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Human Nuclear and Mitochondrial Mt Element-Binding Proteins to Regulatory Regions of the Nuclear Respiratory Genes and to the Mitochondrial Promoter Region

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Summary: We have found that Mt element-binding proteins (MtEBPs) with the same binding capability are present in the nuclei and mitochondria of human, bovine, and rat cells. MtEBPs purified from the human nuclei and mitochondria with Mt element-specific DNA affinity chromatography contained 4 different polypeptides, respectively. A UV-induced DNA cross-linking study showed that 47- or 55-kDa of the nuclear MtEBP recognizes Mt in the 5'-flanking region of the human cytochrome c_1 gene and that both 140- and 180-kDa polypeptides of the mitochondrial (mt) MtEBPs bind Mts in the human mt promoter region. Mt MtEBPs recognized a Mt4 element-like sequence within the mt transcription factor A binding site for the heavy-strand promoter. These results suggest a MtEBP-mediated coordination mechanism between nuclear and mt genetic systems.

Mitochondrial (mt) biogenesis is regulated through a communication between nuclear and mt genetic systems in response to cellular metabolic and energetic states. The number of virtual mitochondria per cell appears to be closely regulated within a given cell type (1). Not translational but transcriptional regulation on the coordinate expression of the nuclear and mt gene for mt biogenesis was reported (2-5).

We have demonstrated that protein factors in a total HeLa cell extract recognizes Mt3 and Mt4, *cis*-elements of nuclear genes, for the coordinate transcription (6). The factors were nominated as Mt element-binding proteins (MtEBPs). MtEBPs were found to recognize Mt3 and Mt4 localized in the displacement loop (D-loop) and promoter regions of human mtDNA (7). The fact suggests that MtEBPs play a role not only in the nucleus but also in mitochondrion for nucleus-to-mitochondrion communication to control the mt gene expression. It has been also reported (8) that mitochondria-derived reactive oxygen intermediates function as signal transducers of TNF-induced nuclear gene expression. Accordingly, Nagley (9) proposed that global and specific mechanisms exist in eukaryotes, ensuring the interorganelle communication between the nucleus and

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mitochondrion at the ranscription and replication levels. Identification of both *cis*-elements as the communication sites and *trans*-acting factors as the communicators involved in regulation of mtDNA replication and transcription, would help us to understand the regulation mechanism of mt gene expression and to provide some insights into impairment of mt functions by genetic disorder (10-12).

Here we report the universal and bipartite presence of MtEBPs in the mammalian nuclei and mitochondria. Both human nuclear and mt MtEBPs recognize Mts, while they have different molecular masses. Interestingly, human mt-MtEBP recognizes a Mt4-like sequence within mt transcription factor A (mtTFA) binding site (13) for the heavy (H) -strand promoter (HSP), but not a Mt4-like sequence within mtTFA binding site (13) for the light (L) -strand promoter (LSP). These results suggest that there exists a MtEBP-mediated mechanism to coordinate the gene expression between nuclear and mt genetic systems.

Materials and Methods

DNA fragments used as probes for gel mobility shift assay and UV cross-linking and as a ligand for DNA-affinity chromatography: The 154-base pair (bp) SP-C1 fragment in the 5'-flanking region of the human cytochrome c_1 gene, and the 64-bp MtP4 and 80-bp MtP34 fragments in the promoter region of the human muscle mtDNA are prepared as described (7). The Mt34T DNA fragment was prepared from the oligo-QPMt3 and oligo-QPMt4 (7). The two oligomers were subcloned separately into *Hinc* II site of pUC19 vector, respectively. The respective insert DNA was excised with *Pst*I and *Xba*I, and ligated to yield *Xba*I-Mt3-*Pst*I-Mt4-*Xba*I which was then subcloned into *Xba*I site of the vector. The insert DNA was excised with *Xba*I and ligated to yield its polymers. The trimer of them was isolated on a 3% NuSieve gel, subcloned into the vector, excised with *Bam*HI and *Sal*I, and used for gel retardation and for DNA affinity chromatography.

The respective mtTFA-binding site immediately upstream from LSP and HSP of the human mt genome has been identified (13). Two 34- and 36-mer DNA oligomers designated mtTFLSP and mtTFHSP (Fig. 1), respectively, representing the binding domains were constructed with complementary synthetic oligonucleotides as described (7).

Extract preparation: From human, bovine and rat liver tissues (100~150g), nuclear extracts were prepared as described by Gorski et al. (14) and mt extracts were prepared according to Chang and Clayton (15) with some modifications. All the following procedures were performed at 4°C. Mt were prepared from liver tissues and further purified by centrifugation in a continuous sucrose density gradient from 1 to 2 M sucrose containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Hepes

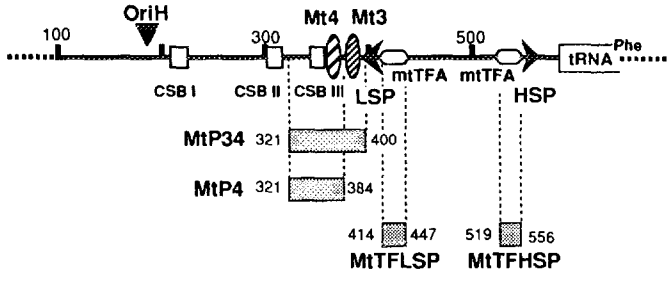

Fig. 1. Schematic presentation of the human promoter region and mtDNA fragments used as probe DNAs. OriH: The origin of H-strand synthesis; Mt3 and Mt4: Mt3 and Mt4s, respectively (7); CSBs I, II, and III: Conserved sequence blocks I, II, and III (20); LSP and HSP: L-and H-strand promoters, respectively; mtTFA: Binding site of mt transcription factor A (21); $tRNA^{Phe}$: Gene for phenylalanine and MtP34, MtP4, MtTFLSP and MtTFHSP: DNA fragments used for gel mobility shift assay and UV cross-linking. Figures are nucleotide numbers of the mtDNA according to Anderson et al. (22).

(pH 7.9). The purified mt (1~1.5 g of protein) collected by centrifugation were suspended in buffer A (20 mM Hepes (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10 % glycerol) to make a suspension of 100~150 mg protein/ml, and then Triton X-100 and KCl were added to 3.5 mg/100 mg protein and 0.2 M, respectively. The suspension was vortexed for 10 s every 5 min for 15 min, centrifuged at 100,000 rpm (250,000 x *g*) for 1 h in a Hitachi Himac RP100AT4-111 rotor, and the supernatant including unsedimented insoluble materials was recovered. Three volumes of buffer A were added to the supernatant to reduce the concentrations of the detergent and KCl, and mixed well. The clear supernatant obtained by re-centrifugation of the suspension as described above, was recovered to yield crude mt extracts.

Purification of Mt element-binding protein (MtEBP): The crude nuclear (50~75 mg of protein) and mt extracts (100~150 mg of protein) were applied at 36 ml/h to a 1.5 x 13-cm column of DEAE-Toyopearl 650M equilibrated with buffer A plus 50 mM KCl. The column was washed with 50 ml of the same buffer until the A_{280} of the effluent was reduced below 0.05. Bound proteins were then eluted from the column with a linear gradient of KCl from 0.05 to 0.5 M (40 ml each) to yield DEAE-purified MtEBP fraction. In this step, large parts of DNase activities were removed from the mt extracts. The DEAE-purified fractions were dialyzed overnight against buffer B (20 mM Hepes (pH 7.9), 0.1 M KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 17% glycerol), and then applied at 12 ml/h to a 1 x 6 cm column of heparin-Sepharose CL-6B equilibrated with buffer B. The column was washed with 30 ml of the same buffer. Bound proteins were eluted with a linear gradient of KCl from 0.1 to 0.7 M (40 ml each) to yield heparin-purified MtEBP fraction (3~7 mg of protein). The fraction was dialyzed overnight against buffer B and used for gel mobility shift assays and UV cross-linking studies. For further purification of mt MtEBPs, Mt34T (~300 μg) is covalently coupled to CNBr-activated Sepharose 4B (1 g) in 10 mM sodium phosphate (pH 8.2) with rotating overnight at room temperature to yield the affinity resin. The heparin-purified fraction was applied at 22 ml/hr to 1 x 4-cm column of the resin equilibrated with buffer B. The column was washed with 20 ml of the buffer. The bound proteins were successively eluted with 20 ml of 0.2, 0.3, 0.4 and 0.5 M KCl in buffer B. 0.3 M-KCl eluate contained MtEBPs (30~60 μg of protein) designated affinity-purified MtEBPs.

Gel mobility shift assays: Gel mobility shift assays were performed as described (16).

UV cross-linking: Probes for UV cross-linking were labeled with PCR. 50 μl of the PCR reaction mixture contained 1~5 ng of the probe DNA as a template, 50 pmol of each primer, 10 nmol of dATP, dGTP and dTTP, 0.1 nmol of dCTP, ~8.25 pmol of ^{32}P-dCTP, 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin. PCR was performed as described (7).

A 50-μl reaction mixture containing 25 μl (5~10 μg of protein) of human heparin-purified (10~20 μg of protein) or affinity-purified (0.2~0.5 μg of protein) MtEBPs in buffer B, 5 μl (20 μg) of poly(dI-dC)-poly(dI-dC), 1 μl (0.5-3 ng, 5 X 10^4 cpm) of ^{32}P-labeled probe DNA, and 19 ml of 10 mM Tris-HCl, pH 8.0 / 1 mM EDTA was incubated in the presence and absence of competitor DNAs at room temperature for 30 min. The mixture was exposed to 312 nm UV (8000mW/cm^2) on ice for 30 min at a distance of 5 cm, digested with 25 units of DNase I at 37°C for 30 min after addition of $CaCl_2$ (10 mM). Proteins were precipitated by 80% ethanol at -20°C for 1 hr. The precipitates were dissolved in 10 μl of a sample buffer containing 0.1% sodium dodecyl sulfate (SDS) and 1 mM DTT, incubated at 37°C for 1 hr, and electrophoresed on a 10% SDS-polyacrylamide gel followed by autoradiography.

Results and Discussion

Bipartite presence of MtEBPs in the vertebrates nuclei and mitochondria . Previously, we demonstrated that protein factors in a total HeLa cell extract bind to both nuclear and mt Mts (7). This led us to investigate the distribution of MtEBPs into the nucleus and mitochondria and their binding properties. Using heparin-Sepharose, we partially purified MtEBPs from the nuclei and mitochondria of human, bovine, and rat liver cells. As shown in Fig. 2, all the heparin-purified MtEBPs from mammalian cells bound to Mt-containing DNA fragments derived from the 5' flanking region of the human cytochrome c_1 gene (SP-C1) and from the human mt promoter region (MtP34, see Fig. 1). The result indicates that MtEBPs with the same binding capability exist in the mammalian nuclei (n-MtEBPs) and mitochondria (mt-MtEBPs) suggesting the evolutional importance for the MtEBP interaction in the regions. Recently, Ghivizzani et al. (17) have identified the sites of protein-mtDNA interaction in the bovine promoter region: one within conserved sequence block I and the others within the putative mtTFA

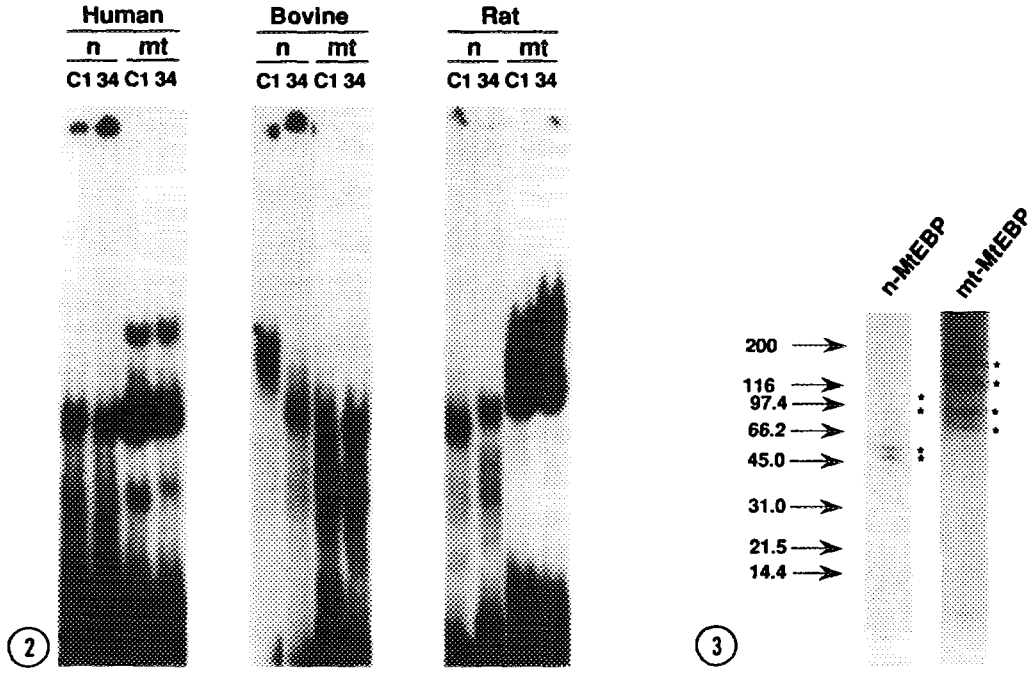

Fig. 2. Occurrence of MtEBPs in the nuclei and mitochondria of human, bovine and rat cells. MtEBPs were partially purified with DEAE and heparin-Sepharose column chromatography, and the binding activities of 2.5 μg of the purified proteins were measured by gel mobility shift assay as described in Materials and Methods. n and mt: Heparin-purified n- and mt-MtEBPs, respectively. C1 and 34: SP-C1 and MtP34 used as probes, respectively.

Fig. 3. Polypeptide compositions of human n- and mt-MtEBPs purified by Mt34T-specific DNA affinity chromatography. The heparin-purified n- and mt-MtEBPs were purified by a Mt34T-Sepharose 4B column chromatography as described in Materials and Methods. Bound proteins were eluted with 0.1, 0.2, 0.3, 0.4 and 0.5 M KCl. The 0.3 M-KCl eluates have the binding activities. 100 μl of the eluates were precipitated by 10% TCA. The precipitates were washed with 80% ethanol and then diethyl ether, dissolved in 5 μl of a sample buffer containing 0.1% SDS and 1 mM DTT and then incubated at 37°C for 1 hr. 1 μl (~0.4 μg of n-MtEBP and ~1 μg of mt-MtEBP) of the samples was electrophoresed on a 8~25% gradient SDS-polyacrylamide gel and visualized by silver staining. Standards of indicated molecular mass in kDa are shown at the left.

binding sites for LSP and HSP. They have found a sequence similar to Mt4 within the mtTFA binding site for LSP. We also found a sequence (186-TGGTCACAG-194) homologous to Mt4 within the conserved sequence block I. Together with our result (Fig. 2), there is a possibility that bovine mt-MtEBPs presented here bind to Mt4-like sequences in the sites identified by Ghivizzani et al.(17).

We purified human n- and mt-MtEBPs with Mt34T-specific DNA affinity chromatography. It should be noted that Mt34T containing only Mt3 and Mt4s is derived from the 5' flanking region of the nuclear gene for the ubiquinone-binding protein of human cytochrome bc_1 complex (16). As visualized by silver-staining in Fig. 3, the purified n- and mt-MtEBPs contained 4 polypeptides but with distinct molecular masses of 47, 55, 85 and 110 kDa, and of 65, 85, 140 and 180 kDa, respectively. As shown in Fig. 4, both the purified n- and mt-MtEBPs exhibited the same binding capability with

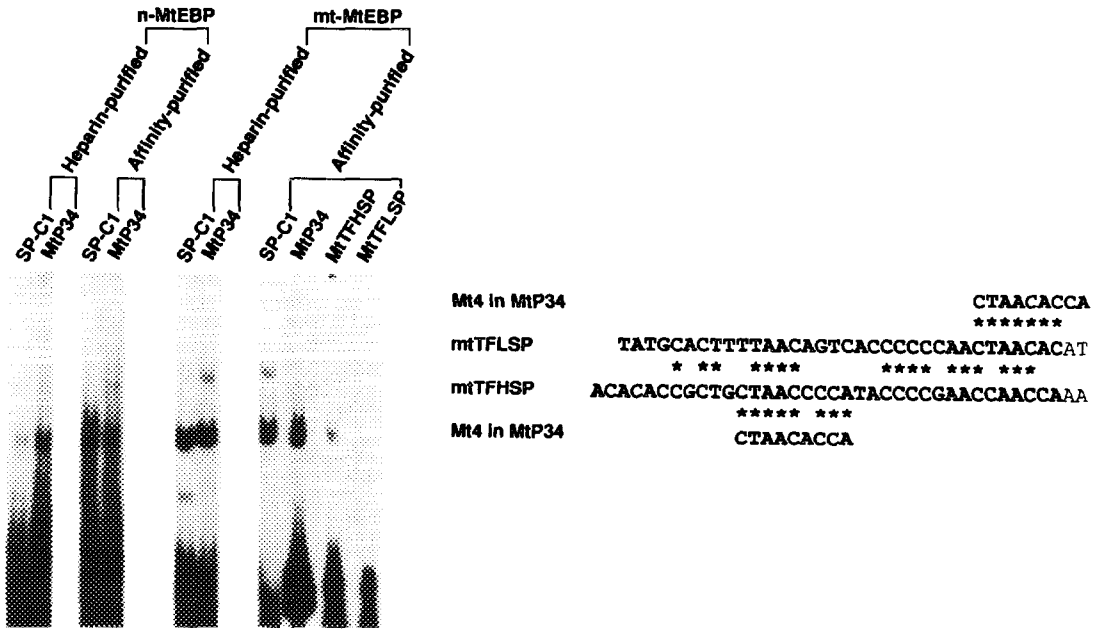

Fig. 4. Binding capability of the affinity-purified MtEBPs. The binding capabilities of the heparin- (2.5 μg of protein) and affinity-purified (~0.1 μg of protein) MtEBPs were measured by gel mobility shift assay using SP-C1, MtP34, mtTFLSP and mtTFHSP as probes. Nucleotide sequences shown at the right represent Mt4 in MtP34 and the binding sites of mtTFA for LSP (mtTFLSP) and HSP (mtTFHSP). *Asterisks* between the sequences show the matched nucleotides.

the heparin-purified ones, respectively. We found a sequence homologous to Mt4 at the 3' end of the mtTFA binding site for LSP and in the middle portion of that for HSP (Fig. 4). The purified mt-MtEBPs bound to mtTFHSP, while any retarded bands of mtTFLSP were not detected. The result indicates that mt-MtEBPs exhibit high binding specificity suggesting the involvement of mt-MtEBPs in transcriptional regulation. No detection of the mt-MtEBP to mtTFLSP binding might be due to the 3' end position of Mt4 in the oligonucleotide used.

Identification of the binding polypeptide. We tried to identify the binding polypeptides to Mts by UV-induced cross-linking using the human heparin-purified n- and mt-MtEBPs, and MtP34, MtP4, SP-C1 and MtTFHSP as probes. As shown in Fig. 5, a polypeptide of n-MtEBP ranging from 45 to 55kDa commonly crosslinked to MtP34, MtP4, and SP-C1. An additional polypeptide around 66kDa also crosslinked to MtP34. Polypeptides of mt-MtEBPs ranging from 120 to 180 kDa crosslinked to MtP34, MtP4, and SP-C1. Based on the molecular masses of polypeptides of the affinity-purified MtEBPs (Fig. 3), it is likely that 47- and/or 55-kDa polypeptide of n-MtEBP and 140- and 180-kDa polypeptides of mt-MtEBP are specific for Mt. When MtP34 was used as a probe, approximately 140- and 180-kDa polypeptides were detected. When MtP4 or SP-C1 was used, however, only the 140-kDa polypeptide weakly crosslinked to the probe. MtP4 contains only Mt4 and is different from MtP34 in

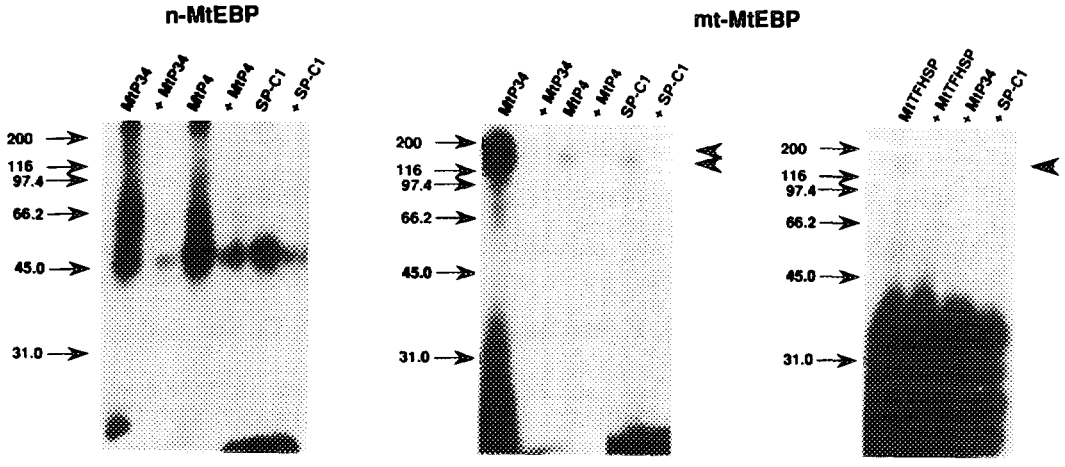

Fig. 5. UV-induced DNA cross-linking of polypeptides of the heparin-purified human MtEBPs. The heparin-purified n- and mt-MtEBPs (12.5 μg of protein) were incubated with ^{32}P-labeled MtP34, MtP4, SP-C1 or mtTFHSP in the presence and absence of 60 ng of the fragments indicated by + as competitors and then irradiated by UV light. The irradiated samples except for samples containing ^{32}P-MtTFHSP as a probe are treated with DNase I and then all the samples were electrophoresed on a 10%-SDS polyacrylamide gel followed by autoradiography as described in Materials and Methods. *Arrow heads* at the right show polypeptides detected by UV cross-linking. Standards of the indicated molecular mass in kDa are shown at the left.

lacking a 16-bp sequence containing Mt3 (see Fig. 1). This agrees with the previous observation (7) showing that a total HeLa cell extract binds more tightly to MtP34 than to MtP4. Hence, the 140-kDa polypeptide is probably specific for Mt4 and thus the 180-kDa one for Mt3. As shown in Fig. 5, the 140-kDa polypeptide cross-linked to Mt4-containing MtTFHSP. We obtained the same results using the affinity-purified n- and mt-MtEBPs (data not shown). Recently human mtDNA polymerase has been purified (17). The polymerase is composed of 140-kDa catalytic subunit and 54-kDa polypeptide. The molecular mass of the catalytic subunit appears to be the same with 140-kDa polypeptide of mt-MtEBPs. The possible coincidence of the catalytic subunit of the polymerase and mt-MtEBPs awaits further elucidation. Mt-MtEBPs are different from 24.4-kDa mtTFA (19), with respect to the molecular mass and recognition site, suggesting that mt-MtEBP is another type of mt regulatory protein, possibly, regulating the initiations of transcription and replication.

On the basis of our findings presented here, we propose a model where n-MtEBP participate in concerted expression of a subset of nuclear respiratory genes such as cytochrome c_1 and ubiquinone-binding protein which are imported into mitochondria to construct oxidative phosphorylation system. Both n-MtEBP and mt-MtEBP are coexpressed in nucleus. mt-MtEBPs are imported into mitochondria, and involved in transcriptional and replicational regulation of the mt gene. Consequently, the MtEBP-mediated communication pathway could synchronize the gene expression between physically separated nuclear and mt genetic systems.

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