM molecules (31).

Here we show that after import of these amounts of wt-TFAM, a 2-fold stimulation of the transcription rate of mitochondrial RNAs transcribed from the HSP(s) occurred (Fig. 2C and Table 1). The synthesis of mRNAs as well as rRNAs was equally affected, indicating that TFAM is not involved in direction of the transcription machinery to the alternative transcription start sites at HSP I versus HSP II (32), reviewed recently in Fernandez-Silva *et al*. (45). Import of ΔC-25-TFAM had no significant effect on transcription rate (Fig. 2D and Table 1), although about the same amount of protein had been synthesized in the reticulocyte lysate and imported, emphasizing the importance of a full-length C-terminus distal to the HMG box region for binding to the promoter sequence and also transcription activation function *in situ* (18). These findings would be hard to reconcile with occupancy of the entire mtDNA molecule by the endogenous protein (41,46), although there are no stringent arguments against the coexistence of both functions, mtDNA packaging as well as regulated binding to promoter sequences. However, by footprinting techniques it had consistently been found that binding sites for TFAM are not fully occupied *in vivo*. It was demonstrated repeatedly that only about 75% of mtDNA molecules are protected at the TFAM binding site near the LSP and that a consistently lower fraction (30–50%) of the binding site close to HSP is occupied, due to its lower affinity for the protein (15,47). This had been shown for mitochondria from bovine brain (48), human placenta (49), HeLa cells (50) and rat liver (51). Thus, even if enough TFAM was present to cover the entire genome in mammalian cells (41,46), binding sites proximal to the promoters are obviously still accessible to

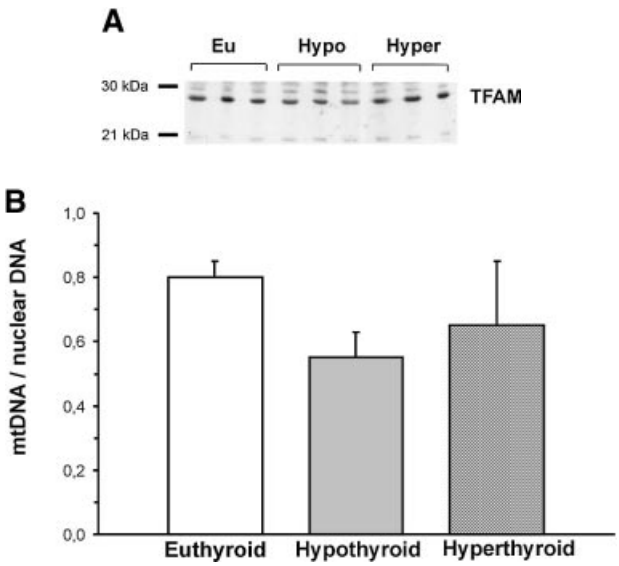

Figure 3. Levels of TFAM protein (**A**) and mtDNA (**B**) in livers of euthyroid, hypothyroid and hyperthyroid rats. TFAM levels were analyzed by western blotting and mtDNA levels were obtained by hybridization of DNA slot blots with a mtDNA probe and normalized to 28S rDNA.

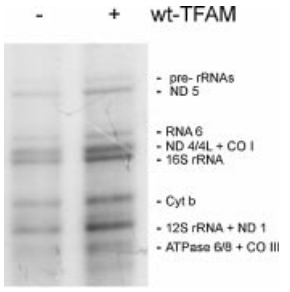

Figure 4. Effect of import of TFAM on mitochondrial transcription rate in isolated liver mitochondria from hypothyroid rats. For legend see Figure 2.

additional molecules, which upon import stimulate synthesis of the primer for 7S DNA from LSP (31) or HSP transcripts (this study). It should be noted that the human TFAM protein and rat liver mitochondria were used here, again emphasizing the promiscuous properties of this protein, which can also replace the mouse (52) and even the yeast homolog (17).

In liver mitochondria isolated from hypothyroid rats, the TFAM footprints at HSP and LSP were clearly weakened, showing that both binding sites seem to be even more subsaturated with TFAM *in vivo* (23), resulting in a lowered transcription activity compared to the euthyroid state (10,23). While addition of T3 to hypothyroid mitochondria could reverse the decreased mRNA/rRNA ratio towards the euthyroid state, it could not increase the general transcription rate; this could only be done by treating animals with the hormone, probably by charging them with TFAM *in vivo*. Alternatively, loading hypothyroid mitochondria with TFAM *in organello* resulted in a clearly greater stimulation of transcription compared to euthyroid mitochondria, thus also rescuing a high transcriptional activity (Fig. 4).

In conclusion, we show here that in freshly isolated mitochondria, the matrix concentration of TFAM indeed regulates the rate of transcription from the mitochondrial HSP promoter. We conclude that regulation of matrix TFAM levels by T3 via the nucleus is probably responsible for the different TFAM occupancy of promoter sites and, consequently, different mitochondrial transcription rates observed in different thyroid states *in vivo*. Not surprisingly, the 5′-region of the human (53) and rat (GenBank accession no. AF264733, nt 42–46) TFAM genes contain a putative thyroid hormone response element (TGACC). We postulate that other factors, like the truncated c-ErbAα1 mitochondrial thyroid hormone receptor p43 (54), would modulate the basal transcription machinery consisting of TFAM, TFBM and POLRMT, e.g. by directing it to different promoter sites (HSP I and II, respectively), leading to altered ratios of initiation from these sites. This would result in the peculiar shift between mRNA/rRNA transcription rates modulated by thyroid hormone (23). Modification of the binding activity of pre-existing TFAM to mitochondrial promoters, as recently shown for diabetic rat hearts, may be an additional mode of control (55). Since transcription of mtDNA is certainly an important step in the biogenesis of the whole organelle, regulation of TFAM levels and its promoter binding is indeed a key event in regulation of the OXPHOS capacity of cells.

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REFERENCES

1. Attardi,G. and Schatz,G. (1988) Biogenesis of mitochondria. *Annu. Rev. Cell. Biol.*, **4**, 289–333.
2. Clayton,D.A. (2000) Vertebrate mitochondrial DNA—a circle of surprises. *Exp. Cell Res.*, **255**, 4–9.
3. Dirmeier,R., O'Brien,K.M., Engle,M., Dodd,A., Spears,E. and Poyton,R.O. (2002) Exposure of yeast cells to anoxia induces transient oxidative stress: implications for the induction of hypoxic genes. *J. Biol. Chem.*, **277**, 34773–34784.
4. Nelson,B.D. (1990) Thyroid hormone regulation of mitochondrial function. Comments on the mechanism of signal transduction. *Biochim. Biophys. Acta*, **1018**, 275–277.
5. Cornwall,G.A., Orgebin,C.M. and Hann,S.R. (1992) Differential expression of the mouse mitochondrial genes and the mitochondrial RNA-processing endoribonuclease RNA by androgens. *Mol. Endocrinol.*, **6**, 1032–1042.
6. Weber,K., Bruck,P., Mikes,Z., Kupper,J.H., Klingenspor,M. and Wiesner,R.J. (2002) Glucocorticoid hormone stimulates mitochondrial biogenesis specifically in skeletal muscle. *Endocrinology*, **143**, 177–184.
7. Adhihetty,P.J., Irrcher,I., Joseph,A.M., Ljubicic,V. and Hood,D.A. (2003) Plasticity of skeletal muscle mitochondria in response to contractile activity. *Exp. Physiol.*, **88**, 99–107.
8. Klingenspor,M., Ivemeyer,M., Wiesinger,H., Haas,K., Heldmaier,G. and Wiesner,R.J. (1996) Biogenesis of thermogenic mitochondria in brown adipose tissue of Djungarian hamsters during cold adaptation. *Biochem. J.*, **316**, 607–613.

9. Gagnon,J., Kurowski,T.T., Wiesner,R.J. and Zak,R. (1991) Correlations between a nuclear and a mitochondrial mRNA of cytochrome c oxidase subunits, enzymatic activity and total mRNA content, in rat tissues. *Mol. Cell. Biochem.*, **107**, 21–29.
10. Cantatore,P., Flagella,Z., Fracasso,F., Lezza,A.M., Gadaleta,M.N. and de Montalvo,A. (1987) Synthesis and turnover rates of four rat liver mitochondrial RNA species. *FEBS Lett.*, **213**, 144–148.
11. Gadaleta,M.N., Di,R.N., Bove,G. and Saccone,C. (1975) Effects of triiodothyronine on rat-liver mitochondrial transcription process. *Eur. J. Biochem.*, **51**, 495–501.
12. Barsano,C.P., Degroot,L.J. and Getz,G.S. (1977) The effect of thyroid hormone on *in vitro* rat liver mitochondrial RNA synthesis. *Endocrinology*, **100**, 52–60.
13. VanItallie,C.M. (1992) Dexamethasone treatment increases mitochondrial RNA synthesis in a rat hepatoma cell line. *Endocrinology*, **130**, 567–576.
14. Shadel,G.S. and Clayton,D.A. (1997) Mitochondrial DNA maintenance in vertebrates. *Annu. Rev. Biochem.*, **66**, 409–435.
15. Falkenberg,M., Gaspari,M., Rantanen,A., Trifunovic,A., Larsson,N.G. and Gustafsson,C.M. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nature Genet.*, **31**, 289–294.
16. McCulloch,V., Seidel-Rogol,B.L. and Shadel,G.S. (2002) A human mitochondrial transcription factor is related to RNA adenine methyltransferases and binds S-adenosylmethionine. *Mol. Cell. Biol.*, **22**, 1116–1125.
17. Parisi,M.A., Xu,B. and Clayton,D.A. (1993) A human mitochondrial transcriptional activator can functionally replace a yeast mitochondrial HMG-box protein both *in vivo* and *in vitro*. *Mol. Cell. Biol.*, **13**, 1951–1961.
18. Dairaghi,D.J., Shadel,G.S. and Clayton,D.A. (1995) Addition of a 29 residue carboxyl-terminal tail converts a simple HMG box-containing protein into a transcriptional activator. *J. Mol. Biol.*, **249**, 11–28.
19. Larsson,N.G., Wang,J., Wilhelmsson,H., Oldfors,A., Rustin,P., Lewandoski,M., Barsh,G.S. and Clayton,D.A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genet.*, **18**, 231–236.
20. Wredenberg,A., Wibom,R., Wilhelmsson,H., Graff,C., Wiener,H.H., Burden,S.J., Oldfors,A., Westerblad,H. and Larsson,N.G. (2002) Increased mitochondrial mass in mitochondrial myopathy mice. *Proc. Natl Acad. Sci. USA*, **99**, 15066–15071.
21. Enriquez,J.A., Fernandez,S.P., Perez,M.A., Lopez,P.M. and Montoya,J. (1996) The synthesis of mRNA in isolated mitochondria can be maintained for several hours and is inhibited by high levels of ATP. *Eur. J. Biochem.*, **237**, 601–610.
22. Wiesner,R.J., Kurowski,T.T. and Zak,R. (1992) Regulation by thyroid hormone of nuclear and mitochondrial genes encoding subunits of cytochrome-c oxidase in rat liver and skeletal muscle. *Mol. Endocrinol.*, **6**, 1458–1467.
23. Enriquez,J.A., Fernandez,S.P., Garrido,P.N. and Montoya,J. (1999) Direct regulation of mitochondrial RNA synthesis by thyroid hormone. *Mol. Cell. Biol.*, **19**, 657–670.
24. Parisi,M.A. and Clayton,D.A. (1991) Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science*, **252**, 965–969.
25. Rittner,K., Stöppler,H., Pawlita,M. and Sczakiel,G. (1991) Versatile eucaryotic vectors for strong and constitutive transient and stable gene expression. *Methods Mol. Cell. Biol.*, **2**, 176–181.
26. Enriquez,J.A., Perez,M.A., Lopez,P.M. and Montoya,J. (1996) *In organello* RNA synthesis system from mammalian liver and brain. *Methods Enzymol.*, **264**, 50–57.
27. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
28. Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
29. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
31. Gensler,S., Weber,K., Schmitt,W.E., Perez-Martos,A., Enriquez,J.A., Montoya,J. and Wiesner,R.J. (2001) Mechanism of mammalian mitochondrial DNA replication: import of mitochondrial transcription factor A into isolated mitochondria stimulates 7S DNA synthesis. *Nucleic Acids Res.*, **29**, 3657–3663.
32. Montoya,J., Gaines,G.L. and Attardi,G. (1983) The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell*, **34**, 151–159.
33. Garstka,H.L., Facke,M., Escribano,J.R. and Wiesner,R.J. (1994) Stoichiometry of mitochondrial transcripts and regulation of gene expression by mitochondrial transcription factor A. *Biochem. Biophys. Res. Commun.*, **200**, 619–626.
34. Rodriguez-Pena,A., Escriva,H., Handler,A.C. and Vallejo,C.G. (2002) Thyroid hormone increases transcription of GA-binding protein/nuclear respiratory factor-2 alpha-subunit in rat liver. *FEBS Lett.*, **514**, 309–314.
35. Antoshechkin,I. and Bogenhagen,D.F. (1995) Distinct roles for two purified factors in transcription of *Xenopus* mitochondrial DNA. *Mol. Cell. Biol.*, **15**, 7032–7042.
36. Dairaghi,D.J., Shadel,G.S. and Clayton,D.A. (1995) Human mitochondrial transcription factor A and promoter spacing integrity are required for transcription initiation. *Biochim. Biophys. Acta*, **1271**, 127–134.
37. Kuhn,A., Voit,R., Stefanovsky,V., Evers,R., Bianchi,M. and Grummt,I. (1994) Functional differences between the two splice variants of the nucleolar transcription factor UBF: the second HMG box determines specificity of DNA binding and transcriptional activity. *EMBO J.*, **13**, 416–424.
38. Fisher,R.P., Lisowsky,T., Breen,G.A. and Clayton,D.A. (1991) A rapid, efficient method for purifying DNA-binding proteins. Denaturation-renaturation chromatography of human and yeast mitochondrial extracts. *J. Biol. Chem.*, **266**, 9153–9160.
39. Shen,E.L. and Bogenhagen,D.F. (2001) Developmentally-regulated packaging of mitochondrial DNA by the HMG-box protein mtTFA during *Xenopus* oogenesis. *Nucleic Acids Res.*, **29**, 2822–2828.
40. Diffley,J.F. and Stillman,B. (1992) DNA binding properties of an HMG1-related protein from yeast mitochondria. *J. Biol. Chem.*, **267**, 3368–3374.
41. Takamatsu,C., Umeda,S., Ohsato,T., Ohno,T., Abe,Y., Fukuoh,A., Shinagawa,H., Hamasaki,N. and Kang,D. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep.*, **3**, 451–456.
42. Spelbrink,J.N., Li,F.Y., Tiranti,V., Nikali,K., Yuan,Q.P., Tariq,M., Wanrooij,S., Garrido,N., Comi,G., Morandi,L., Santoro,L., Toscano,A., Fabrizi,G.M., Somer,H., Croxen,R., Beeson,D., Poulton,J., Suomalainen,A., Jacobs,H.T., Zeviani,M. and Larsson,C. (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nature Genet.*, **28**, 223–231.
43. Seidel-Rogol,B.L. and Shadel,G.S. (2002) Modulation of mitochondrial transcription in response to mtDNA depletion and repletion in HeLa cells. *Nucleic Acids Res.*, **30**, 1929–1934.
44. Montoya,J., Perez,M.A., Garstka,H.L. and Wiesner,R.J. (1997) Regulation of mitochondrial transcription by mitochondrial transcription factor A. *Mol. Cell. Biochem.*, **174**, 227–230
45. Fernandez-Silva,P., Enriquez,J.A. and Montoya,J. (2003) Replication and transcription of mammalian mitochondrial DNA. *Exp. Physiol.*, **88**, 41–56.
46. Alam,T.I., Kanki,T., Muta,T., Ukaji,K., Abe,Y., Nakayama,H., Takio,K., Hamasaki,N. and Kang,D. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res.*, **31**, 1640–1645.
47. Fisher,R.P., Topper,J.N. and Clayton,D.A. (1987) Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. *Cell*, **50**, 247–258.
48. Ghivizzani,S.C., Madsen,C.S. and Hauswirth,W.W. (1993) *In organello* footprinting. Analysis of protein binding at regulatory regions in bovine mitochondrial DNA. *J. Biol. Chem.*, **268**, 8675–8682.
49. Ghivizzani,S.C., Madsen,C.S., Nelen,M.R., Ammini,C.V. and Hauswirth,W.W. (1994) *In organello* footprint analysis of human mitochondrial DNA: human mitochondrial transcription factor A interactions at the origin of replication. *Mol. Cell. Biol.*, **14**, 7717–7730.
50. Micol,V., Fernandez,S.P. and Attardi,G. (1997) Functional analysis of *in vivo* and *in organello* footprinting of HeLa cell mitochondrial DNA in relationship to ATP and ethidium bromide effects on transcription. *J. Biol. Chem.*, **272**, 18896–18904.
51. Cantatore,P., Daddabbo,L., Fracasso,F. and Gadaleta,M.N. (1995) Identification by in organello footprinting of protein contact sites and of

single-stranded DNA sequences in the regulatory region of rat mitochondrial DNA. Protein binding sites and single-stranded DNA regions in isolated rat liver mitochondria. *J. Biol. Chem.*, **270**, 25020–25027.

52. Fisher,R.P., Parisi,M.A. and Clayton,D.A. (1989) Flexible recognition of rapidly evolving promoter sequences by mitochondrial transcription factor 1. *Genes Dev.*, **3**, 2202–2217.
53. Tominaga,K., Akiyama,S., Kagawa,Y. and Ohta,S. (1992) Upstream region of a genomic gene for human mitochondrial transcription factor 1. *Biochim. Biophys. Acta*, **1131**, 217–219.
54. Wrutniak,C., Cassar,M.I., Marchal,S., Rascle,A., Heusser,S., Keller,J.M., Flechon,J., Dauca,M., Samarut,J., Ghysdael,J. and Cabello,G. (1995) A 43-kDa protein related to c-Erb A alpha 1 is located in the mitochondrial matrix of rat liver. *J. Biol. Chem.*, **270**, 16347–16354.
55. Kanazawa,A., Nishio,Y., Kashiwagi,A., Inagaki,H., Kikkawa,R. and Horiike,K. (2002) Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart. *Am. J. Physiol. Endocrinol. Metab.*, **282**, E778–E785.