Genes for the Human Mitochondrial Trifunctional Protein 
\( \alpha \)- and \( \beta \)-Subunits Are Divergently Transcribed from a Common Promoter Region* 

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Human \( \text{HADHA} \) and \( \text{HADHB} \) genes encode the subunits of an enzyme complex, the trifunctional protein, involved in mitochondrial \( \beta \)-oxidation of fatty acids. Both genes are located in the same region of chromosome 2p23. We isolated genomic clones, including 5' flanking regions, for \( \text{HADHA} \) and \( \text{HADHB} \). Sequencing revealed that both of these genes are linked in a head-to-head arrangement on opposite strands and have in common a 350-bp 5' flanking region. The 5' flanking region has bidirectional promoter activity within this region; two cis elements proved critical for the activity. Transcription factor Sp1 functions as an activator for the bidirectional promoter by binding to both elements. Therefore, expression of trifunctional protein subunits are probably coordinate regulated by a common promoter and by Sp1.

Mitochondrial fatty acid \( \beta \)-oxidation is a major energy-producing pathway, and at least 10 different enzymes are involved in this function. A mitochondrial trifunctional protein (TP) catalyzes the last three steps of the \( \beta \)-oxidation of long-chain fatty acids (1–3). This enzyme complex is composed of four \( \alpha \)- and four \( \beta \)-subunits; the \( \alpha \)-subunit harbors the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase domains, whereas the 3-ketoacyl-CoA thiolase domain is located on the \( \beta \)-subunit (3). These two subunits are encoded in independent genes, \( \text{HADHA} \) and \( \text{HADHB} \) (3, 4). In humans, genetic organization of the \( \alpha \)-subunit (\( \text{HADHA} \)) was determined by Zhang and Baldwin (5), and that of the \( \beta \)-subunit (\( \text{HADHB} \)) was determined in our laboratories (6). Both \( \text{HADHA} \) and \( \text{HADHB} \) are located on chromosome 2p23 (6, 7). Additionally, the distance between the two loci is short, which suggests that the two genes exist side by side (8), as do the two genes of the subunits of the bacterial fatty acid \( \beta \)-oxidation multienzyme complex (9).

Deficiencies in this enzyme complex can cause sudden, unexplained death in children, acute hepatic encephalopathy, skeletal myopathy, and cardiomyopathy (10, 11). TP deficiency was classified into two biochemical phenotypes. The most common phenotype is long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, with normal or near-normal activities of long-chain enoyl-CoA hydratase and long-chain 3-ketoacyl-CoA thiolase (10, 11). The other phenotype is characterized by decreased activity of all three enzyme activities (12, 13). These analyses showed that formation of the enzyme complex is important for related functions (14, 15). Formation of the enzyme complex apparently requires the presence of a similar number of molecules of \( \alpha \)- and \( \beta \)-subunits in mitochondria, which raises the possibility of a synchronized regulation of the expression of the both subunits. We isolated clones of the two genes, including their 5' flanking regions, and characterized their structures and transcriptional regulation.

EXPERIMENTAL PROCEDURES

Materials—A Sau3A1 human leukocyte genomic library containing \( 1 \times 10^6 \) AEMBL3 recombinant phages was purchased from CLONTECH. The radioactive nucleotides [\( \gamma \]32P]dATP and [\( \alpha \]32P]dATP were purchased from Amersham Pharmacia Biotech. The DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA), TaKaRa (Tokyo, Japan), and Toyobo (Tokyo, Japan). Moloney murine leukemia virus reverse transcriptase and culture medium were purchased from Life Technologies, Inc. AmpliTaq DNA polymerase and DNA sequencing kits were purchased from Applied Biosystems Japan (Tokyo, Japan). The LA PCR kit was purchased from TaKaRa. HEK293 cells were obtained from Dr. S. Asano (Department of Geriatrics, Shinshu University School of Medicine). pCMV-Sp1 expression vector was a generous gift from Dr. R. Tjian (Howard Hughes Medical Institute Investigator at University of California, Berkeley, CA). The CAT assay kit was purchased from CLONTECH. The PicaGene luciferase assay system was purchased from Wako (Osaka, Japan).

Isolation and Characterization of Genomic Clones—The human leukocyte genomic library was screened at a density of 40,000 plaques/200-mm plate using as probes [\( \alpha \]32P]dATP-labeled cDNA fragments of human \( \text{HADHA} \) (~23 to ~428, 451 bp) or \( \text{HADHB} \) (~26 to ~204, 230 bp) (6). Approximately 2 \times 106 plaques were screened. Positive clones were purified by repeated screening, and three different clones were obtained (Fig. 1). The phage DNAs from the purified clones were isolated using standard methods (16). EcoRI-BamHI fragments from clone 1 (5 kb) and clone 3 (5 kb) and a BamHI fragment from clone 2 (406 bp) were subcloned into pBluescript KS+ (Stratagene) and sequenced with M13 primers and with primers derived from the human \( \text{HADHA} \) and \( \text{HADHB} \) cDNA sequences (4).

Primer Extension—The appropriate oligonucleotides (primers 1 and 2; Table I) were end labeled with [\( \gamma \]32P]dATP, using Multimize DNA
Regions of fragments A and B were used for DNase I footprint analysis. A and B mutants are mutated fragments. These mutated positions are represented by small characters and are underlined.

| No. | Sequence |
|-----|----------|
| 1   | 5'-AGCGGAGGCTCTGAGGAGCA-3' |
| 2   | 5'-CTTATGTCGAGCTGAGGACAG-3' |
| 3   | 5'-CTTATGTCGAGCTGAGGACAG-3' |
| 4   | 5'-CTTATGTCGAGCTGAGGACAG-3' |
| 5   | 5'-CTTATGTCGAGCTGAGGACAG-3' |

Ribo nucleotides used

Regulation of Trifunctional Protein Gene Expression

Cell Culture Media and Conditions—HePGe, HeLa, and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Drosophila cell line Schneider S2 was maintained in Schneider’s Drosophila medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Transfections and CAT Assays—At 20–24 h before transfection, cells were replated into 60-mm dishes. Cells were cotransfected with 5 μg of reporter construct and 1 μg of CMV-β-galactosidase using the calcium phosphate precipitation technique (16). Duplicate tissue culture dishes for each construct were transfected in each experiment. CAT activities were corrected for β-galactosidase activities. Aliquots of cell extracts were used for the CAT and luciferase assays. The CAT assay was performed using Quanti CAT assay kits (Amersham Pharmacia Bio-Tech). CAT activity was counted in a Beckman (Fullerton, CA) LS6000SC liquid scintillation counter. The luciferase activity was measured using a Promega luciferase assay kit and a Lumat LB 9507 (EG&G, Berthold, Germany).

Electrophoretic Mobility Shift and Antibody Supershift Assays—Nuclear extracts from HeLa cells were then prepared (17). Two double-stranded synthetic oligonucleotides corresponding to nucleotides –210 to –182 and –239 to –210 of a common promoter of HADHA and HADHB containing 5'-GC overhangs were used as probes (Fig. 1, fragment A). Electrophoretic mobility shift assays were done as described, but with minor modification (18). 32P-End-labeled probes (5 fmol of oligonucleotide, 15,000–20,000 cpm) were incubated with 5 μg of nuclear extract from HeLa cells for 15 min at room temperature in 10 μl of reaction mixture containing 15 mM Hepes-KOH (pH 7.1) buffer, 0.3 mM EDTA, 50 mM KCl, 5 mM MgCl2, 1 mM CaCl2, and 30 units of RNAse inhibitor (16). Control reactions were run using the same amount of yeast RNA.

The hybridized oligonucleotide with RNA was dissolved with 20 μl of reverse transcription buffer; 50 μl of Monely murine leukemia virus reverse transcriptase was added, followed by incubation at 37 °C for 2 h. After RNAase A treatment, the products were purified by phenol-chloroform extraction, recovered by ethanol precipitation, and then electro- phoresed, using a 6% polyacrylamide gel containing 8 μm urea. The gels were exposed to x-ray films for 18 h. Size of the labeled products was determined by comparison with DNA-sequencing reactions run using the same primer.

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DNase I Footprinting—The 276-bp-length probe was prepared by PCR amplification using primers 19 and 20, including BamHI and PsI sites, respectively. After BamHI and PsI treatment, the probe was end labeled by 32P. Footprinting reaction was performed in 50 μl of 20 mM
**RESULTS**

**Head-to-Head Arrangement of Human HADHA and HADHB Genes—** Chromosomal localization of the human HADHA and HADHB genes was previously identified in the same region, 2p23 (6, 7). Because the distance between the two loci was short (8), we examined the structure of the region between the two loci. When λEMBL3 recombinant phages from a Sau3AI human leukocyte genomic library were screened using as probe a human leukocyte genomic DNA clone, the genomic DNA clones were isolated. Sequencing revealed that these clones partially overlapped and covered approximately a 15-kb region. Clones 2 and 3 carried both exon 1 of HADHA and exon 1 of HADHB, on opposite strands in approximately a 400-bp region, respectively (Fig. 1A), thereby indicating that human HADHA and HADHB genes exist side by side in a head-to-head arrangement.

The sequence between exons 1 of the two genes is shown in Fig. 1B. The nucleotide sequence data reported in this paper will appear in the DNA Data Bank of Japan-EMBL-GenBank nucleotide sequence data bases under accession number AB020811. The translation initiation codon ATG of HADHA was present in exon 1, whereas that of HADHB was present in exon 2. The sequence of exon 1 of HADHB and 5′ flanking region (Fig. 1B, −369 to −333) corresponded to part of the 5′ untranslated region of the cDNA clone (Ref. 4; −45 to −9).

**Determination of Transcription Initiation Sites—** To determine the transcription initiation sites of the HADHA and HADHB genes, primer extension experiments were done using primers located in exon 1 of HADHA or in exon 2 of HADHB (Fig. 1B, Oligo 1 and Oligo 2, respectively). Using oligonucleotide 1 with total RNAs from HeLa, HepG2, and HEK293 cells, respectively, three bands of the extended cDNAs were detected at 86, 97, and 104 cpm; the 86-bp band is the major product in these cell lines (Fig. 1B, left panel). The major transcription initiation site of HADHA corresponded to the 5′ end of the cDNA clone (4), as shown by arrow +1 in Fig. 1B. Two other sites corresponded to positions −11 and −35, respectively (Fig. 1B).
Six bands of extended cDNAs (60, 77, 86, 92, 100, and 107 bp) appeared when using oligonucleotide 2 (Fig. 2, right panel); the positions of 5′ ends of these fragments corresponded to nucleotide positions +20, +1, −9, −15, −23, and −30, respectively, in the HADHB cDNA clone (4). The main transcription initiation site of HADHB seems to be position of the 77-bp band (Fig. 2, right panel), which is nucleotide position −351 relative to the major transcription start site of the HADHA gene (Fig. 1B). These findings was confirmed by reverse transcription-PCR and ribonuclease protection assay (data not shown).

Determination of the Promoter Region(s) for HADHA and HADHB Genes—To examine promoter activities of the 5′ flanking regions of HADHA and HADHB genes, 1.2-kb (−1200 to +25, pCAT-α1) and 2.2-kb (+1850 to −366, pCAT-β1) fragments were connected to CAT cDNA, as shown in Fig. 3, and the constructs were transfected to HeLa or HEK293 cells. The construct pCAT-α1 showed a 10- and 7-fold higher level of the reporter gene activity in HeLa and HEK293 cells, respectively, when compared with the pCAT-Basic with no promoter. pCAT-β1 exhibited a 12-fold higher expression of CAT in both cell lines. Because promoter activity of the 5′ flanking region of HADHB exon 2 (pCAT-β8 and 9; Fig. 3) was low compared with that of pCAT-β1 in both cell lines, the transcription band of HADHB mRNA initiated at the 5′ end of exon 2 in some degree (Fig. 2 right panel) might be derived from a small amount of nuclear precursor RNA, retaining intron 1, in the total RNA sample.

Deletion analyses of the 5′ flanking regions of the both genes were done to define the active promoter regions. The promoter activity of pCAT-α1 was at a similar level or increased until truncating between positions −366 and −253 (pCAT-α5) in both cell lines. Furthermore, significant decreases occurred when truncation to position −169 was tested (pCAT-α9). On the other hand, the activity of pCAT-β1 was completely retained by truncation to position +25 (pCAT-β4). Truncation to position −238 led to a significant decrease in the activity. Activities of internal deletion (−101 to −275) mutants (pCAT-α10 and β11) were not above background in both cases. These observations suggest that the region between positions +25 and −366, being common to both genes, has promoter activity for both directions, whereas the more limited region (−169 to −238) is important for bidirectional promoter function.

Transcriptional Factors Binding to the Bidirectional Promoter Region—DNase I footprint analysis for the −169 to −238 region was done using nuclear extract from HeLa cells (Fig. 4). Five protected regions appeared when the 276-bp (−90 to −366) fragment was digested with DNase I in the presence of the nuclear extract: region I, positions −185 to −190; region II, −192 to −203; region III, −213 to −224; region IV, −230 to −240; and region V, −250 to −265. Protection in these regions was enhanced by increasing the amount of nuclear extract.

To confirm which protected regions have promoter activity for both genes, we further analyzed several deletion mutants (pCAT-α4–9 and pCAT-β6–10) (Fig. 5). Significant decreases of promoter activities occurred when truncation to region II for HADHA (pCAT-α8) and region IV for HADHB (pCAT-β10) genes. These findings suggested that regions II and IV are important for HADHA and HADHB, respectively.

To confirm the binding of nuclear proteins to regions II and IV, gel mobility shift assays were done using 32P-labeled synthetic oligonucleotides, fragments A and B for regions II and IV, respectively. The major complexes, C1 and C2, formed with fragments A and B, respectively, disappeared in a dose-dependent manner when unlabeled probes were added (Fig. 6). When the unlabeled oligonucleotides containing mutations in regions II and IV were used as competitors, the intensity of complexes...
C1 and C2 remained unchanged (Fig. 6).

Both sites contain consensus binding sites for transcription factors Sp1 and AP2 (Fig. 1). When 400-fold molar excess oligonucleotides of the Sp1, AP1, and AP2 consensus sites were used as competitors, the Sp1 consensus oligonucleotide specifically competed in formation of complexes C1 and C2. The AP1 consensus oligonucleotide did not compete, and the AP2 consensus oligonucleotide slightly inhibited the binding but only in fragment B (Fig. 7A). Binding of Sp1 to sites A and B was confirmed in supershift experiments using a polyclonal anti-Sp1 antibody. Electrophoretic mobilities of both complexes C1 and C2 were slower in the presence of an antibody (Fig. 7B).

**Regulative Function of Sp1 on the Bidirectional Promoter**—Mutations were introduced at regions II and IV in pCAT-α3 and pCAT-β4, respectively. Mutations at regions II and IV were the same as for Fig. 6, to which Sp1 did not bind. Each of the recombinants was transfected into HEK293 cells, and reporter gene expression in the transfected cells was examined (Fig. 8). The promoter activity of pCAT-α3 decreased to 25, 40, and 25% in constructs mutated at regions II, IV, and both, respectively. Similarly, the activity of pCAT-β4 was reduced to 50, 40, and 40% by mutation at regions II, IV, and both, respectively. Drosophila Schneider S2 cells lacking Sp1 were used to examine the function of Sp1 on the bidirectional promoter (Fig. 9). Luciferase constructs were transfected into the S2 cells with or without Sp1-expressing vector (pCMV-Sp1). Without Sp1-expressing vector, luciferase constructs of pLUC-α3, β4, αm3, and βm3 did not have promoter activities, as seen in the control wild type Wilms’ tumor-1 gene, the promoter activity of which depends on Sp1 (19). Co-transfection of pCMV-Sp1 with normal constructs led to an 11- and 10-fold increase in expression of pLUC-α3 and -β4, respectively, but to a <2-fold increase in the expression of pLUC-αm3 and -βm3 (Fig. 9). These findings suggest that expressing of HADHA and HADHB genes depends on Sp1, and that regions II and IV are “minimal” elements required for expression.

**DISCUSSION**

We obtained evidence that human HADHA and HADHB genes encoding subunits of the multienzyme complex TP exist side by side in a head-to-head arrangement on chromosome 2p23. The multienzyme complex of fatty acid β oxidation in various organisms has been identified. The complex consists of two subunits with high amino acid sequence homology to human TP (3, 20, 21). In prokaryotes, two genes for the enzyme complex fadA and B in Escherichia coli and faoA and B in Pseudomonas fragi, which are multienzyme complexes similar to human TP, are tandemly arranged on the bacterial operon (9, 20, 21). Head-to-head arrangement of human TP genes differs from these cases and may have occurred by gene inversion during processes of evolution. The structure of the HADHA and HADHB locus is unique.

The intergenic region between exons 1 of HADHA and HADHB has bidirectional promoter function regulated by transcription factor Sp1. The two Sp1 sites are critical for promoter expression of both genes, but they are not the minimal elements required for expression. Thus, this region probably functions as a common promoter of the two genes. In our foregoing
Gel mobility shift assays of regions II and IV. To confirm the binding of nuclear proteins to regions II and IV, gel mobility shift assays were done using 32P-labeled synthetic oligonucleotides, fragments A and B for regions II and IV, respectively. Five micrograms of protein of nuclear extract from HeLa cells were used. Sequences of oligonucleotides used as probes (Normal) and competitors (Normal and Mutant) are given at the bottom. The substituted bases in mutants are shown by small letters. Amounts of competitors used are 10-, 100-, and 400-fold molar excess, respectively. The positions of major complexes C1 and C2 are indicated on the right.

Identification of transcription factors binding to the regions II and IV. A, 400-fold molar excess of unlabeled oligonucleotides of fragments A and B for regions II and IV, respectively, and consensus binding sites of Sp1, Ap1, and Ap2 were used as competitors. Regions II and IV bound with nuclear proteins. Its major complexes, C1 and C2, respectively, disappeared by adding unlabeled the Sp1-consensus oligonucleotide specifically. B, supershift assay was performed using 1 μg of anti-human Sp1 antibody, which recognizes Jun family proteins (c-Jun, JunB, and JunD). The positions of complexes C1 and C2 are indicated on the right. Open arrowheads indicate the complexes with antibody.
Fig. 8. Mutational evidence for the roles of regions II and IV on the promoter. The -366 to +25 region was mutated at regions II and IV using mutant oligonucleotides described in Fig. 6. The CAT constructs represented on the left were independently transfected into HEK293 cells, and CAT activity in the transfected cells was assayed. Normal and mutant sequence within regions II and IV are indicated by the open box and X within the open box, respectively. Transfection of constructs into HEK293 was performed in triplicate, and means of CAT activities were compared with that of pCAT-Basic.

Fig. 9. Effect of Sp1 on promoter activity. The constructs pLUC-α3, B4, Am3, and Bm3 were transfected into Drosophila Schneider S2 cells without (open bar) or with pCMV-Sp1 (solid bar). Transfection of the constructs was triplicated, and means of luciferase activities were compared with that of control vector pLUC-WT, Wilms’ tumor-1 gene, regulated by Sp1.

reports, we stated that the association of both subunits is required for stabilization in mitochondria (14, 15). The coordinated gene expression of HADHA and HADHB, regulated by the common promoter, seems appropriate for the association and for subsequent stabilization. However, levels of individual subunit mRNA in organs are not always similar (3, 13). Promoter activities of HADHA constructs are different in HeLa and HEK293 cell lines (Fig. 3). Therefore, other regions and/or other transcription factors may be involved in regulating the cell type-specific expression of the two genes.

Promoters having bidirectional functions have been identified in surf-1 and surf-2 of the surfeit locus (22), the murine and human collagen IV genes α1 and α2 (23, 24), histone H2A and H2B genes (25), DHFR and Rep-1 genes (26, 27), and the Wilms’ tumor locus (28). These pairs of genes are coordinately expressed in general, and their gene products have similar functions. HADHA and HADHB genes code for proteins different in structure and function, although the proteins belong to the same metabolic system, fatty acid β oxidation, and form an enzyme complex. This character differs from the gene pairs described above; hence, the HADHA-HADHB gene pair is unique.

Expression of the mitochondrial medium-chain acyl-CoA dehydrogenase gene is regulated by nuclear receptor response elements, which bind with orphan members of the steroid/thyroid nuclear receptor superfamily, and Sp1 (29). In addition, it is known that several genes in fatty acid β oxidation systems, such as peroxisomal acyl-CoA oxidase (30), peroxisomal bifunctional enzyme (31), and mitochondrial acetyl-CoA thiolase (32), have GC boxes and lack typical TATA and/or CAAT boxes in their putative promoter regions (29). Thus the expression of many genes at β oxidation of fatty acids seems to relate to Sp1.

It was reported that induction of TP in rat liver by the administration of di-(2-ethylhexyl) phthalate, one of the peroxisome proliferators, was 10-fold (1). Using peroxisome proliferator-activated receptor α null mice, it has just been reported that expression of HADHA and HADHB genes in the liver is enhanced coordinately by administration of a potent hypolipidemic reagent, Wy-14,643, and that these enhancements are mediated by peroxisome proliferator-activated receptor α (33). In our numerous experiments done to identify peroxisome proliferator responsive elements on both the HADHA and HADHB genes, we found no active element binding with peroxisome proliferator-activated receptor α, at least in 15-kb regions sandwiching the common promoter region (data not shown). Thus, the mechanism of peroxisome proliferator-activated receptor α-dependent induction of HADHA and HADHB expression remains to be determined.

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