Mitochondrial Transcription Factor A and Its Downstream Targets Are Upregulated in a Rat Hepatoma*

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ABSTRACT

Mitochondrial transcription factor A is a key regulator involved in mitochondrial DNA (mtDNA) transcription and replication. In a poorly differentiated rat hepatoma, Morris hepatoma 3924A, the mRNA and protein levels of this factor were elevated about 10 and 11 fold, respectively, relative to the host liver. The mRNA levels for the hepatoma cytochrome c oxidase I, II, and NADH dehydrogenase 5, 6, the downstream targets of Tfam, were augmented 10, 8, 5, and 3 fold, respectively. Interestingly, Tfam was also found in the hepatoma nucleus. The mRNA levels for nuclear respiratory factor 1 and 2 (NRF-1, -2), the proteins that are known to interact with specific regulatory elements on human TFAM promoter, were 5 and 3 fold higher, respectively, in the hepatoma relative to the host liver. Unlike the human promoter, the rat Tfam promoter did not form a specific complex with the NRF-1 in the liver or hepatoma nuclear extracts, which is consistent with the absence of an NRF-1 consensus sequence in the proximal rat promoter. A single specific complex formed between the rat promoter and the NRF-2 protein was comparable in the two extracts. The DNA binding activity of Sp1 in the hepatoma nuclear extract was 4 fold greater than that in the liver extract. In vivo genomic footprinting showed occupancy of NRF-2 and Sp1 consensus sites on the promoter of rat Tfam gene. Tfam was also upregulated in other hepatoma cells. Together, these results show upregulation of Tfam in some tumors, particularly the liver tumors. Further, relatively high level of Sp1 binding to the
promoter in the hepatoma could play a major role in the upregulation of *Tfam* in these tumor cells.

**INTRODUCTION**

Mammalian cells contain two distinct genomes that are localized in nuclear and mitochondrial compartments, respectively. The maintenance of mitochondrial DNA (mtDNA) requires factors encoded by nuclear DNA. Unlike nuclear DNA, mtDNA contains limited genetic information. In fact, it encodes just 2 rRNAs, 22 tRNAs, and 13 polypeptides including cytochrome c oxidase I–III (COX I-III), NADH dehydrogenase subunits 1-6 (ND1-6), cytochrome b (Cytb), and ATPase 6, 8. These polypeptides are essential components of the mitochondrial electron transport chain (ETC). Consequently, the majority of the mitochondrial proteins, including most of the ETC subunits are encoded by the nuclear genome. It is, therefore, not surprising that the mammalian mitochondrial transcription is directed by a limited number of proteins. The mitochondrial transcription machinery is composed of at least two trans-acting factors: a core RNA polymerase and a dissociable transcription factor. RNA polymerase is relatively non-selective on the promoter sequence, while the dissociable factor confers the promoter specificity and transcription efficiency (1, 2). The human dissociable factor was later purified and cloned, designated human mitochondrial transcription factor A (TFAM) (3). The full-length cDNA of this nuclear gene encodes 246 amino acids of TFAM (precursor form). The very N-terminal 42 amino acids function as mitochondrial targeting signal and are cleaved during mitochondrial translocation. Thus the mature functional form of TFAM is comprised of 204 amino acids (24,400 daltons). TFAM is a member of high mobility group (HMG)-box protein family that
contains two HMG box DNA-binding domains. The HMG-box proteins in the nucleus are involved in transcription enhancement and chromatin packaging, and exhibit a dual DNA-binding specificity that includes recognition of unique DNA sequence and common DNA conformation (4). TFAM binds upstream of the transcriptional control elements in both heavy-strand and light-strand promoters (HSP and LSP) of mitochondrial DNA and initiates its transcription (5). The sequence specificity of TFAM binding is relatively less stringent since the sequences of these two promoters are only partially similar (2). The mouse Tfas−/− knockout embryos died at the early stage (E10.5) of embryonic development with mtDNA depletion and abolished oxidative phosphorylation (6). In Tfat+/− heterozygous knockout mice, the mtDNA copy number decreased by 34±7% in all tissues analysed, and the mitochondrial transcript levels were reduced by 22±10% in heart and kidney (6). This observation demonstrated that Tfas is essential for the mtDNA maintenance and embryonic development. Cloning and characterization of the mouse homologue of TFAM (Tfas) resulted in the identification of a nuclear counterpart of the protein (TS-HMG) in the mouse testis, but the physiological function of this nuclear isoform is not yet clear (7).

Since mitochondria provide the energy for the cellular processes including cell growth, proliferation, it is not uncommon that the alterations of mitochondrial gene expression often occur in tumors (8, 9). Nuclear respiratory factors (NRF-1 and NRF-2) are the two major trans-acting factors that have been suggested to play a key role in the transcription of human TFAM gene (10, 11). As the name implies these two proteins are also involved in the expression of several human and rodent cytochrome oxidase subunits (10) that are important for cellular respiration. NRF-1 binds DNA as a homodimer, which is the active form of the factor. The activation domain resides in the C-terminal end of the protein downstream from the DNA
binding domain (12) that does not belong to any known domain classes. On the other hand, NRF-2 is a multisubunit protein that can bind to GGAA sequence motif in the human TFAM promoter and activate its transcription activity as demonstrated by in vitro transcription or in vivo transfection studies (11). NRF-2 is composed of five subunits: \( \alpha, \beta_1, \beta_2, \gamma_1, \gamma_2 \), of which the \( \alpha \) subunit contains the DNA binding domain (ETS domain). Initially, the sequence analysis of human TFAM promoter showed the absence of a typical TATA box in this promoter (13). Later, the promoter of human TFAM gene was characterized by mutational analysis, and the finding showed that there are at least three DNA-binding motifs in the proximal promoter: NRF-1, NRF-2, and Sp1 (11). The promoter sequence alignments showed that the mouse and rat Tfam promoters also contain well-conserved Sp1 and NRF-2 recognition sites, but neither of them exhibited the consensus binding site for NRF-1 (14).

Mitochondria play very important roles in cellular metabolism, generation of reactive oxygen species, and apoptosis (15, 16). Since TFAM is coded by nuclear DNA but controls the synthesis of mitochondrial respiratory chain components, this protein has been suggested to play the role of a key mediator between nuclear and mitochondrial genomes (11). For its dual role in replication as well as transcription of mtDNA, it was of considerable interest to investigate the role of Tfam in a rapidly growing tumor. Here, we explored the level of expression, localization, and regulation of Tfam gene, and its effect on the downstream target genes in the rat liver and Morris hepatoma transplanted into the same animal.
EXPERIMENTAL PROCEDURES

Maintenance of Morris Hepatoma 3924A. Morris hepatoma 3924A is a poorly differentiated, rapidly growing tumor. The tumor was maintained in the hind leg of rats (ACI strain) as described previously (17).

cDNA Library Construction and Screening. To isolate the full-length cDNA of rat Tfam, we constructed rat Morris hepatoma 3924A cDNA library using the cDNA synthesis kit and Gigapack III Gold Packaging Extract (Stratagene). Briefly, total RNA was isolated from the hepatoma by the single-step method (18), and the poly(A)+ RNA was isolated from the total RNA using the PolyATtract mRNA isolation system (Promega). The cDNA synthesis and packaging reactions were performed as described in the manufacturer’s protocol (Stratagene). The cDNA library screening was carried out following the standard protocol (19) using KpnI/PstI fragment of mouse Tfam cDNA (pN26 clone, a generous gift from Dr. Nils-Göran Larsson, Karolinska Institute, Stockholm, Sweden) as the probe. Ten positive clones (pHepTFA #1-#10) were picked randomly and sequenced, and the DNA sequence was analyzed using the MacVector software (Oxford Molecular Group PLC).

5' Rapid Amplification of cDNA Ends (5' RACE). Since the clones isolated from the rat hepatoma cDNA library lack 5'-end sequence, we performed 5' RACE to complete the cDNA full-length sequence using the 5' RACE System (Life Technologies). Briefly, the first strand of cDNA was synthesized from poly(A)+ RNA of Morris hepatoma 3924A using rat Tfam gene-specific primer Hep-GSP1 (5'-GTACACCTCCACTCAG-3'), and was purified using the GlassMax DNA isolation spin cartridge supplied with the kit. Following oligo-dC tail addition by TdT enzyme, PCR amplification was performed to amplify the specific product with the Abridged
Anchor Primer (AAP: 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGIIG) and a gene-specific primer Hep-GSP2 (5'-AGCTCCCTCCACATGGCTGCAAT). The primary PCR product was re-amplified with an Abridged Universal Amplification Primer (AUAP: 5'-GGCCACGCGTCGACTAGTAC-3') and a nested gene-specific primer Hep-GSP4 (5'-TCAGTTCTGAAACTTTTGCATCTGGGTG-3'). The amplified 5' cDNA end was confirmed by a nested PCR reaction with the primer Hep-GSP2 and another nested gene-specific primer Hep-GSP3 (5'-TGTATTCCGAAGTGTTTTTCCAGCTTG-3'). The 5' RACE cDNAs were sequenced and subcloned into the hepatoma cDNA clone pHepTFA#1 to make a full-length cDNA clone designated pHepTFA-FL.

**Purification of Recombinant Rat Hepatoma Tfam.** The coding region corresponding to the mature form of the rat hepatoma Tfam (without the mitochondrial targeting signal peptide) was obtained by PCR amplification of the above Tfam full-length cDNA clone pHepTFA-FL with the primers Hep-ExF2 (5'-CGGGATCCAGCTTGGGTAATTATCCA-3') and Hep-ExB1 (5'-GGGGTACCAATGACAACTCTGTCTTCAATC-3'). The amplified DNA was then subcloned into the sites BamHI/KpnI of bacterial expression vector pQE30 (Qiagen), which contains 6-histidine tag sequence at the 5' end. The recombinant plasmid was transformed into M15 bacteria. The expression of the recombinant Tfam protein was induced with 1mM IPTG added to the bacterial culture in log phase and was grown for an additional 4 hours. The protein was purified by chromatography on Ni-NTA Sepharose column under denaturing conditions as described by the manufacturer (Qiagen).

**Generation of Antibodies Against rat Tfam.** Purified recombinant Tfam (1 mg) was separated on 12% SDS-polyacrylamide gel. The protein was visualized by staining with ice-cold 0.25 M KCl for 5 min. The gel was then rinsed with cold ddH2O, and the protein band was cut out and rinsed
again with cold ddH₂O. The gel slice containing Tfam was fragmented by passing through two 5-ml syringes, and the protein was resuspended in 2 ml of PBS buffer. Prior to injection into rabbits, 1 ml of antigen solution was mixed with 1 ml of complete Freund’s adjuvant (CFA, Sigma) to prepare the emulsion. The standard procedure (20) for immunization was followed. The antibody specificity was determined by Western blot analysis.

**Isolation of RNA, Northern blot and RT-PCR Analyses.** The procedure for poly(A)^+ mRNA isolation was as described above. For Northern blot analysis, 5 µg of poly(A)^+ mRNA was separated on 1.2% formaldehyde-agarose gel and transferred to Hybond N+ membrane (Amersham)). The membrane was hybridized with [α-³²P]dCTP-labeled mouse Tfam cDNA (KpnI/PstI fragment) from pN26 clone and MBD2 cDNA (PCR amplified with the primers: MBD2-F: 5′-GCTGTTGACCTTAGCAGTTTTGAC-3′, MBD2-B: 5′-TTACGCCTCATCTCCTGCTG-3′) sequentially. Then the membrane was exposed to X-ray film (Kodak). For RT-PCR analysis, the 20 ng of each poly(A)^+ mRNA sample was used for reverse transcription. The PCR reactions were carried out under the following conditions: 4 min at 94 °C; specified cycles of 30 sec at 94 °C, 30 sec at particular annealing temperature, and 1 min at 72 °C for each gene (see Table 1). The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide, the signal was quantified by Kodak Digital Science™ 1D image analysis software (Eastman Kodak Company).

**Subcellular Fractionation, immunoprecipitation and Western Blot Analysis.** The rat liver and hepatoma nuclei were isolated as described (17). The mitochondria from rat liver and hepatoma were isolated as described (21, 22) with some modifications. Briefly, rat livers were homogenized in the mitochondria isolation medium (225 mM Mannitol, 75 mM sucrose, 500
mM EDTA, 2 mM MOPS, pH 7.4). The homogenate was centrifuged at 800 × g for 10 min, and the supernatant thus obtained was centrifuged again at 800 × g for 10 min. The mitochondrial supernatant was centrifuged at 6,500 × g for 15 min to sediment the crude mitochondrial pellet. The mitochondrial pellet was resuspended in a small volume of the isolation medium, disaggregated using a Dounce homogenizer, and centrifuged at 6,500 × g for 10 min. The above wash step was repeated once. The mitochondrial pellet was resuspended in the isolation medium and then layered onto a step gradient containing 15-ml of 1.5 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA and a 15-ml of 1.0 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA. The gradient was centrifuged at 80,000 × g for 1 hr and the phase between the layers of 1.0 M sucrose and 1.5 M sucrose was collected. For the preparation of hepatoma mitochondria, the tissue was homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose and 1 mM EDTA. The crude mitochondria were resuspended in sucrose-TE buffer (585 mM sucrose, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA) before it was layered on sucrose gradient. The gradient was then centrifuged, the mitochondrial layer was collected as above, and the purified mitochondria were resuspended in mitochondrial lysis buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 mM DTT, 1mM PMSF, 15% glycerol, 0.1 µg/ml leupeptin, 0.1 µg/ml pepstatin A). For further purification, Triton X-100 was added to a final concentration of 0.5%, the suspension was homogenized with a tightly fitting motor-driven Teflon pestle, 4 M KCl was then added to a final concentration of 0.35 M, and the homogenization was repeated. The mitochondrial lysate was centrifuged for 60 min at 130,000 × g in a Ti 70 rotor (Beckman) and the supernatant (mitochondrial extract) was carefully removed and saved for analyses.

The immunoprecipitation was performed according to the procedure described by Harlow and Lane (23). Briefly, the purified nuclei were resuspended in RIPA lysis buffer (150 mM sodium
chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A), incubated on ice for 30 min and sonicated briefly to shear the DNA. The lysate was centrifuged for 10 min at 10,000 × g at 4 °C. Either the preimmune serum or rat Tfam antiserum was added to aliquots of the lysate (0.5 ml per aliquot) after the lysate was precleared with normal rabbit serum. The immune complexes were collected by adding protein A-Sepharose beads (Life Technologies) and analyzed by SDS-PAGE followed by Western blotting.

For Western blot analysis of Tfam protein, the protein samples were resolved on the SDS-PAGE and transferred to ECL membrane (Amersham). The blot was incubated with rabbit anti-rat Tfam serum (1:6000) for 1 h at room temperature, followed by donkey anti-rabbit IgG-peroxidase conjugate (Amersham). The detection was performed with ECL™ Western Blotting Detection Reagents (Amersham) following the manufacturer’s protocol. For internal control, the blot was reprobed with mouse monoclonal anti-COX I antibodies at 0.4 µg/ml (Molecular Probes), followed by sheep anti-mouse IgG-peroxidase conjugate (Amersham) and ECL™ detection. The quantitation of protein amount was performed using a densitometer (Shimadzu). For Western blot analysis of NRF-1, the blot was incubated with rabbit anti-NRF-1 serum (1:6000) for 1 h at room temperature, followed by donkey anti-rabbit IgG-peroxidase conjugate (Amersham). The detection was performed as described above. For internal control, the blot was reprobed with mouse monoclonal anti-Ku70 antibodies (Neomarker, 1:1000), followed by sheep anti-mouse IgG-peroxidase conjugate (Amersham) and ECL™ detection.

*Electrophoretic Mobility Shift Assay (EMSA).* The DNA binding reactions for NRFs were performed as described previously (24). Briefly, oligonucleotides were labeled with [γ-32P]ATP by using polynucleotide kinase, then annealed to double-strand oligonucleotides. Binding
reactions were carried out in 20 µl of reaction mixture containing 20 µg of nuclear extract, 4 µg of poly(dI-dC), 0.2 ng of labeled oligonucleotide, 25 mM Tris, pH 7.9, 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM KCl, and 10% (vol/vol) glycerol. For competition, specified molar excess of unlabeled oligonucleotide was incubated with the extract at RT for 15 min prior to the addition of labeled oligonucleotide. For supershift assays, 1 µl of antiserum was added first and incubated for 30 min prior to addition of labeled oligonucleotide. The binding reactions were incubated at RT for 15 min. The samples were electrophoresed on 5% polyacrylamide gel (acrylamide : bis = 58 : 1) in 0.5 × TBE for 2.5 hr at 10V/cm. The gel was dried and then exposed to a PhosphorImager screen.

The binding reaction for Sp1 was performed as described (17). Briefly, Sp1 consensus oligonucleotide was labeled with [γ-32P]ATP by using polynucleotide kinase, and then annealed into double-strand oligonucleotides. For the binding reaction, 10 µg of nuclear extract was incubated with 0.2 ng of labeled oligonucleotide in the buffer containing 2 µg of poly(dI-dC), 10 mM Heps, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 10% glycerol. For competition, the extract was incubated with 100 fold molar excess of unlabeled Sp1 consensus or mutant oligonucleotide for 15 min on ice prior to the addition of labeled Sp1 consensus oligonucleotide. The binding reaction was incubated on ice for 30 min, and electrophoresed on 4% polyacrylamide gel (acrylamide:bis = 38.7:1.3) in 0.25 × TBE buffer. The gel was then dried and exposed to X-ray film (Kodak).

The DNA sequences of oligonucleotides used for the EMSA were as follows:

RC4 -173/-147: 5'-GATCATGCTAGCCCGCATGCGCGCACCTT-3'

3'-TACGATCGGGCGTACGCGCGCGTGGAATCGA-5'

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In vivo Genomic Footprinting. The procedure was essentially as described (17). Briefly, nuclei were isolated as described above. For in vivo footprinting, the nuclei (1 × 10⁸) were treated with 0.2% dimethylsulfate (DMS) for 2 min at RT followed by 3 washes in ice-cold PBS. Then the nuclei were resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂ followed by addition of an equal volume of 2X TNESK solution (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM EDTA, 2% SDS, 200 μg/ml proteinase K). The lysis of nuclei was carried out for overnight at RT. Genomic DNA was then isolated following the standard protocol. Meanwhile, genomic DNA was also isolated from control nuclei that were not treated with DMS. Then the control
DNA samples were treated with 0.2% DMS for 2 min at RT, immediately followed by addition of DMS stop solution (1.5 M Sodium acetate, pH 7.0, 1 M β-mercaptoethanol, 10 µg yeast tRNA) and 3 volume of cold absolute ethanol. The DMS-treated DNA samples were cleaved by piperidine for 30 min at 90 °C, followed by five times of lyophilization in a Speed Vac without heat. Then the DNA pellets were dissolved in dH2O, and precipitated by addition of 0.1 vol. of 3 M sodium acetate, pH 7.0, 10 µg yeast tRNA, and 2.5 vol. absolute ethanol for 15 min at –20 °C. Finally, the DNA pellets were dissolved in TE buffer, and quantified by measuring OD$_{260}$ absorbance.

For reactions of ligation-mediated PCR (LM-PCR), amplification, and labeling, two sets of three nested primers were designed following the criteria optimal for IVGF. The minus-strand primers are: TFAFP3'-1: 5'-CCTACACACAGCCACGAAAC-3' (annealing temp: 57 °C), TFAFP3'-2: 5'-GGTACTCCAGGGGGCTTGTTATC-3' (annealing temp: 59 °C), TFAFP3'-3: 5'-CTCCAGGGGCTTGTTATCATG-3' (annealing temp: 63 °C). The plus-strand primers are: TFAFP5'-1: 5'-CCTTCCAGCAGAATACTCAGAG-3' (annealing temp: 56 °C), TFAFP5'-2: 5'-AGCAACACCCTTGCCAAAC-3' (annealing temp: 59 °C), TFAFP5'-3: 5'-CAACACCCTTGCCAAACTAAAAGCG-3' (annealing temp: 64 °C). Each sample of 2.5 µg of DNA was used for the reaction that was performed as described previously (17). The reactions were resolved on 6% sequencing gel, dried, and then exposed to PhosphorImager screen.

**Immunocytochemistry and Microscopy.** The hepatoma nuclei prepared as above were used for immunocytochemistry in order to remove the mitochondrial background. The immunofluorescence procedure was essentially as described by Iborra et al (25). Briefly, the
nuclei purified by centrifuging through sucrose cushion twice and washing with the nuclear wash buffer (0.34 M sucrose, 1 mM MgCl$_2$, 0.3% Triton X-100, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A) were resuspended in PBS containing 100 mg/ml BSA, then fixed with 4% paraformaldehyde on coverslips for 15 min at 4 °C, followed by 8% paraformaldehyde for 20 min at room temperature. The fixed nuclei were then treated with 0.3% Triton X-100. The coverslips were blocked with 1% BSA for 1 hr at room temperature, then incubated with affinity purified polyclonal rabbit anti-Tfam (1 µg/ml) and mouse anti-COX I (2 µg/ml, Molecular Probes) antibodies for 1 hr at room temperature. After three washes in PBS, the coverslips were incubated with Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, 1:200) and FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, 1:200) for 1 hr at room temperature. After three more washes with PBS, the coverslips were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). The specificity of staining was assessed with three different controls: 1) substitution of the primary antibody with normal rabbit IgG (1 µg/ml, Sigma), 2) omission of the primary antibody, and 3) using the nuclei not washed with the nuclear wash buffer as a positive control for COX I detection. Standard microscopy was performed using Nikon E800 microscope equipped with HiQ FITC and TRITC/DAPI dual wavelength filter sets (Chroma Technology) and nuclei were photographed under the ×60 oil-immersion lens. The immunostained pure nuclei were also examined under a Bio-Rad MRC-600 confocal microscope and photographed under the ×60 oil-immersion lens. The z-series images were captured and converted into TIFF files using the Confocal Assistant™ software (Bio-Rad). The confocal microscopy was performed at the Campus Microscopy and Imaging Facility at the Ohio State University.
Citrate Synthase Assay. The citrate synthase was used as a mitochondrial marker to check the purity of rat hepatoma nuclear extract. The assay was performed as described by Srere and Kosicki (26). Briefly, the assay mixture contained 67 mM Tris-HCl, pH 8.0, 0.4 mM oxaloacetic acid (OAA), and 0.15 mM acetyl-CoA in a final volume of 1.5 ml. The reaction was initiated by adding specified amount of either nuclear or mitochondrial extract. The absorbance at 233 nm was measured kinetically using a Beckman DU 640B spectrophotometer.

RESULTS

Tfam Gene Expression Is Upregulated in the Rat Hepatoma

Since Morris hepatoma 3924A is a rapidly growing tumor, we examined the mRNA level of Tfam in the hepatoma and compared with that of the host liver. Northern blot analysis with mouse Tfam as a probe suggested higher level (~8 fold) of Tfam expression in the hepatoma relative to the host liver (Fig. 1A). To confirm and extend this observation, we studied the role of Tfam in the mitochondrial function in this tumor (see Experimental Procedures for the generation of this solid tumor). First, we constructed the rat hepatoma cDNA library and generated Tfam cDNA clone. The full-length rat Tfam cDNA clone was nearly identical to the Tfam entry (accession no. AB014089) in the GenBank. We then overexpressed Tfam in the bacteria and purified the recombinant protein by affinity chromatography. The recombinant mature form of Tfam was ~28 kDa on SDS-polyacrylamide gel, which is slightly greater than the molecular size of the recombinant mouse Tfam (7). Polyclonal antibodies generated against the
recombinant Tfam reacted specifically with rat, mouse, and human Tfam in a Western blot analysis (data not shown). Western blot analysis showed significantly higher Tfam protein level in rat hepatoma (about 11 fold) relative to the host liver (Figure 1B, C). These data suggest that the transcription of its gene and/or its translation is elevated in the hepatoma. To semi-quantify the expression of its gene at mRNA level in the hepatoma, RT-PCR analysis was performed using poly(A)^+ RNA with gene-specific primers. The data showed that the steady state level of Tfam transcripts was at least 10 fold higher in the hepatoma than that in the liver (Figure 1D, E), implying that Tfam gene is upregulated at the transcriptional level or its mRNA is relatively more stable in the hepatoma compared to the host liver.

*Expressions of the Regulatory Factors of Tfam Gene Are Upregulated in the Hepatoma*

Since the Tfam gene expression is significantly elevated in the rat hepatoma relative to the host liver, we predicted that the key regulatory factors such as NRF-1 and NRF-2 involved in humanTFAM gene transcription are likely to be upregulated in the hepatoma. The steady-state levels of NRF-1 and NRF-2 mRNAs were analysed by semi-quantitative RT-PCR. As shown in Figure 2A, NRF-1 and NRF-2 mRNA levels increased about 5 and 3 fold, respectively, in the hepatoma compared to the levels in control liver. We then determined the NRF-1 protein level in the liver and tumor using specific antibodies against this protein. The NRF-1 protein level was at least three fold higher than that in the host liver, which suggests transcriptional control of NRF-1 gene expression as well in the tumor (Figure 2B). NRF-1 is known to undergo phosphorylation, and serine phosphorylation in the amino-terminal domain can enhance DNA binding activity (27). It was present predominantly in the phosphorylated state (the upper band in the Fig. 2B) in
both liver and hepatoma nuclei. NRF-2 protein level was not measured due to unavailability of specific antibodies.

**DNA Binding Activities of the Transcription Factors NRF-1 and Sp1 But Not of NRF-2 Are Augmented in the Hepatoma**

Human TFAM promoter is known to contain three major DNA-binding elements (11). It was of interest to determine the conservation of these sequence elements between rat and human TFAM gene promoters. The sequence alignment showed well conserved binding sites for Sp1 and NRF-2, but no consensus NRF-1 binding site was observed in the proximal rat Tfam promoter (Figure 3A). Further analysis of rat Tfam promoter sequence of −462 to +100 (the longest promoter sequence we can get from the GenBank database) could not reveal any NRF-1 consensus sequence.Interestingly, sequence analysis revealed two other Sp1 binding sites upstream of the conserved Sp1 site in the rat Tfam promoter. For the sake of convenience, these three Sp1 sites are referred to as Sp1-A (−64 to −57), Sp1-B (−108 to −101), and Sp1-C (−121 to −116), respectively, in the order from 3' to 5' orientation. To measure the DNA binding activity of these factors in the rat hepatoma and host liver, electrophoretic mobility shift assay was performed in the nuclear extracts prepared from the hepatoma and host liver. We used ³²P labeled Sp1 consensus oligonucleotide as a probe for measuring Sp1 DNA binding activity. Two Sp1 complexes were formed with either the liver or the hepatoma nuclear extract (Fig. 3B). These complexes could be disrupted by the 100 fold molar excess of unlabeled Sp1 consensus but not mutant oligonucleotide, which confirmed the specificity of the complex formation. The Sp1 DNA binding activity was four fold higher in the hepatoma relative to the liver.
We also measured NRF-1 and NRF-2 binding activities in the rat hepatoma and host liver nuclear extracts. For measurement of the DNA binding activity of NRF-2, the nuclear extracts prepared from liver and hepatoma were incubated with \(^{32}\)P labeled NRF-2 oligonucleotide corresponding to the human TFAM promoter (hTFAM –34/-13). The DNA binding activity of NRF-2 was comparable in the liver and hepatoma (compare lanes 1 and 2 in Fig. 3C). Two complexes were formed with human \(TFAM\) promoter sequence. Both complexes could be competed by 50 fold molar excess of unlabeled \(TFAM\) promoter oligonucleotide (Fig. 3C, lanes 3 and 4). Interestingly, only the upper complex could be competed by the oligonucleotide corresponding to NRF-2 element in the rat \(Tfam\) promoter at a concentration as low as 50 fold molar excess (Fig. 3C, 7-10). The lower complex was not competed even at 100 fold molar excess of rat NRF-2 oligonucleotide. Thus, only one NRF-2 complex can be formed with the rat \(Tfam\) promoter sequence although NRF-2 forms two major complexes with human \(TFAM\) promoter sequence. The specificity of the NRF-2 complexes was confirmed further by the lack of competition with two unrelated oligonucleotides: the NRF-1 element of rat somatic cytochrome c gene (RC4 –173/-147) and rat \(Tfam\) –95/-70, the sequence that aligned with the human NRF-1 element (lanes 5, 6, 11, and 12 in Fig.3C).

For NRF-1 EMSA, similar assay was performed using the rat hepatoma and liver nuclear extracts and the NRF-1 element from \(RC4\) promoter (-173/-147) as a probe. The DNA binding activity of NRF-1 in the hepatoma was about 4 fold higher than that in the liver (Fig. 3D, compare lanes 2 and 3). The specific complex could be competed by 50 fold molar excess of unlabeled NRF-1 element of an unrelated promoter, RC4 –173/-147 (lanes 4 and 5). The complex could not, however, be competed out with 100 fold molar excess of rat \(Tfam\) promoter-based oligonucleotide (rTfam –52/-27) that corresponds to NRF-1 element located in the human.
TFAM promoter (based on a sequence alignment). This observation suggests that unlike the corresponding region in the human TFAM promoter, rat Tfam –52/-27 sequence does not harbor any NRF-1 binding site. The DNA binding data show that all the three major trans-acting factors known to be involved in human TFAM gene expression are abundant in the hepatoma and may play critical role in the upregulation of Tfam expression in the rat hepatoma.

NRF-2 and Sp1 Binding Sites on Rat Tfam Promoter Are Occupied in vivo

To explore the involvement of NRF-1, -2 and Sp1 in rat Tfam promoter activation in vivo, we performed in vivo genomic footprinting. We designed rat Tfam gene specific primers for LM-PCR that would allow us to analyze the proximal promoter region (-141 to –3 bp with respect to transcription start site). Nuclei isolated from rat liver and Morris hepatoma were subjected to dimethyl sulfate treatment, genomic DNA was isolated and cleaved with piperidine, and Tfam promoter region was amplified as described in Experimental Procedures. Naked genomic DNA from both liver and hepatoma was also treated in a similar manner as intact nuclei and similar PCR reactions were performed to provide the genomic G ladder. DNA-protein interaction protecting a G residue at a cis-element was shown as a less intense band on the sequencing gel, whereas more intense bands indicate hypersensitive G residues due to factor binding when compared with naked DNA ladder. The footprinting data of the upper strand of hepatoma Tfam promoter showed that two G residues in NRF-2 consensus binding site (-42 GGAA –39) were protected by factor binding. Four G residues in and adjacent to NRF2 binding sites became hypersensitive of which two are at the 3' end boundary, one inside the binding site, and another is at the 5' end boundary (Fig. 4B). Distinct footprinting was also observed at Sp1-A site as shown
in the Fig. 4B. One G residue was protected in the Sp1-A binding site (-64 GCCCGCC –57), and two G residues were hypersensitive of which one is in the Sp1 consensus sequence, and another at the immediate upstream of the Sp1-A site. The footprinting analysis of the lower strand detected two protected sites (underlined G residues) at the Sp1-A (-57 GGCGGGGC –64) and Sp1-B (-101 GGCGGGGC –108) sites, respectively (Fig. 4A and B). A few hypersensitive G residues were also observed on the lower strand. Three of them are located at the Sp1-C site (-116 GGGCGG –121). Two other hypersensitive G residues are located at the 5' end boundary of Sp1-A and Sp1-B binding site, respectively (Fig. 4A and B). No footprint was observed in the sequence from –70 to –95 bp on rat Tfam promoter, which corresponds to the NRF-1 site in the human TFAM promoter based on the alignment of these two promoter sequences (see Fig. 3A). This is consistent with the electrophoretic mobility shift data where oligonucleotide corresponding to putative rat NRF-1 element could not interact with the NRF-1 protein (Fig. 3D). These data suggest the probable involvement of NRF-2 and Sp1 in the rat Tfam gene expression.

The Expressions of Mitochondrial Genes Regulated by Tfam Are Upregulated in the Rat Hepatoma

Because Tfam is the major regulator of mitochondrial genome transcription in mammalian cells, it is likely that the mitochondria-encoded genes are upregulated in the rat hepatoma in response to enhanced Tfam expression. RT-PCR analyses were performed to explore the differential expression of the mitochondrial genes such as COX I, II, ND1, 5, 6, and ATPase 6 in the hepatoma and liver. Among these genes, the expressions of COXI, II and ND5 were elevated 10,
8, and 5 fold, respectively, in the hepatoma whereas the expression of ND6 was moderately high (3 fold increase). On the other hand, the mRNA levels of ND1 and ATPase 6 were almost identical in the tumor and liver (Figure 5). These data show that several proteins coded by mtDNA are upregulated in the hepatoma at the mRNA level. This observation is consistent with the upregulation of Tfam in the tumor.

*Increased Expression of Tfam Occurs in Other Rodent Hepatoma Cells*

To determine whether the upregulation of *Tfam* gene expression is a common event in the hepatoma cells, we analyzed both mRNA and protein levels of Tfam in two other lines of rodent hepatomas: rat H4-II-E-C3 and mouse Hepa cells. We performed RT-PCR analysis of the Tfam gene expression using the same set of primers and identical PCR conditions for both cell lines and control liver tissue. The RT-PCR data showed increased Tfam mRNA levels in both tumor cells compared to the corresponding normal liver tissue. Interestingly, the mouse liver exhibited higher basal expression of Tfam than rat liver (Figure 6A). The elevated expression of Tfam was also observed at the protein level in the hepatoma cells as shown by Western blot analysis (Figure 6B). It was also noted that the molecular size of rat Tfam (~28 kDa) is slightly larger than that of mouse Tfam (~25 kDa). The molecular weight difference is probably due to the amino acid composition. These data show that the upregulation of Tfam expression may be a common phenomenon in at least hepatoma cells.
Tfam Exhibits Two Subcellular Locations in the Rat Hepatoma

In mouse, two forms of Tfam have been observed, one is the mitochondrial form and the other one is a testis-specific nuclear form (7). Overexpression of this protein in the hepatoma prompted us to investigate its subcellular localization in this tumor tissue. To determine the subcellular distribution, pure nuclear and mitochondrial fractions from both tissues were isolated followed by Western blot analysis of the proteins in the two subcellular fractions using anti-Tfam antibodies. Tfam was indeed present in the hepatoma nuclei but not in the liver nuclei (Fig. 7A). We also performed immunoprecipitation with anti-Tfam antibody using liver and hepatoma nuclear extract. Western blot analysis of the protein immunoprecipitated with anti-Tfam antibody (Fig. 7B) showed that Tfam was pulled down selectively from the hepatoma nuclear extract which further confirmed enrichment of Tfam only in the hepatoma nuclear extract. To rule out the possibility of mitochondrial contamination of the nuclear extract, we reprobed the same blot with the antibody against the mitochondrial protein COX I. No COX I was detected in the hepatoma nuclear extract, although it was very abundant in both liver and hepatoma mitochondria (Fig. 7A). We also assayed the activity of citrate synthase in the hepatoma nuclear and mitochondrial extracts, as the citrate synthase localized in the mitochondrial matrix is commonly used as a mitochondrial marker. The citrate synthase activity increased with increasing protein concentration when assayed with the hepatoma mitochondrial extract but was barely detectable in the nuclear extract prepared from the same tissue (Fig. 7C). These results further confirmed expression of an isoform of mitochondrial Tfam in the hepatoma nucleus.

To demonstrate further this unique phenomenon of dual subcellular localization of Tfam in the rat hepatoma, we performed immunocytochemistry using isolated nuclei to eliminate the mitochondrial background. The nuclei were purified by passing twice through sucrose cushion
followed by washing with the nuclear wash buffer containing 0.3% Triton X-100 to get rid of all mitochondrial contaminants. Fluorescence microscopy showed that the nuclei were stained with anti-Tfam IgG (Fig. 8A, panel a) but not by normal rabbit IgG (panel c), which suggested the specificity of immunostaining. To confirm that the observed nuclear immunostaining was indeed due to nuclear Tfam, we used antibodies specific for a mitochondrial marker enzyme, COX I. The negative staining with the monoclonal anti-COX I antibody ruled out again the possibility of any mitochondrial contamination (panels b and d). This finding strongly favors dual locations of Tfam in the rat hepatoma. To examine further the distribution of Tfam within the nucleus, confocal microscopy was performed with pure nuclei. With the aid of analysis software (Bio-Rad Confocal Assistant™ software), the serial sections were captured from the top to the bottom of the nucleus. The equatorial view indeed demonstrated localization of Tfam in the nucleus, and its general distribution throughout the nucleus (Fig. 8B).

**DISCUSSION**

Mitochondria are involved in multiple cellular processes such as energy metabolism, apoptosis, and generation of reactive oxygen species (ROS). The alteration of mitochondrial functions has been reported in a variety of tumors, and is believed to play an important role in tumorigenesis and tumor proliferation (8, 28-32). HeLa cells devoid of mitochondria (rho⁰) lose the capability to form tumors when injected subcutaneously in nude mice. The tumorigenicity was restored after the normal human fibroblast mtDNA was introduced (33). The rho⁰ cells derived from human glioblastomas and breast cancer cells lose the capacity for anchorage-independent growth, and this capability was restored upon introduction of the normal DNA-containing
mitochondria (34). As a key mitochondrial transcription factor, Tfam is essential for mitochondrial biogenesis. The $Tfam^{-/-}$ knockout embryos die at the early embryonic stage with severe mtDNA depletion. The heterozygous $Tfam^{+/+}$ knockout mice can survive but exhibit reduced number of mtDNA and decreased mitochondrial gene expression in several organs (6). In the fast growing tumors like Morris Hepatoma 3924A (doubling time: 4-5 days; tumor growth: attaining 15-20 g within 4-5 weeks), not only more energy is needed to meet the rapid proliferation rate, but very active mitogenesis is required to maintain relatively stable number of mitochondria in the tumor cells. The present study revealed a significant increase in the overall expression of Tfam in the hepatoma (about 10 and 11 fold in the levels of mRNA and protein relative to the liver, respectively). The increased expression of Tfam was also observed in two other hepatoma cell lines, H4-II-E-C3 and Hepa. These data suggest a common biochemical phenotype with respect to interaction between nucleus and mitochondria in the hepatoma cells or perhaps in rapidly growing tumors.

The increased expression of Tfam implies that the hepatoma contains critical factors in the nuclei that can markedly activate the promoter of $Tfam$. Nuclear respiratory factors (NRF-1 and NRF-2) have been suggested to play a key role in the transcription of human $TFAM$ (10, 11). The increase in mRNA and protein levels for NRF-1 as well as its binding to the respective cis element and the increase in mRNA level for NRF-2 in the rat hepatoma relative to host liver (Figure 2) are consistent with this notion. The augmented expression of these transcription factors may be responsible for the upregulation of $Tfam$ gene in the hepatoma. Sp1 is involved in the transcriptional activation of numerous genes. The glutamine-rich activation domains of Sp1 are very important for protein-protein interaction. In Huntington's disease, this interaction could
be disrupted by the mutant huntingtin that contains an expanded glutamine tract (35). Specifically, this mutant protein associates with Sp1 and TAFII130 and interferes with the interaction of the latter two proteins. In this study, we found that the binding of Sp1 to the cis element was also elevated in the hepatoma relative to the host liver, which suggests the contribution of Sp1 to the transcription activity of Tfam gene in the rat hepatoma. These findings agree with the mutational analysis of the TFAM promoter, which showed 4.4-fold reduction in the reporter activity of human TFAM promoter following mutations in the Sp1 site (11).

Binding of NRF-1 to its consensus element was not competed by the rat Tfam promoter sequence –95/-70 (rTfam –95/-70) which is located in the similar position on the promoter to the human NRF-1 element by sequence alignment analysis (Fig. 3D). Moreover, no conserved NRF-1 recognition site was observed in the rat Tfam promoter spanning from –1 to -132 (Fig. 3A).

The in vivo genomic footprinting data further suggested that NRF-2 and Sp1 are probably involved in the regulation of Tfam gene. To our knowledge, this is the first report regarding the occupancy of the binding sites of these transcription factors on Tfam promoter in the chromatin context. The NRF-2 recognition site contains the consensus GGAA motif, and the G residues (underlined) in the motif were significantly protected on the rat Tfam promoter in vivo, which is consistent with the methylation interference footprinting for NRF-2 site on human TFAM promoter (11). Three Sp1 recognition sites (Sp1-A, B, C) were identified in the rat Tfam promoter (Fig. 3A). All of them were found to be footprinted as indicated by either protection at particular G residues or hypersensitivity of G residues along the binding sites. Together with the EMSA, these data demonstrate that the binding sites for Sp1 and NRF-2 are functional in rat, which is consistent with the sequence analyses by us and others (14). Because no conserved
NRF-1 recognition site was observed in the proximal promoter of rat Tfam gene, no footprint for NRF-1 was identified by IVGF at least in the region analyzed. In human, the region spanning –55 to –72 demonstrated NRF-1 consensus site. No consensus NRF-1 site was detected within this region of the rat Tfam promoter. The lack of footprinting at this site further reinforce the notion that rat Tfam promoter does not harbor any NRF-1 site within the first 141 bp upstream promoter region. In addition, analysis of the available rat Tfam promoter sequence up to –462 bp did not reveal any NRF-1 consensus element. In this respect, the human TFAM promoter differs from the corresponding rat promoter, as the latter promoter contains an NRF-1 consensus sequence to which NRF-1 binds and trans-activates the human gene.

Since mitochondria provide the energy in the form of ATP for the cellular processes including cell growth and proliferation, it is not unexpected to observe alterations of mitochondrial gene expression in the fast-growing tumors. The elevated mitochondria-encoded gene expression has been reported in a variety of solid tumors including cancers of breast, colon, liver, kidney, and lung in human (8). The mRNA level of COX II increased in the breast cancer samples compared to non-malignant tissue. No changes were observed in the mRNA levels for ND2, ND4, and ATPase 6 (9). In rat Zajdela hepatoma cells, the steady-state mRNAs for COX I, II, and ND2 were elevated by five fold compared to the resting liver cells. In addition, it was also found that the mitochondrial number decreased in this hepatoma (36). In another chemically induced rat hepatoma, the elevated transcripts for ND5, COX II, and 16S rRNA were observed. No differential expression of ND1 was found between the hepatoma and normal hepatocytes (37). The steady state levels of transcripts for COX I, COX II, ND5, ND6 are elevated in the Morris hepatoma 3924A compared to the host liver, which is consistent with the increased expression of
Tfam. No changes in the expression of ND1 and ATPase 6 were, however, observed. This observation is consistent with mitochondrial gene transcription in other tumors including cancers of breast, colon, liver, and some other rat hepatomas (9, 36-38). The present study has shown a correlation between Tfam activity/level and the expression of some proteins coded by mitochondrial DNA.

Interestingly, a nuclear isoform of Tfam was detected in the rat hepatoma. This unusual localization was confirmed by different techniques including subcellular fractionation, Western blot analysis, and immunocytochemistry. What is the probable function(s) of the nuclear form of Tfam? The presence of HMG boxes in the Tfam protein suggests its potential role in the nuclear transcription. The actual function of this nuclear form has not been established. A nuclear isoform of Tfam from mouse testis (TS-HMG) has also been reported (7). The TS-HMG is a splicing variant of Tfam, which is different from Tfam only in the first exon. Its physiological function is, however, not clear, although a possible role as a nuclear transcription activator or as a structural protein in the compaction of the nuclear DNA during spermatogenesis has been suggested. The TS-HMG was not, however, detected in the human or rat testis (14). Further study is necessary to establish the role of the nuclear isoform in the rat hepatoma.
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FOOTNOTES

The nucleotide sequence reported in this paper has been deposited in the GenBank™ under accession number AF377866.

1 The abbreviations used are: mtDNA, mitochondrial DNA; TFAM, human mitochondrial transcription factor A; Tfam, rodent mitochondrial transcription factor A; HMG, high mobility group; NRF, nuclear respiratory factor; LSP, light-strand promoter; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; IVGF, in vivo genomic footprinting; RACE, rapid amplification of cDNA ends; CS, citrate synthase; RT, reverse transcriptase; PCR, polymerase chain reaction.
LEGENDS FOR FIGURES

FIGURE 1: Increased expression of *Tfam* gene in the rat hepatoma. (A) Northern blot analysis of the mRNA levels of Tfam in the rat liver and hepatoma. Five µg each of rat liver and hepatoma poly(A)⁺ mRNAs were resolved on 1.2% formaldehyde-agarose gel and transferred to membrane. The membrane was hybridized with mouse Tfam and MBD-2 cDNAs sequentially. MBD-2 was chosen as an internal control, as previous study showed that its mRNA levels are comparable in the rat hepatoma and host liver (39). Lanes 1 and 2 represent rat liver (L) and hepatoma (H) mRNA samples, respectively. (B) Immunoblotting of Tfam in the rat liver and hepatoma. Mitochondrial extract (15 µg protein) from rat liver (L) or hepatoma (H) was resolved on 12% SDS-polyacrylamide gel. The lower portion (below 50 kDa marker) of the gel was transferred to ECL membrane. The blot was analysed with anti-Tfam antibodies. To show equal loading of protein, a non-specific protein signal (NS) from the same blot and the Coomassie Brilliant Blue R250 staining of the upper portion of the gel are included. (C) Semi-quantitation of Tfam protein level. The protein signal from the ECL detection was quantified using a densitometer, the ratio of Tfam/NS was then calculated. The data were derived from three independent experiments, and are plotted as mean ± standard error. (D) Tfam mRNA levels for liver (L) and hepatoma (H) were analysed by RT-PCR. The generated PCR products were electrophoresed on 1.5% agarose gel, and stained with ethidium bromide. As an internal control, the RT-PCR for rat β-actin mRNA was also performed using the same amount of mRNA. (E) Relative quantitation of Tfam mRNA. The band signals from the agarose gel images in panel D were quantified using Kodak Digital Science™ 1D image analysis software (Eastman Kodak...
Company). The mRNA ratios of Tfam/β-actin were then calculated. The data were derived from three independent sets of mRNA samples, and are plotted as mean ± standard error.

**Figure 2. Upregulation of transcription factors NRF-1 and NRF-2 in the rat hepatoma relative to the host liver.** (A) The steady level of mRNAs for NRF-1 and NRF-2 were analysed by RT-PCR. Each sample (20 ng of poly(A)+ mRNA) was reversely transcribed, and amplified by gene-specific primers. The PCR products were separated on 1.5% agarose gel, and the image was captured by a digital camera (Kodak). Lanes 1 and 2 represent rat liver (L) and hepatoma (H) mRNA, respectively. (B) The protein level of NRF-1 was analysed by immunoblotting with anti-NRF-1 antibodies. Nuclear extracts (90 µg protein) from rat liver and hepatoma were separated on 12% SDS-PAGE, transferred to nitrocellulose, and the protein detected by anti-NRF-1 using anti-Ku70 antibodies as a control. Lanes 1 and 2 correspond to rat liver and hepatoma nuclear extracts, respectively.

**Figure 3. The DNA binding activity of Sp1 and NRFs in the rat liver and hepatoma.** (A) The cis-elements in the proximal promoter of rat Tfam gene. The proximal promoter of rat Tfam gene (Accession no. AF264733) was aligned with the corresponding human sequence (13). The defined cis-elements in human TFAM promoter are underlined, and the identical residues are bolded and indicated by asterisks. The cis-elements on the rat Tfam promoter are indicated by dashed line above the sequence. The transcription initiation sites for human (13) and rat Tfam (14) genes are indicated by bent arrows. (B) The DNA binding activity of Sp1 in the rat liver and hepatoma nuclear extracts. The 32P-labeled Sp1 consensus oligonucleotides were incubated with 10 µg of protein under optimal binding conditions. Lanes 1-2 indicate the reactions with liver and hepatoma nuclear extract (LNE, HNE) in the absence of competitor, respectively. Lanes 3-4 represent reactions with LNE and HNE, respectively, in the presence of 100 fold
molar excess of unlabeled Sp1 consensus oligonucleotide (wt-Sp1). Lanes 5-6 represent the reactions with LNE and HNE in the presence of Sp1 mutant oligonucleotide (mut-Sp1). The non-specific complex (NS) is also indicated. (C) The DNA binding activity of NRF-2 in the rat liver and hepatoma nuclear extracts. The $^{32}$P-labeled NRF-2 recognition site in the human TFAM promoter (hTFAM –34/-13) was incubated with either rat liver or hepatoma nuclear extract (20 µg protein) for the binding assays. Lanes 1-2, no competitor; lanes 3-4, in the presence of 50 fold molar excess of unlabeled hTFAM –34/-13 oligonucleotide; lanes 5-6, in the presence of 50 fold molar excess of NRF-1 recognition site in the rat RC4 gene promoter (RC4 –173/-147); lanes 7-10, in the presence of 50 or 100 fold molar excess of NRF-2 recognition site in the rat Tfam promoter (rTfam –52/-27), respectively; lanes 11-12, in the presence of 50 fold molar excess of the rTfam –95/-70 oligonucleotide. The formed complexes (I and II) are indicated by the arrows. (D) The DNA binding activity of NRF-1 in the rat liver and hepatoma. The $^{32}$P-labeled NRF-1 oligonucleotide of RC4 gene (RC4 –173/-147) was used as a probe for the binding assays. Lane 1, free probe; lane 2-3, binding with liver and hepatoma nuclear extracts (LNE and HNE), respectively; lanes 4-7, in the presence of 50 or 100 fold molar excess of unlabeled RC4 –173/-147 oligonucleotide, respectively; lanes 8-11, in the presence of 50 or 100 fold molar excess of rTfam –95/-70, respectively; lanes 12-13, in the presence of anti-NRF-1 antibody. The specific complex, non-specific complex and supershift complex are represented by CX, NS and SS, respectively.

**Figure 4. In vivo genomic footprinting of the proximal promoter of rat Tfam gene.** (A) The overview of IVGF profile on the rat Tfam promoter. The sequence of –32 to –131 bp of rat Tfam promoter is shown. The recognition sites for Sp1 and NRF-2 are outlined by the boxes. The protected G residues on the upper and lower strands of the Tfam promoter are indicated by
arrows. The hypersensitive G residues on the upper and lower strands are marked by asterisks.

(B) In vivo genomic footprinting of the rat Trafam promoter. The in vivo footprinting was performed to check the occupancy of binding sites of the transcription factors on the rat Trafam gene promoter in vivo. Lanes 1-4 and 5-8 indicate the reactions for the upper and lower strand of rat Trafam promoter, respectively. Lanes 1, 5 and 2, 6 represent in vitro (N) and in vivo (V) DMS-treated liver genomic DNA, respectively. Lanes 3, 7 and 4, 8 indicate in vitro and in vivo DMS-treated hepatoma genomic DNA, respectively. The recognition sites for Sp1 and NRF-2 are indicated by the vertical lines. The protected G residues are pointed by the arrows, and the hypersensitive G residues are marked with the asterisks.

**Figure 5:** Increased expression of mitochondrial genes in the rat hepatoma. The mRNA levels of mitochondria-encoded genes including COX I, II, ND1, 5, 6, and ATPase 6 were analysed by RT-PCR. The same amount of cDNA for rat liver and hepatoma (lanes 1 and 2, respectively) was used for the PCR reactions. The PCR products were resolved on 1.5% agarose in TAE buffer. The β-actin gene was used as internal control for RT-PCR.

**Figure 6:** Upregulation of Trafam in other hepatoma cells. (A) The mRNA levels of Trafam in the rat hepatoma H4-II-E-C3 and mouse hepatoma cells were analysed by RT-PCR. The same amount of cDNA of each sample was used for the PCR reactions. The RT-PCR for β-actin was also performed as internal control. (B) The protein levels of Trafam in the above two lines of hepatomas were also analysed by immunoblotting. Mitochondrial extracts (30 µg each) were resolved on 12% SDS-polyacrylamide gel, and Trafam identified by Western blot using anti-Trafam antibodies. Anti-COX I antibodies were used as a control. A non-specific protein signal (NS) was also included to indicate equal loading of proteins.
FIGURE 7: The dual subcellular localization of Tfam in the hepatoma. (A) Detection of Tfam in the hepatoma nuclear extract by Western blot analysis. Each sample of 30 µg of protein extract was resolved on 12% SDS polyacrylamide gel and transferred to ECL membrane (Amersham). The blot was detected with polyclonal anti-rat Tfam. To rule out the possibility of mitochondrial contamination in the nuclear extracts, the same blot was reprobed with mouse monoclonal anti-COX I antibody. A non-specific protein signal from either nuclear or mitochondrial extract was also included for loading control. Lanes 1, 2, correspond to liver and hepatoma nuclear extracts (LNE, HNE), respectively; lanes 3, 4, represent liver and hepatoma mitochondrial extracts (LME, HME), respectively. (B) Immunoprecipitation of Tfam from hepatoma nuclear extract. Nuclear extracts (500 µg) from liver (LNE) and hepatoma (HNE) were used for immunoprecipitation. The immune complexes were analysed by SDS-PAGE and Western blot using anti-rat Tfam antibodies. Lanes 1, 3, immunoprecipitation controls in the absence of antibody for the liver and hepatoma nuclear extracts, respectively; lanes 2, 4, immunoprecipitation of liver and hepatoma nuclear extracts, respectively, with anti-rat Tfam antibodies. (C) Total citrate synthase (CS) activities in the hepatoma nuclear and mitochondrial extracts. Varying amount of the extracts (25, 50, 75, 100 µg protein) were used for the assay. Total CS activity in each extract was represented by the substrate turnover in nmols per minute. The data were derived from three different extracts, and are plotted as mean ± standard error.

FIGURE 8: Detection of Tfam in the rat hepatoma nucleus by fluorescence microscopy. (A) Either pure (with Triton X-100 wash) or partially pure (without Triton X-100 wash) nuclei were fixed with paraformaldehyde, and blocked with 1% BSA, then incubated with affinity purified anti-Tfam (1 µg/ml) and mouse monoclonal anti-COX I (2 µg/ml) antibodies. After three washes in PBS, the coverslips were incubated with Cy3-conjugated anti-rabbit IgG (1:200) and FITC-
conjugated anti-mouse IgG (1:200). The microscopy was performed using Nikon E800 microscope and nuclei were photographed under the ×60 oil immersion lens. The total magnification for the captured images is 600 ×. In all panels, Cy3 (pink), DAPI (blue), and FITC (green) indicate staining for Tfam, DNA, and COX I, respectively. Panel a, Tfam staining in pure nuclei; panel c, the negative control for rabbit anti-Tfam antibodies using normal rabbit IgG; panel e, Tfam staining in partially pure nuclei; panels b, d, COX I staining in the pure nuclei; panel f, COX I staining in the partially pure nuclei. (B) The confocal microscopy of hepatoma nuclei. Pure nuclei treated as above were also examined under a Bio-Rad MRC-600 confocal microscope and photographed under the ×60 oil immersion lens. The total magnification for the image is 600 ×. The equatorial section is shown here.

Table 1: RT-PCR primers and reaction conditions

| Primer names | Sequences                                      | Annealing temperature | Cycle number |
|--------------|------------------------------------------------|-----------------------|--------------|
| Tfam-F       | 5'-AGTTTCATACCTCGATTTTC-3'                     | 52 °C                 | 30           |
| Tfam-B       | 5'-TGACTTGGAGTTAGCTGC-3'                       |                       |              |
| COX I-F      | 5'-CCCCC TGCTATAACCCAATATCAG-3'                | 60 °C                 | 22           |
| COX I-B      | 5'-TCCTCCATGTAGTGAGCGAGTCAG-3'                 |                       |              |
| Primer   | Sequence                                    | Tm  | Anneal |
|----------|---------------------------------------------|-----|--------|
| COX II-F | 5'-GGCTTACCACATTCAACTTGGC-3'                | 60 °C | 25     |
| COX II-B | 5'-CACCTGGTTTAGGTCATTGTTG-3'                |      |        |
| ND1-F    | 5'-TTCGCCCTATTCTCATAGCCG-3'                 | 60 °C | 22     |
| ND1-B    | 5'-GGAGGTGCATTAGTTGGTCATATCG-3'             |      |        |
| ND5-F    | 5'-CTACCTTGCTTTCTCCACATTGG-3'               | 60 °C | 25     |
| ND5-B    | 5'-AAGTGATTATTAGGGCTCAGGCG-3'               |      |        |
| ND6-F    | 5'-CTGCTATGGCTACTGAGGAATATC-3'              | 58 °C | 30     |
| ND6-B    | 5'-GCAAAACAATGACCACCCAGC-3'                 |      |        |
| ATP6-F   | 5'-TCACACACCAAAAGGACGAACC-3'                | 60 °C | 22     |
| ATP6-B   | 5'-CTAGGGTAGCTCCTCGATTAG-3'                 |      |        |
| NRF1-F   | 5'-GTATGCTAAGTGGCTAGAAT-3'                  | 58 °C | 35     |
| NRF1-B   | 5'-GGGTTTGGAGGGTGAGAT-3'                    |      |        |
| NRF2-F   | 5'-GCACAGAAAGAAGCATGG-3'                    | 56 °C | 35     |
| NRF2-B   | 5'-AGTGAGGTGGTCATGTC-3'                     |      |        |
| β-actin-F| 5'-TTGTCCCTGTATGCTGCTGGTC-3'                | 62 °C | 22     |
| β-actin-B| 5'-TTGATCTTCTAGTGCTAGGAGC-3'                |      |        |
A. Northern blot

B. Immunoblotting

C. Relative quantitation of Tfam protein

D. RT-PCR

E. Relative quantitation of Tfam mRNA

Figure 1
Figure 2

A  RT-PCR

L  H

NRF-1

NRF-2

β-actin

1  2

B  Immunoblotting

L  H

NRF-1

Ku-70

1  2
Figure 3A
Figure 3B-D
Figure 4A
Figure 4B
Figure 5
A. RT-PCR

|     | rLiver | H4    | mLiver | Hepa |
|-----|--------|-------|--------|------|
| Tfam|        |       |        |      |
| β-actin|      |       |        |      |

B. Immunoblotting

|     | rLiver | H4    | mLiver | Hepa |
|-----|--------|-------|--------|------|
| Tfam|        |       |        |      |
| COX I|       |       |        |      |
| NS   |        |       |        |      |

Figure 6
A. Immunoblotting

|     | LNE | HNE | LME | HME |
|-----|-----|-----|-----|-----|
| Tfam | ![Image](Tfam.png) | ![Image](Tfam.png) | ![Image](Tfam.png) | ![Image](Tfam.png) |
| COXI | ![Image](COXI.png) | ![Image](COXI.png) | ![Image](COXI.png) | ![Image](COXI.png) |
| NS   | ![Image](NS.png) | ![Image](NS.png) | ![Image](NS.png) | ![Image](NS.png) |

B. Immunoprecipitation

|     | LNE | HNE |
|-----|-----|-----|
| anti-Tfam | - | + |
| IgG | ![Image](IgG.png) |
| Tfam | ![Image](Tfam.png) |

C. Total CS Activity

![Graph showing total CS activity vs. amount of extracts (μg)](Graph.png)

**Figure 7**
Mitochondrial transcription factor A and its downstream targets are upregulated in a rat hepatoma
Xiaocheng Dong, Kalpana Ghoshal, Sarmila Majumder, Satya P. Yadav and Samson T. Jacob

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