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# SMALLER ISOFORM OF HUMAN MITOCHONDRIAL TRANSCRIPTION FACTOR 1: ITS WIDE DISTRIBUTION AND PRODUCTION BY ALTERNATIVE SPLICING

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**SUMMARY:** Mitochondrial transcriptional factor 1 (mtTF1) is required for both transcription and replication of mammalian mitochondrial DNA (mtDNA) and it has two consensus sequences of HMG (high mobility group) boxes. In studies on the regulation of gene expression of mtTF1, we examined the steady state level of the mRNA in cultured HeLa cells. We found that in addition to the major mRNA, 30% of the mRNA of mtTF1 in the cells was a smaller isoform with a 96 base deletion. This smaller mRNA was also found in most human tissues. The region of the deletion corresponds to the second HMG-box, which may interact directly with DNA. We examined the structure of the genomic gene encoding the human mtTF1 to determine the mechanism of the deletion. We found that the gene is composed of 7 exons spanning over 10 kilobase-pairs and that its 5th exon is identical to the 96 bases skipped in the shorter mRNA. Therefore, the shorter mtTF1 is concluded to be generated by alternative splicing. 

Mammalian mitochondrial DNA (mtDNA) is bidirectionally transcribed from a heavy strand promoter(s) and a light strand one (1, 2). Mitochondrial transcriptional factor 1(mtTF1) binds to upstream regions of both promoters and activates transcription of mtDNA *in vitro* (3, 4). Human mtTF1 has been purified (5) and its cDNA has also been cloned and sequenced (6). We have also cloned its human genomic gene and reported its partial sequence (7). The deduced amino acid sequence revealed that mtTF1 has two regions with consensus sequences for high mobility group (HMG) proteins. The HMG family was initially defined as chromosomal proteins that modulate the structure of distinct regions in chromatin by wrapping to and bending DNA (8). Known functions of HMG-proteins are to stimulate transcription of the ribosomal RNA gene in the case of UBF ( upstream binding factor) (9) and to bend DNA and facilitate assembly of the functional nucleoprotein structure in the case of LEF-1 (lymphoid enhancer factor 1) (10). In addition, HMG-I(Y) stimulates transcription of the

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interferon β gene, probably by modulating the DNA structure of the binding site for a transcriptional factor (11). Moreover, mtTF1 causes supercoiling of circular mtDNA as other HMG-proteins do (12).

Here, we report a shorter form of mtTF1 which was found to be generated by alternative splicing of exon 5 coding the second HMG-box.

## MATERIALS AND METHODS

*Cultured cells*--- Cultured cells lacking mtDNA (EB8) were isolated by treating HeLa Cot cells with ethidium bromide as described previously (13). Cells (CP8) with a 5 kbp-deletion of mtDNA were obtained by fusing enucleated fibroblasts from a patient with CPEO (chronic progressive external ophthalmoplegia) with HeLa cells lacking mtDNA as described previously (14). The content of the deleted mtDNA in CP8 cells was 80% and their capacity for oxidative phosphorylation was nearly completely lost as described (14). As controls, CP5 cells were made by transferring normal mitochondria to EB8 cells.

*Isolation of RNA*---- Total cellular RNA from cultured cells was isolated by the guanidium isothiocyanate method with centrifugation through a cesium chloride cushion (15). Autopsied human tissues were obtained with the consent of the patient's family. Poly (A)+ RNAs from human tissue were isolated using a QuickPrepTM mRNA purification kit (Pharmacia).

*Ribonuclease protection assay* --- The coding region of human mtTF1 was amplified by RT-PCR using total RNA from HeLa cells as a template [from nucleotide 133 to 875 numbered according to the cDNA reported by Parisi et al. (6)]. The fragment was subcloned into Bluescript SK(+) (Startagene, CA). An antisense RNA probe for mtTF1 was synthesized with T7 RNA polymerase and [ $\alpha$ - $^{32}$P]UTP (the RNA contained 801 bases originating from the vector). The antisense RNA was purified by electrophoresis in polyacrylamide gel containing 8M urea, followed by elution with buffer containing 0.5M ammonium acetate, 1mM EDTA and 0.2% SDS. The total RNA(10μg) was hybridized with $1\times10^5$ c.p.m. of the radioactive antisense RNA for 15 h and then digested with a mixture of RNaseA and RNaseT1. Protected fragments were isolated by electrophoresis in 5% polyacrylamide gel containing 8M urea. Autoradiograms were obtained by exposing the gel to an imaging plate (Fuji Photo Film, Co., Tokyo, Japan) and visualized with a Bioimaging analyzer BAS2000 (Fuji Photo Film, Co.).

*Polymerase chain reaction coupled with reverse transcriptase* (RT-PCR)--- Samples of 1μg of total RNA from cultured cells or of several ng of poly (A)+RNAs isolated from various tissues were primed with 200ng of random hexamer with 10 units of reverse transcriptase (Avian Myeloblastosis Virus Enzyme XL, Life Sciences, FL) in a volume of 20 $\mu$ l. Then DNA fragments of mtTF1 in 1μl of the reaction mixtures were amplified in a volume of 100 $\mu$ l by PCR. The primers used were 5'-TATCAAGATGCTTATAGGGC (from nucleotide 427 to 446) and 5'-CACTCCTCAGCACCATATTTTCG (from nucleotide 847 to 869).

*Southern blot analysis of total human genomes* --- Human total DNA was isolated from peripheral blood cells by the proteinase K method. Samples of 10 $\mu$ g of the DNA were digested with various restriction endonucleases as indicated. The digested DNAs were fractionated in 0.8% agarose gel, transferred to Hybond-N+ nylon membranes (Amersham) after alkaline denaturation, and then hybridized with the probes indicated in the legend of Fig.5 in rapid hybridization buffer (Amersham) at 65°C for 2 h. Finally the membranes were washed with 1× SSC and 0.1% SDS at 65°C, and autoradiographed.

## RESULTS

*Gene expression of mtTF1* : mtTF1 regulates mitochondrial transcription and plays an important role in replication since the synthesized RNA acts as a primer for replication (16).

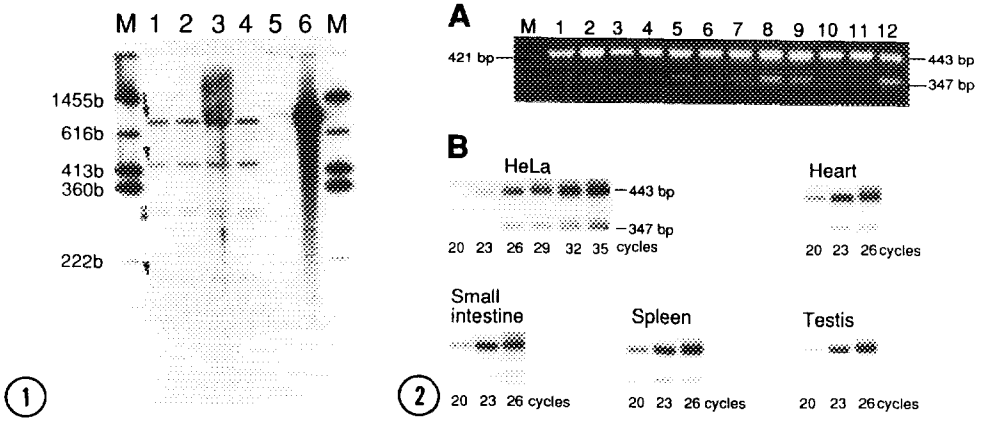


Figure 1. Ribonuclease protection assay for estimation of mtTF1 mRNA. Total RNA was isolated from 4 different cultured cell lines and RNase protection assay was performed as described in the **Materials and Methods**.
Lane M: radiolabeled DNA fragment markers. Lanes 1-6: RNAs from 1, parental HeLa Cot cells; 2, EB8 lacking mtDNA derived from HeLa Cot cells; 3, CP8, cybrid clonal cells obtained by fusing EB8 and cytoplasm with normal mtDNA; 4, CP5, cybrid clonal cells obtained by fusing EB8 and cytoplasm with deleted mtDNA (approximately 80% of the mtDNA had the deletion); 5, yeast RNA as a control; 6, labeled RNA without RNase treatment (20% of the sample was loaded). The heavy arrow indicates the expected length of hybridized mtTF1 mRNA. Arrowheads and asterisks indicate the additional bands.

Figure 2. Distribution of the shorter mRNA in various human tissues. PCR was performed using a set of primers flanking the region deleted in the shorter form.
(A) Short mRNA obtained from various tissues (lanes 1-11) by RT-PCR and detected with EtBr staining under ultraviolet light. Lanes: M, marker (421 bp); 1, cerebrum; 2, cerebellum; 3, heart; 4, skeletal muscle; 5, liver; 6, pancreas; 7, kidney; 8, spleen; 9, testis; 10, stomach; 11, small intestine and 12, HeLa cells.
(B) Short mRNA of mtTF1 detected by RT-PCR. Samples of 1μg of total RNA from HeLa cells or of several ng of poly(A)+RNA isolated from heart, small intestine, spleen and testis were primed with 200ng of random hexamer and 10 units of Avian Myeloblastosis Virus reverse transcriptase XL in a volume of 20 μl. DNA fragments of mtTF1 in aliquots of 1μl of the reaction mixtures were amplified by PCR in 100 μl of solution containing 10 μCi of [α-$^{32}$P]dCTP. The amplified fragments at each cycle were separated in 5% polyacrylamide gel. The gels were dried and autoradiographed with intensifying screens at -80$^{o}$C.

We are interested in the regulation of gene expression of mtTF1, especially in mutant cells lacking mtDNA and those with defective oxidative phosphorylation caused by a deletion of mtDNA. In studies on this problem, we examined the content of the mRNA by an RNase protection assay. As shown in Figure 1, we found no difference in the mRNA contents in wild type and mutant cells indicating that nuclear gene expression of the mRNA is independent mtDNA and oxidative phosphorylation ability.

*Shorter species of mRNA of mtTF1* : Lanes 1 to 4 of Figure 1 show two minor bands (indicated by arrowheads) in addition to the major band of 742b. Moreover, two additional faint bands (indicated by asterisks) were detected by RNase protection assay. To examine whether these additional bands were generated by deletions in mRNA, we amplified the cDNA by the PCR method using several sets of primers. Two bands were detected and cloned. The

nucleotide sequence of the larger fragment was identical to that of mtTF1 cDNA reported, but the smaller fragment lacked 96 base-pairs. The deleted amino acid sequence corresponded to half the second HMG-box. Therefore, we concluded that the two additional bands in Figure 1 found by RNase protection assay originated from the mRNA with the 96 base deletion, owing to looping out of the sequence deleted. In contrast to the bands indicated by arrowheads, the short mRNAs that may correspond to the two bands marked by asterisks in Figure 1 could not be detected by RT-PCR using suitable sets of primers.

Next we tested for the presence of the shorter PCR product in various human tissues. As shown in Figure 2A, this shorter mRNA was detected in most of the human tissues examined. The PCR was performed by incorporating a radioactive nucleotide to allow detection of small amounts of the fragments in the exponentially amplifying phase. As shown in Figure 2B, there was no marked difference in the ratio of the shorter mRNA to the normal one in the tissues examined, although the exact ratios were not determined by this method. Interestingly, the longer fragment was more easily amplified than the shorter one for some unknown reasons. Judging from the results of RNase protection assay, the smaller mRNA constituted 30 % of the total mtTF1 mRNA. These results show that the smaller mRNA originating from the mtTF1 gene is widely distributed in human tissues.

Figure 3 shows the alignment of high mobility group boxes. As reported by Parisi et al.(6), mtTF1 contains two HMG boxes. The deleted sequence corresponds to half the second HMG box.

*Gene structure of mtTF1* : To understand the mechanism of formation of the shorter mRNA of mtTF1, we determined the gene structure by cloning and sequencing. As reported previously (7), the genomic gene was isolated from a λ Dash II human genomic library. The gene structure and the exons are shown in Fig. 4A and the exon/intron junctions, in Fig. 4B. The gene is composed of 7 exons spanning over 10 kbp and all the exon/intron junctions conform to the GT-AG rule. In addition, we cloned a pseudogene which had no open reading frame and no intron.

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mtTF1 box 1     PKKPVSSYLRFSKEQLPIFKAQNPDAKT--TELIRRIAQRWRELPDSKKKIYQDAYRAEWQVYKEEISRFKEQLT
mtTF1 box 2    GKPKRPRSAYNVYVAERFQEAKGDSPQEKL--KTV----KENWKNLSDSEKELYIQHAKEDETRYHNEMKSWEEQMI
hHMG1 box A  PKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKG
hHMG1 box B  PNAPKRPPSAFFLFCSEYRPKIKGEHPGLSI--GDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYRAK-G
hUBF box 1   PDFPKKPLTPYFRFFMEKRAKYAKLHPEMSN--LDLTKILSKKYKELPEKKKMKYIQDFQREKQEFERNLARFREDHP
hUBF box 2a   GRPTKPPPNSYSLYCAELMANMKDVP---S--TERMVLCSQQWKLLSQKEKDAYHKKCDQKKKDYEVELLRFLESLP
                 **   *    *           *        *                *  *         *      *
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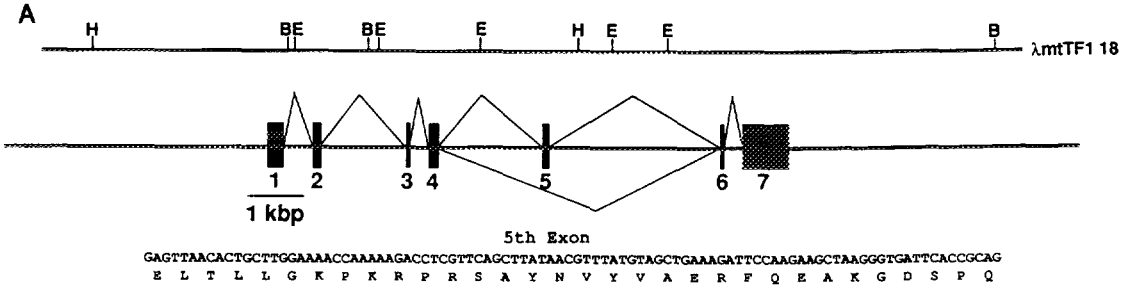
Figure 3. Alignment of HMG boxes. hHMG1 and hUBF indicate human high mobility group protein and human upstream binding factor for RNA polymerase I , respectively. The region deleted in the shorter form of mtTF1 is boxed. Asterisks indicate sequences conserved in all the boxes except the hUBF box2a.




B

| | | |
|---|---|---|
| | | intron |
| 1st exon( 1-233) | TTCAG gtaggc•••••••(0.6kb)••••••• | tttgttatatgtag TTTTG |
| 2nd exon(234-352) | CCCAG gtaagg••••••• | (1.8kb)••••••• atttcattccatag ATGCA |
| 3rd exon(353-423) | AAAAA gtaagc••••••• | (0.4kb)••••••• tttttttatttatag ATATA |
| 4th exon(424-573) | AAAAA gtgagt••••••• | (1.9kb)••••••• tttttttaattcag GAGTT |
| 5th exon(574-669) | CGCAG gtaaag••••••• | (3.5kb)••••••• atattgttttacag GAAAA |
| 6th exon(670-726) | AGGAA gtgagt••••••• | (0.5kb)••••••• atgctttttctcag TTATA |
| 7th exon(727- ) | | |
| | AG gtragt•••••••••••••••••••• | yyttyyyyyyncag G |
| | consensus sequence | |

Figure 4. Gene structure of mtTF1.
(A) Organization of the genomic human mtTF1 gene. The gene was sequenced and compared with that of cDNA to determine its exon/intron junctions. The exons are boxed. The lengths of introns were determined by PCR amplification. The 5th exon sequence is indicated. H, B and E indicate the positions of restriction enzyme digestion sites of *Hind* III, *Bam* HI, and *Eco* RI, respectively.
(B) Nucleotide sequences of exon/intron junctions. Capital and small letters show the nucleotide sequences of the exons and introns, respectively. Nucleotides are numbered according to those of cDNA of mtTF1(6).

The 96 bases deleted in the shorter mRNA corresponded to the 5th exon. This finding strongly suggests that half the second HMG box shown in Fig. 4A is skipped by alternative splicing to generate the shorter mtTF1.

Figure 5 shows an autoradiogram of total Southern blot analysis. Several strong bands observed in each lane, and those corresponding to the functional gene identified from the restriction pattern are marked with arrowheads. The strengths of the bands were nearly the same in a single copy of the gene per haploid, and two-fold in a mixture of the cloned DNA fragments and the total DNA, indicating that the functional gene is a single copy per haploid.

## DISCUSSION

We found that a short mRNA of an mtTF1 isoform is widely distributed in human tissues. Gene cloning and sequencing revealed that the deleted sequence of 96 nucleotides in this isoform was identical with exon 5, which corresponds to half the second HMG-box. Some other HMG-proteins have also been reported to have isoforms. For examples, UBF has a shorter isoform that is generated by skipping exon 8 in splicing and HMG-I also has an isoform that lacks 11 amino acids by alternative splicing (8, 17). However, the functional differences of these isoforms have yet to be elucidated.

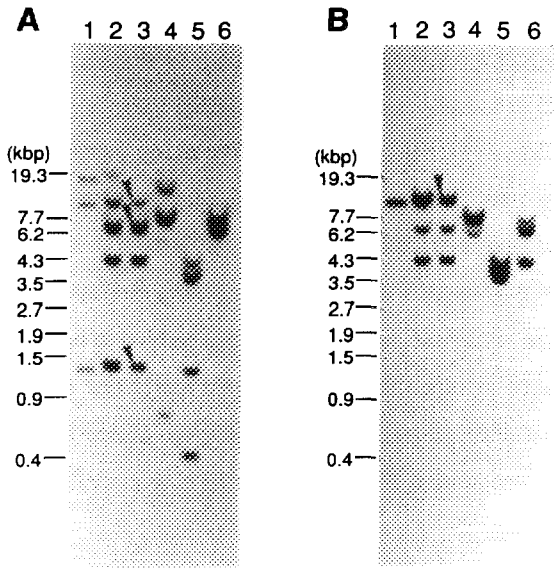



Figure 5. Total Southern blots hybridized with two different probes.
Genomic DNA was isolated from the Japanese man whose DNA was used to construct our genomic library. Samples of 10μg of DNA were digested with the indicated restriction endonucleases, subjected to 0.8 % agarose electrophoresis and transferred to a nylon membrane. The hybridization probes used for Panels A and B were DNA fragments from 133 to 417 (from exon 1 to exon 3) and from 574 to 869 (from exon 5 to exon 7), respectively [nucleotides are numbered according to those of cDNA of mtTF1. (6)].
Lanes 1: sample from a cloned phage DNA digested with *Bam* HI (147pg); 2, a mixture of DNA and phage DNA digested with *Bam* HI; 3, 4 , 5 and 6, DNA digested with *Bam* HI, *Hin*dIII, *Pst* I and *Sac* I , respectively. Arrowheads indicate the bands originating from the functional gene.

Mammalian mitochondrial DNA encodes 13 polypeptides, which are assembled with nuclear-encoded subunits into functional complexes (18). The mechanism of the coordinated expression of nuclear and mitochondrial genomes is not yet understood. Transient inhibition of mtRNA synthesis with ethidium bromide did not affect the transcription of some nuclear genes for mitochondrial proteins (19). Even in cells permanently lacking mtDNA, the content of mRNA for mtTF1 examined by RNase protection assay was not changed by removing mtDNA. Thus at least, no signal from mitochondrial DNA seems to regulate the nuclear gene expression of mtTF1. Recently, Liao et al. reported that the expression of peroxisome proteins is affected by defective yeast mtDNA (20). Inter-organelle interaction may play an important role in expression of these genes.

Many transcriptional factors have multiple isoforms that are generated by alternative splicing (21-24). Often the expression of these isoforms depend on the tissue and developmental stage. In some cases, the functional significance of alternative splicing is not clear. For example, the isoforms of NF-YA (one of the subunits of NF-Y) show no significant difference in binding affinity to the target sequence (25). At least some isoforms of CREB

(cyclic AMP responsive element binding protein) shows similar transcription activation potentials (26). CREM (cAMP-responsive element modulator) has at least three isoforms produced by alternative splicing and individual forms act as negative regulators by competing with CREB (27). Since CREB and CREM have many isoforms, they must form many different resulting dimers composed of multiple combinations of these isoforms which are expressed in a tissue- and/or development-specific manner.

Shorter isoforms generated by alternative splicing are reported to function as negative regulators (28). Some of the isoforms lack the ability to bind to their target sequence and other isoforms lack affinity to other transcriptional factors. For example, ΔFosB forms a heterodimer with c-Jun to prevent it from forming an active Fos-c-Jun heterodimer (29). mtTF1 may form a homodimer and a heterodimer with its shorter isoform, as other isoforms of transcriptional factors do.

mtTF1 was originally isolated as an activator of transcription of mtDNA *in vitro*. Sequencing of the cDNA revealed that the protein has two HMG-boxes and has ability to supercoil circular DNA. Recently, the counterpart of mtTF1 was isolated from yeast (designated as ABF2 or p19/HM) and found to be present at high concentration in mitochondria (30, 31). It binds non-specifically to mtDNA, and is not required for mitochondrial RNA polymerase, at least *in vitro* (32). These findings suggest an additional role of mtTF1. Possibly, the transcription and replication of mtDNA are regulated by the shorter mtTF1 generated by alternative splicing.

## ACKNOWLEDGMENTS

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