Gene Expression of Subunit c(P1), Subunit c(P2), and Oligomycin Sensitivity-conferring Protein May Play a Key Role in Biogenesis of H⁺-ATP Synthase in Various Rat Tissues*

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Mammalian H⁺-ATP synthase is a supramolecule composed of at least 14 subunits that have a constant stoichiometry. Nevertheless the coordinate regulation of the gene expressions of various subunits remains obscure. To clarify the coordinate transcriptional regulatory system of mammalian H⁺-ATP synthase, we determined the absolute amount of nine species of mRNAs for eight nuclear-encoded subunits of H⁺-ATP synthase in different tissues of 8-week-old rats by use of the synthetic mRNAs and 32P-labeled DNA probes for each mRNA. Our quantitative analyses of the transcripts of H⁺-ATP synthase revealed that nine species of the subunits in different tissues of 8-week-old rats were divided into two groups: a high transcript gene (HTG) group (β-subunit, subunit b, subunit d, subunit e, and Factor 6) and a low transcript gene (LTG) group (subunit c(P1), subunit c(P2), IF1, and oligomycin sensitivity-conferring protein). The transcription step of LTG could constitute a bottleneck in the biogenesis of H⁺-ATP synthase. Thus, the transcriptional regulatory system of the LTG may play a key role in the biogenesis of mammalian H⁺-ATP synthase. The HTG were transcribed in a tissue-specific manner that corresponds with energy demand in the tissues. However, there was no tissue specificity in subunit c(P2). Furthermore, the tissue specificity of the transcript of IF1 differed substantially from that of HTG, suggesting that it could be crucial in the protection of mitochondrial membrane under abnormal conditions.

The regulation of the biosynthesis and assembly of multisubunit mitochondrial enzyme complexes is still obscure, especially when a concerted regulation of the expression of genes encoded by both nuclear and mitochondrial genomes is to be expected (1–4). Of particular interest is the biogenesis of H⁺-ATP synthase, which is the key enzyme in oxidative phosphorylation and thus is responsible for the production of most of the ATP in mammalian organisms.

The mammalian H⁺-ATP synthase is a supramolecule composed of at least 14 subunits (5–9) that have a constant stoichiometry (10, 11), 6 of which construct the catalytic site of ATP synthase called F₁ (subunits α, β, γ, δ, and ε and the loosely attached ATPase inhibitor protein IF1) and 8 of which construct an energy transduction part called F₅₆ (subunits a, b, c, d, e, f, OSCP,1 and A6L). The additional subunits f and g were also reported in bovine heart H⁺-ATP synthase (6). Two subunits of the F₅₆ (subunit a and A6L) are encoded by mitochondrial genome, and all the other subunits are separately encoded by nuclear chromosomes (12), but it is still unknown how the gene expression of each subunit is coordinately regulated.

To clarify the coordinate transcriptional regulatory system(s) of the mammalian H⁺-ATP synthase, we have developed a simple and rapid purification method of H⁺-ATP synthase from rat (8, 9) that can carry out the physiological experiments. We then determined the primary sequences of the synthase subunits by the protein sequence and cDNA cloning techniques (13–17).

We report here the absolute amount of nine species of mRNAs for eight nuclear-encoded subunits of H⁺-ATP synthase in different tissues of 8-week-old rats, which we determined using the mRNAs synthesized by an in vitro transcriptional system and applying 32P-labeled DNA probes to each. This is the first demonstration of the absolute amount of the mRNAs of multisubunits of H⁺-ATP synthase in different mammalian tissues.

EXPERIMENTAL PROCEDURES

Purification of Poly(A)+ RNA from Rat Tissues—Eight-week-old male Wistar strain rats were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). The rats were decapitated, and tissues of the brain, liver, heart, kidney, and muscle were rapidly removed. The poly(A)+ RNA was isolated from the tissues by the BioMag mRNA purification kit (Japan PerSeptive Biosystems Ltd., Tokyo) as described in the manual and in Refs. 18 and 19 with the exception that the poly(A)+ RNA eluted from the BioMag Oligo(dT)20 with 50 μl of DEPC water was further purified as follows: RNase inhibitor (64.2 units, 2.5 μl) and RNase-free DNase I (2.5 units, 2.5 μl) were added to the obtained poly(A)+ RNA solution (50 μl) and then incubated at 37 °C for 1 h. DEPC water was prepared by adding DEPC to deionized water (Milli-Q) at 0.2% (w/v), stirring vigorously for 12 h, and then autoclaving for 30 min. DEPC water (55 μl) and 100 μl of a mixture of phenol, chloroform, and isoamyl alcohol (50:48:2) were further added to the poly(A)+ RNA solution, which was then treated with a Vortex mixer and centrifuged at 12,000 × g for 5 min. Sodium acetate (3.6 μl of 3 M), 1 μl of Ethanchinmate (Nippon Gene Ltd., Tokyo), and 2.5 volumes of ethanol were mixed into the obtained supernatant, which was again centrifuged at 12,000 × g for 5 min. The supernatant was discarded, and 500 μl of 70% ethanol was added and centrifuged at 12,000 × g for 3 min at 4 °C. The obtained pellet (the purified poly(A)+ RNA) was dried and then resuspended with 50 μl of DEPC water.

Preparation of 32P-Labeled Probe DNAs—As shown in Fig. 1, the probes used were a Xho-BglII fragment of cDNA for subunit b (13), an Accl-AvaII fragment of cDNA for subunit d (17), a HindIII-HindIII fragment of cDNA for subunit e (15), a HindIII-ApoLI fragment of cDNA for OSCP (16), a HindIII-ApoLI fragment of cDNA for IF1 (16), a HindIII-HindIII fragment of cDNA for F₅₆ (14), a Xho-EcoRI fragment

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1 The abbreviations used are: OSCP, oligomycin sensitivity-confering protein; DEPC, diethyl pyrocarbonate; HTG, high transcript gene; LTG, low transcript gene; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s); SSSPE, saline/sodium/phosphate/EDTA.
of cDNA for subunit c(P1) (16), and an AccI-EcoO191I fragment of cDNA for subunit c(P2) (16). The probe for β-subunit mRNA was a fragment of 634–925 bp of β-subunit cDNA (21) that was synthesized by a nested reverse transcription-polymerase chain reaction method using four primers (outer primers: 5′-ATAAGGTTGGATCTGCTGGCC-3′ and 5′-AATCTGCGATAGCCTGCACTGAG-3′; inner primers: 5′-GGATATCGCGTTGGATATATGGGCAGATGA-3′ and 5′-GGGGGCGCAGCACATAGATAGCCTGCACTGAG-3′).

The primers' design was carried out by Genetyx-Mac (Version 7.0, Software Development Co., Tokyo). The obtained DNA was a fragment of 634–925 bp of β-subunit cDNA, which was confirmed by sequence analysis using the dye primer method in a Pharmacia Biotech Inc. Automated Laser Fluorescent DNA sequence.

A fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA was obtained from Clontech (catalog no. 9805). The probe DNAs were labeled by the random-prime DNA labeling method (22), using [α-32P]dCTP.

**mRNA Synthesis by In Vitro Transcriptional System**—The mRNAs of H+-ATP synthase subunits were synthesized by the MAXiScrip In Vitro Transcription Kit (Ambion, catalog no. 1318) as described in the manual. Phagemid expression vector (Bluescript KS M13+) (Stratagene), which contains one of the cDNAs of the H+-ATP synthase subunits b, c(P1), c(P2), d, e, Factor 6, OSCP, or β-subunit (634–925 bp), was linearized by cutting with NotI. The mRNAs of these subunits were synthesized at 37°C for 1 h by adding 25 units of triiodothyronine RNA polymerase and for 1 h more by further addition of the enzyme. The obtained mRNAs (50 μl) were incubated at 37°C for 1 h by addition of 2.5 μl of RNase inhibitor (25 units/ml) and 2.5 μl of RNase-free DNase I (2 units/ml). After adding 55 μl of DEPC water, the phenolchloroform extraction was carried out once. 3.6 μl of 5 M sodium acetate, 1 μl of EtBr (Ethachinmate, Nippon Gene Ltd., Tokyo), and 2.5 volumes of ethanol were then added, mixed thoroughly with a Vortex mixer, and centrifuged at 10,000 × g for 3 min. The supernatant was discarded, 70% ethanol was added, and the tube was centrifuged at 10,000 × g for 3 min. The supernatant was again discarded, and the pellet was dried and ethanol precipitation was repeated once. 3.6 μl of 3 M sodium acetate, 1 μl of 20 M MOPS buffer (pH 7.0) containing 6% formaldehyde, 50% deionized formamide, 5 mM sodium acetate, and 1 mM EDTA at 65°C for 5 min, cooled in ice water, and then diluted by adding 106.7 μl of 20 × SSPE cooled on ice. The RNA solutions (213.4 μg) of the synthetic mRNAs containing 20–60 μg of poly(A)+ RNA (200 ng) purified from the various rat tissues were dotted onto nylon membranes (presoaked in 10 × SSPE solution) using the Bio-Dot Slot Format blotting apparatus (Bio-Rad). The resulting nylon membranes were laid RNA side up on Whatman No. 3MM paper previously dampened with 0.05 mM NaOH solution for 5 min, rinsed with 2 × SSPE, blotted with 3MM paper, dried at 80°C for 10 min, and then cross-linked by a Funakoshi UV Linker, model FS-1500.

Before hybridization, the nylon membrane was prehybridized in 50% formamide, 5 × SSPE (0.75 mM NaCl, 50 mM NaH2PO4, and 5 mM EDTA at pH 7.4), 200 μg/ml sonicated salmon sperm DNA, 0.5% SDS, and 5 × Denhardt’s solution at 42°C for 5 h. Hybridization was performed at 42°C for 14 h in the same solution containing 10% dextran sulfate and labeled probe DNA (5 × 106 cpm/ml). After hybridization, the nylon membrane was washed four times with 2 × SSPE containing 0.1% SDS for 15 min at 42°C and then with 1 × SSPE containing 0.1% SDS for 30 min at 42°C. The membrane was left with a Fuji imaging plate in the cassette at room temperature for 6 h and then scanned and analyzed by a bioimaging analyzer (BAS1500Mac, Fuji Film Co., Tokyo).

**RESULTS**

The amounts of 9 species of mRNAs for 8 subunits of rat H+-ATP synthase were determined by dot blot analysis of each

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**Fig. 1.** Sequence map of cDNAs and probe DNAs for H+-ATP synthase subunits.

**Fig. 2.** In vitro synthesis of mRNAs of rat H+-ATP synthase subunits. The mRNAs of H+-ATP synthase subunits were synthesized by the MAXiScrip In Vitro Transcription Kit as described under "Experimental Procedures." The obtained synthetic mRNAs were denatured in a solution of 20 mM MOPS buffer (pH 7.0) containing 6% formaldehyde, 50% deionized formamide, 5 mM sodium acetate, and 1 mM EDTA at 65°C for 5 min, electrophoresed on 1.2% agarose gel containing 6% formaldehyde as described by Lahrach et al. (20), and then stained with ethidium bromide.
synthetic mRNA and of poly(A) RNAs purified from rat brain, liver, heart, kidney, and muscle. Each synthetic mRNA was transcribed from NotI-linearized pBluescript IIKS, which inserted cDNA from the H+-ATP synthase subunit into its EcoRV-NotI site, using triiodothyronine phage RNA polymerase as described under “Experimental Procedures.” The lengths of all synthetic mRNAs were the same as those of the corresponding H+-ATP synthase subunit cDNAs and a fragment of β-subunit cDNA (634–925 bp), as confirmed by agarose gel electrophoresis (Fig. 2).

Fig. 3 shows radio signals of dot blots of the nine species of synthetic mRNAs and poly(A)+ RNAs purified from different tissues of 8-week-old rats that were detected with each corresponding [32P]DNA probe. The probe DNAs for subunit c(P1) and c(P2) specifically hybridized with the synthetic mRNAs of subunit c(P1) and c(P2), respectively. There was no cross-reaction between them.

Linear relationships were derived between the strength of radio signals analyzed by a Fuji Film bioimaging analyzer, model BAS1500Mac, and the dotted amount of each synthetic mRNA (cf. an example of Factor 6 in Fig. 4). From these dose-response curves, the molar number of mRNA in 200 ng of poly(A)+ RNAs purified from the rat tissues was determined for each subunit of H+-ATP synthase (Fig. 5).

As these data make clear, the nine subunits of H+-ATP synthase in various tissues of 8-week-old rat were classified into the following two groups: a high transcript gene group and a low transcript gene group (Fig. 5). The high transcript gene (HTG) group consisted of β-subunit, subunit b, subunit d, subunit e, and Factor 6, and the low transcript gene (LTG) group included subunit c(P1), subunit c(P2), IF1, and OSCP.

The HTG were transcribed in a tissue-specific manner. The expression levels of mRNAs in the HTG in each tissue were nearly identical (Fig. 5). Therefore, the transcription of the HTG could be regulated by a common transcriptional regulatory system. This is in good accord with previous findings (23–28) in which the regulation of the expression of nuclear-encoded proteins involved in mitochondrial energy metabolism has been described mostly at the transcriptional level.

Although the steady-state levels of the transcripts of subunit c(P1), c(P2), IF1, and OSCP were very much lower than those of the HTG, they were of the same order. Thus, the LTG could also be regulated by an additional common transcriptional regulatory system that differs from that in the HTG. Subunit c(P1) and OSCP were also transcribed in a tissue-specific manner, but there was no tissue specificity in the transcriptional level of subunit c(P2) in the different tissues. Subunit c(P1) and subunit c(P2) are isomers composed of different signal peptides and the same mature protein that were found in bovine (29), rat (16), and human (30). Furthermore, the transcriptional level of IF1 in the rat tissues differed substantially from those of the other subunits according to the following hierarchy: brain > kidney > heart > muscle > liver.
LTG could constitute a bottleneck in the biogenesis of Hc(P1), subunit c(P2), IF1, and OSCP. The transcription step of Factor 6 and a low transcript gene (LTG) group (subunit c(P1), subunit c(P2), IF1, and OSCP) facilitates the biogenesis of H⁺-ATP synthase subunits and of glyceraldehyde-3-phosphate dehydrogenase in different tissues of 8-week-old Wistar rats. Thus, the transcriptional regulatory system of the tissue specificity in the tissues under abnormal conditions. Hence, the tissue specificity of the decay step of the transcripts as well as the decay of the mRNAs of H⁺-ATP synthase subunits and of glyceraldehyde-3-phosphate dehydrogenase in different tissues of 8-week-old Wistar rats was determined as described under "Experimental Procedures." Data were the average values of two experiments. Maximal difference between any two replicate measurements was 8%. Attomole is 10⁻¹⁸ mol.

DISCUSSION

Our quantitative analyses of the transcripts of H⁺-ATP synthase revealed that nine species of the subunits of H⁺-ATP synthase in different tissues of 8-week-old rats were clearly divisible into the following two groups: a high transcript gene (HTG) group (β-subunit, subunit b, subunit d, subunit e, and Factor 6) and a low transcript gene (LTG) group (subunit c(P1), subunit c(P2), IF1, and OSCP). The transcription step of LTG could constitute a bottleneck in the biogenesis of H⁺-ATP synthase. Thus, the transcriptional regulatory system of the LTG may play a key role in the biogenesis of mammalian H⁺-ATP synthase. Such a hypothesis would be in good accord with previous findings (31). Nevertheless further studies examining the decay step of the transcripts (32) as well as the real rate of the transcription in the nucleus will be required to confirm this.

A possible reason for the differential gene expression could be to prevent formation of proton leaks during an assembly of H⁺-ATP synthase, which has been examined in detail in prokaryotes (33–35). The tissue specificity of the transcripts of HTG may correspond with energy demand in the tissues, but the tissue specificity of the transcript of IF1 differed substantially from those of HTG. Because IF1 reversibly binds to F₁ and inhibits ATP hydrolysis to prevent ATP loss in the tissues under ischemia, anoxia, and other energy-deficient conditions (36), it seems likely that IF1 serves to protect mitochondrial membrane under conditions of energy deficiency. Thus, the tissue specificity of IF1 could be crucial in the protection of mitochondrial membrane under abnormal conditions.

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