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Alternative Usages of Multiple Promoters of the Acetyl-CoA Carboxylase β Gene Are Related to Differential Transcriptional Regulation in Human and Rodent Tissues*

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Acetyl-CoA carboxylase β (ACC β) is a critical enzyme in the regulation of fatty acid oxidation and is dominantly expressed in the skeletal muscle, heart, and liver. It has been established that two promoters, P-I and P-II, control the transcription of the ACC β gene. However, the precise mechanism involved in controlling tissue-specific gene expression of ACC β is largely unknown yet. In this study we revealed that promoter P-I, active in the skeletal muscle and heart but not in the liver, could be activated by myogenic regulatory factors and retinoid X receptors in a synergistic manner. Moreover, P-I was also activated markedly by the cardiac-specific transcription factors, Csx/Nkx2.5 and GATA4. These results suggest that the proper stimulation of P-I by these tissue-specific transcription factors is important for the expression of ACC β according to the tissue types. In addition, CpG sites around human exon 1a transcribed by P-I are half-methylated in muscle but completely methylated in the liver, where P-I is absolutely inactive. In humans, the skeletal muscle uses P-II as well as P-I, whereas only P-I is active in rat skeletal muscle. The proximal myogenic regulatory factor-binding sites in human P-II, which are not conserved in rat P-II, might contribute to this difference in P-II usage between human and rat skeletal muscle. Hepatoma-derived cell lines primarily use another novel promoter located about 3 kilobases upstream of P-I, designated as P-O. This study is the first to explain the mechanisms underlying the differential regulation of ACC β gene expression between tissues in living organisms.

In mammals, acetyl-CoA carboxylase (ACC)¹ is a critical enzyme in fatty acid metabolism. ACC exists as two isoforms, α and β , that are encoded by the separate genes and show different tissue distribution (1–5). Because ACC β is associated with the mitochondrial outer membranes, the changes in its activity

affect the concentration of malonyl-CoA around the mitochondria (3, 6). Malonyl-CoA is a negative modulator of carnitine palmitoyltransferase-I (CPT-I), which is the rate-limiting enzyme in the fatty acyl-CoA transport system for fatty acid β -oxidation. Therefore, ACC β plays a critical role for regulating mitochondrial fatty acid oxidation.

ACC β is expressed abundantly in heart, skeletal muscle, and liver, all places in which fatty acid oxidation actively occurs (2, 7, 8). ACC β transcripts contain two species of 5'-UTRs, which contain either the sequence of exon 1a or of exon 1b via the alternative usage of two promoters, *i.e.* P-I and P-II. Exon 1a and exon 1b are located ~15 kilobases apart in human genome but are both connected to the common exon 2 in mRNA after splicing. However, the two transcripts encode for the same protein because they both use the same ATG start codon for translation, which resides in exon 2 (3, 9).

In skeletal and cardiac muscles, ACC β activities are reported to be rapidly regulated via phosphorylation by AMP-activated protein kinase in response to exercise, resulting in increases in fatty acid β -oxidation (4, 10–14). The liver is another organ that actively oxidizes fatty acids, although the purpose of fatty acid oxidation in the liver differs from its functions in skeletal and cardiac muscles. Hepatic fatty acid oxidation provides acetyl-CoA for the production of ketone bodies during periods of fasting. Recently, we reported that hepatic ACC β is regulated by sterol regulatory element-binding protein-1 in response to feeding status, through the P-II (15). The metabolic changes in the liver in response to environmental stimuli are not as rapid as those in skeletal and cardiac muscles. This implies that the change in ACC β amounts by transcriptional regulation is important in the liver, although the rapid regulation of enzyme activity by phosphorylation/dephosphorylation is the major control in skeletal and cardiac muscles.

P-II is also active in human skeletal muscle and is regulated by myogenic regulatory factors (MRFs) (9). MRFs, including Myf5, MyoD, myogenin, and MRF4, are basic helix-loop-helix transcription factors involved in myogenic differentiation. Although these factors all recognize the common consensus sequence, E-box (CANNTG), four MRFs are expressed in a temporally distinct pattern during myocyte differentiation. Myf5 and MyoD have been shown to establish the myogenic lineage during embryogenesis, whereas myogenin and MRF4 play a major role in the expression of muscle genes in fully differentiated myotubes (16–19). These factors physically interact with retinoid acid receptors and act as transcriptional activators during differentiation (20–22). The synergistic action between MRF4 and RXR, which are the abundant members of their families in fully differentiated myocytes, is most effective in the

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¹ The abbreviations used are: ACC, acetyl-CoA carboxylase; MRF, myogenic regulatory factor; 5'-UTR, 5'-untranslated region; RXR, retinoid X receptors; RAR, retinoic acid receptors; PIPES, 1,4-piperazine diethanesulfonic acid.

TABLE I
Sequences of oligonucleotides used in the experiments

Name	Sequences
prP-II β -(-1864/+14) -1864_S +14_AS	5'-CTC CTC CCT ACG CGT GGT TCT CTC TCA-3' 5'-TGA GTG GCA GCA GTG ACC TA-3'
phP-I β -(-1735/+100) -1735_S +100_AS GATA4_S GATA4_AS 1a_AS 1o_AS	5'-TGA GGC AGG AGG TAC CTT TGA GCC CA-3' 5'-TAA CCC TGA ATG CAC GGT GG 3' 5'-AGA GGG ATC CGC GAT GTA CCA AAG-3' 5'-GAA GGC TCG AGT GAT TAC GCG GT-3' 5'-TTT CAA GCT CCT CTG TGG CT-3' 5'-ACA GGA ATC ATT AGG CCA GGT-3'
phP-O β -(-1143/+191) -1143_S +191_AS CpG_S CpG_AS hexon1o_S hexon1b_S hexon2_S hexon2_AS β -Actin_S β -Actin_AS	5'-AGC AGT GAG CTC CAA GTT TCC A-3' 5'-ACA GGA ATC ATT AGG CCA GGT-3' 5'-GGT AGA GTA AGT AGT TAG TAG G-3' 5'-GGG TCT TCT AAT TAA CTT CCT TCA-3' 5'-AGC CTG CCT CTG CAA AGG CAG GAC C-3' 5'-TGT GGG CGC CTG TCA GCC TCA CTC A-3' 5'-GCA AAC CTC ATC CCG AGC CAG GAG C-3' 5'-CCA GCA ACA GAG TCC TCG TCG GAG G-3' 5'-ATG GAA TCC TGT GGC ATC CA-3' 5'-ACC AGA CAG CAC TGT GTT GG-3'

activation of ACC β P-II activity in humans (23).

The level of ACC β is higher in the heart than in skeletal muscle. However, it is currently not clear as to which promoter directs ACC β expression in the heart. Cardiomyocyte-specific transcription factors, such as Csx/Nkx2.5, GATA4, MEF2, and eHand but not MRFs, have been implicated in cardiac development and cardiac gene expression. The cardiac-specific homeobox protein, Csx/Nkx2.5, and the zinc finger protein, GATA4, function as critical transcription factors in cardiac development (24–27) and synergistically activate a number of cardiac genes, such as the atrial natriuretic factor gene, the iodothyronine deiodinase gene, and the α -actin gene (28–31). In the present study our intention was to identify the promoter that directs ACC β expression in the heart and to prove that the ACC β promoter is indeed activated by the cardiac-specific transcription factors, Csx/Nkx2.5 and GATA4.

ACC β is actively expressed in the skeletal muscle, the heart, and the liver, and its gene expression is differentially regulated in the respective organs. The mechanisms underlying this phenomenon remain an enigma. In the present study we proved that ACC β levels change drastically in liver as a response to feeding status, whereas they are maintained at a constant level both in skeletal muscle and in the heart. This differential regulation of ACC β gene expression originates in the alternative usage of promoters, such as P-I and P-II. P-I is the sole promoter found in the heart and skeletal muscle of rats, although both P-I and P-II are active in human skeletal muscle. We demonstrated the activation of P-I via synergistic action between MRF4 and the retinoid X-receptor as well as Csx/Nkx2.5 and GATA4, which explains the tissue-specific activation of P-I in both the skeletal muscle and the heart. We also elucidate that the CpG sites around exon 1a are half-methylated in skeletal muscle, in contrast to their complete methylation in the liver, resulting in the silencing of P-I. This study is the first to explain the mechanisms underlying the differential regulation of ACC β gene expression in human and rodent tissues.

EXPERIMENTAL PROCEDURES

Animals and Diets—Male Sprague-Dawley rats, weighing 150–200 g, were used for all experiments. For the fasting and refeeding study, rats were put on a fast for 48 h and then refed with a fat-free high carbohydrate diet for 0, 24, or 48 h. All experiments were performed at least three times. The fat-free high carbohydrate diet contained 82% (w/w) carbohydrates (74% starch, 8% sucrose), 18% (w/w) casein, 1% (w/w) vitamin mix, and 4% (w/w) mineral mix. All the materials for the diet were purchased from Harlan Teklad Co. (Madison, WI).

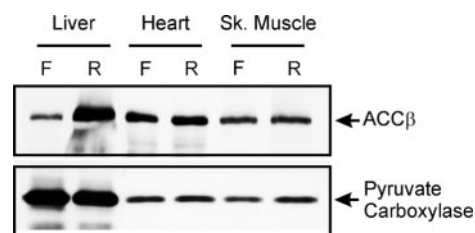


FIG. 1. The different regulation of ACC β expression in the liver, heart, and skeletal muscle. Proteins were extracted from the livers, hearts, and skeletal (*Sk.*) muscles of Sprague-Dawley rat groups, which had fasted for 48 h (*F*) or fasted and then were refed for 48 h (*R*), as described under "Experimental Procedures." Fifty micrograms of extract proteins were run on 5% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were stained using polyclonal anti-ACC β antibody and streptavidin-conjugated horseradish peroxidase for the detection of ACC β and internal control, pyruvate carboxylases, respectively. The bands for ACC β and pyruvate carboxylase were indicated.

Western Blot Analysis—Rat tissues were homogenized in 50 mM sodium phosphate buffer, pH 7.4, containing 10% (v/v) glycerol, 10 mM β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 \times protease inhibitor mixture with glass pestles and then centrifuged at 5000 rpm at 4 °C for 10 min. Supernatants were precipitated in 12.5% polyethylene glycol. Precipitated proteins were dissolved in 1/5 initial volume of homogenization buffer, and the concentration of soluble protein was determined by the Bradford assay (Bio-Rad). Extracts were separated in 5% SDS-polyacrylamide gel and transferred onto Protran nitrocellulose membranes (Schleicher & Schuell). Immunoblot analysis was carried out with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) and polyclonal anti-ACC β antibody, and specific bands were visualized using a SuperSignal West Pico Trial Kit (Pierce).

RNAse Protection Assays—Rat cRNA probes were synthesized from the sequences of either exon 1a (90 bp) or exon 1b (52 bp) extending to exon 2 (69 bp) by *in vitro* transcription. Human cRNA probes I, II, and O were established from pCRII plasmids containing sequences of exon 1a (58 bp), 1b (60 bp), and 1o (56 bp) extending to 100 bp of exon 2. After linearization of each plasmid (1 μ g) by HindIII digestion, ³²P-labeled cRNA was synthesized by T7 RNA polymerase (Ambion, Austin, TX). Probes were purified by gel elution after electrophoresis with 6% polyacrylamide, 6 M urea gel. RNase protection assays with purified probes were performed with the RPAIII kit (Ambion, Austin, TX). The total RNA (20 μ g) isolated from rat livers was hybridized with a probe (1.6 \times 10⁵ cpm) in 30 μ l of hybridization buffer at 42 °C for 12–16 h. The unhybridized RNA was digested by adding 150 μ l of the diluted solution (1:100) of RNase A/T1 at 37 °C for 30 min. Probes protected from RNase were precipitated by the addition of 225 μ l of RNase inactivation/precipitation III solution followed by 15 min of centrifugation at 12,000 rpm. Precipitates were washed with 70% ethanol and then denatured

FIG. 2. Analysis of 5'-UTR of ACC β transcripts expressed in skeletal muscle, heart, and liver. RNase protection assays were performed using total RNAs prepared from skeletal muscles, hearts (A), and livers (B) of rats that had fasted for 48 h (F) or had fasted and then been refed on a high carbohydrate diet for 24 h hours *ad libitum* (R). Antisense RNA probe I and probe II, consisting of 90 bp of exon 1a or 52 bp of exon 1b, respectively, and the common 69 bp of exon 2 were prepared as described under "Experimental Procedures." After total RNA was hybridized with each probe, the unhybridized parts of the probes were removed by treatment with RNase A/T1 mix. The sizes of the protected probes were analyzed by electrophoresis on 6% denaturing polyacrylamide gel. A negative control using yeast tRNA instead of total RNA and the full length of probes by omission of RNase A/T1 addition are indicated by a - and -RNase, respectively.

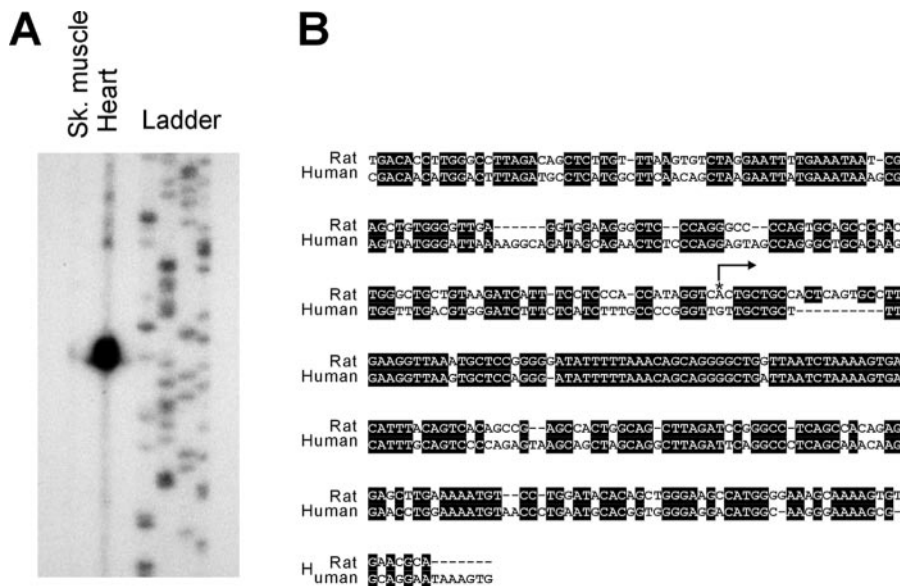
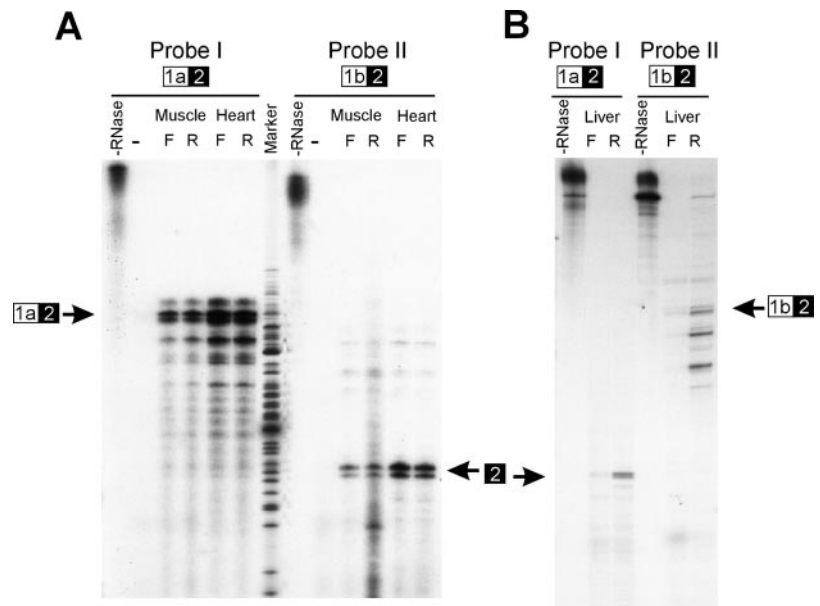


FIG. 3. Primer extension analysis revealed the length of exon 1a to be 201 bp. A, transcription start site in ACC β P-I was identified by primer extension analysis using rat skeletal (*Sk.*) muscle and heart RNAs. Total RNA was hybridized with antisense oligonucleotide probe, locating the region of exon 1a, and then reverse transcription was performed. RNA was digested with RNase A/H mix, and final primer-extended products were resolved on 6% denaturing polyacrylamide gel. The sequence ladder is shown for size determination. B is the sequence comparison of ACC β P-I and exon 1a between rat and human. An asterisk (*) indicates the transcription start site of rat ACC β P-I.

with 4 μ l of sequencing gel-loading buffer at 95 $^{\circ}$ C for 3 min, then resolved on 6% polyacrylamide, 6 M urea gel. Gels were dried and exposed to Kodak BioMax film at -70 $^{\circ}$ C with intensifying screens. A sequencing ladder was loaded in the adjacent lane to determine the size of the products.

Primer Extension Analysis—Primer extension was performed as described by Kim *et al.* (32). Antisense oligonucleotides of rat exon 1a and human exon 1a, 1a_AS, and 1a_AS were labeled with [γ - 32 P]ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase. The labeled oligonucleotides (2×10^5 cpm) were mixed with 50 μ g of rat skeletal muscle, heart, and HepG2 RNAs in 100 μ l of hybridization buffer (40 mM PIPES, pH 6.8, 1 mM EDTA, 0.4 M NaCl, 80% deionized formamide). The mixtures were incubated at 90 $^{\circ}$ C for 3 min and hybridized overnight at 42 $^{\circ}$ C. Annealed mixtures were precipitated by ethanol and used for the extension reaction. These mixtures were extended with SuperScriptTMII (Invitrogen) at 42 $^{\circ}$ C for 1 h under buffer conditions specified by the manufacturer's instructions. After phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation, the sizes of the products were determined by 6% denaturing polyacrylamide gel electrophoresis. The lengths of rat exon 1a and human exon 1a were determined by comparing to sequencing products of the cloned promoter region in both rats and humans.

Construction of Plasmids—The luciferase constructs of human ACC β P-II, pH-P-II β (-569/+65), and pH-P-II β (-93/+65), were described by Lee *et al.* (9). The oligonucleotides used in promoter construction are shown in Table I. pH-P-I β (-1735/+100) was constructed by amplifying

the human ACC β promoter region and introducing it into the SmaI site of the pGL3-Basic vector. Constructs of prP-I β (-1864/+14), prP-II β (-485/+65), and prP-II β (-90/+65) were generated from the rat ACC β promoter region and cloned in the SmaI sites of pGL3basic. pH-P-O β (-1143/+191) was constructed by amplifying the upstream region containing human ACC β exon 1a and introducing it into the SacI/SmaI site of the pGL3-Basic vector. Rat GATA4 cDNA was amplified by PCR and inserted into the HindIII/XhoI site of pcDNA3. The plasmid of pcDNA3-mycCSX was a generous gift from Dr. Issei Komuro (Chiba University, Chiba, Japan).

Cell Culture and Transient Transfection—All reagents for cell cultures and Lipofectamine PLUS reagents were purchased from Invitrogen. NIH3T3 (Dulbecco's modified essential medium (DMEM)), C2C12 (DMEM), Alexander (minimal essential medium (MEM)), HepG2 (MEM), Hep3B (RPMI1640), and PLC/PRF5 (RPMI1640) cells were cultured in medium supplemented with 10% (v/v) fetal bovine serum and 100 μ g/ml antibiotics/antimycotics at 37 $^{\circ}$ C in an 80 ~ 90% humidified CO $_2$ incubator. Rat primary hepatocyte culture and transfection were performed as describe by Ahn *et al.* (33). Cells were prepared for experiments on 6-well plates at $2.5 \times 10^5 \sim 1 \times 10^6$ cells per well and then incubated for about 20 h. When cells were 80% confluent, cells were transfected with the indicated plasmids using Lipofectamine PLUS according to the manufacturer's protocols. The plasmid DNA and 3 μ l of PLUS reagent were mixed in 100 μ l of serum-free media and then added to 100 μ l of serum-free media containing 2 μ l of Lipofectamine reagent. The total amounts of DNA per well were adjusted to

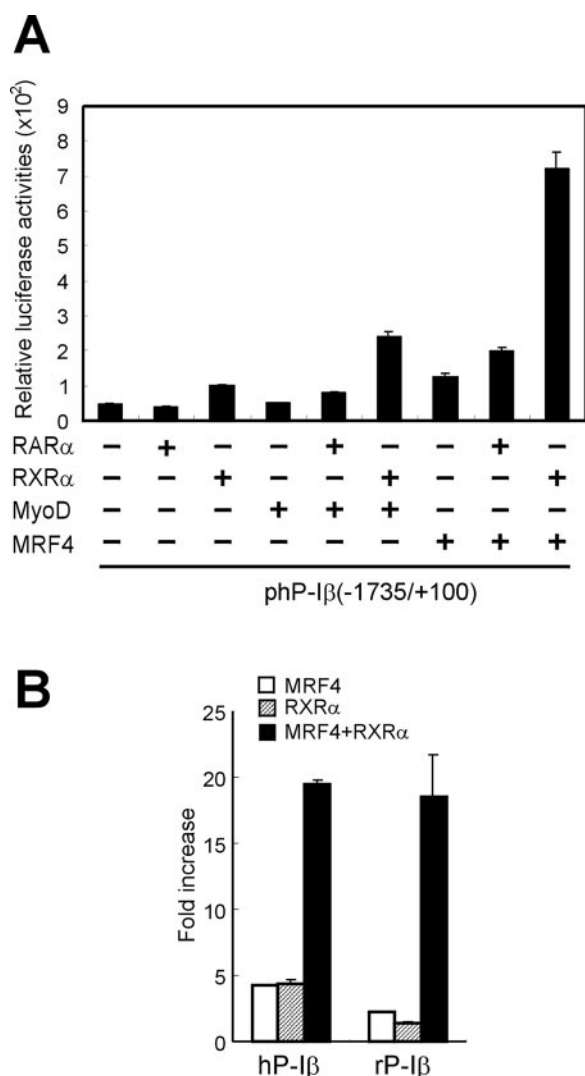


FIG. 4. Activation of ACC β P-I by MRFs and retinoic acid receptors (RAR and RXR). In *A* the phP-I β (-1735/+100) reporter construct (0.4 μ g) and pCMV- β -gal reference construct (0.1 μ g) were transfected into NIH3T3 cells in company with overexpression vectors (0.1 μ g) of pcDNA3, pcRAR α , pcRXR α , pcMyoD, and pcMRF4, as indicated in the figure. In *B* the responsiveness of human and rat ACC β P-I to MRF4 and RXR α was assayed with phP-I β (-1735/+100) and rP-I β (-1864/+14) reporter constructs. Total amounts of transfected DNA were adjusted to 0.7 μ g with pcDNA3. In the groups overexpressing RAR α or RXR α , the respective ligand of all-*trans*- or 9-*cis*-retinoic acid was added 24 h after transfection to a final concentration of 1 μ M. Luciferase activities were measured 48 h after transfection and normalized by β -galactosidase activities. The data are represented as the mean \pm S.D. of three independent experiments, each performed three times.

the same amounts by the addition of mock vector plasmid. The cells were washed with PBS, and supplied with 800 μ l of serum-free media during incubation. After 15 min, Lipofectamine-DNA mixture was added to the wells. The cells which had been transfected for 3 h were washed twice with phosphate-buffered saline then grown for 48 h in media supplemented with 10% fetal bovine serum and 100 μ g/ml antibiotics/antimycotics. RXR and RAR ligands, 1 μ M 9-*cis*-retinoic acid and all-*trans*-retinoic acid, were treated after 20 h since cells were transfected and cultured further for additional 24 h. Cells were harvested and lysed with 200 μ l of reporter lysis buffer (Promega, Madison, WI), and cell debris was removed by centrifugation. Luciferase activities were measured using 10 μ l of cell extract and 50 μ l of luciferase assay reagent (Promega). For the β -galactosidase assay, the color changes of extracts by hydrolysis of *o*-nitrophenol- β -D-galactopyranoside (Sigma-Aldrich) were detected as kinetics at 420 nm at 37 $^{\circ}$ C for 5 min.

Methylation Analysis of CpG Islands—These genomic DNA were prepared from human muscle, liver, and HepG2 cell lines. Each tissue

was ground using liquid nitrogen and lysed in lysis buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS, 20 μ g/ml RNase A, 1 mg/ml proteinase K) at 50 $^{\circ}$ C for 5 h. After phenol:chloroform:isoamyl alcohol extraction, DNA precipitated by ethanol was picked up and was dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). After 10 μ g of genomic DNA was digested by 10 units of EcoRI at 37 $^{\circ}$ C for 5 h, unmethylated C residues were converted into U residues via the bisulfite reaction. In brief, 2 μ g of linearized DNA were denatured in 0.3 M NaOH at 37 $^{\circ}$ C for 20 min and treated with 550 μ l of converting solution (10 mM hydroxyquinone, 2.8 M sodium bisulfite, pH 5.0) then incubated in 55 $^{\circ}$ C for 16 h in darkness. Sulfonated single-strand DNA fragments were purified using the Wizard DNA Clean-Up system (Promega). Sulfonated C residues were desulfonated and deaminated with 0.3 M NaOH at 37 $^{\circ}$ C for 15 min and neutralized with 3 M ammonium acetate, pH 7.0. The converted DNA in which C residues had been converted to U residues was precipitated with ethanol and dissolved in 50 μ l of TE buffer. The primers were designed according to C-to-T converted sequence of the region surrounding exon 1a as denoted in Table I as CpG_S and CpG_AS. The PCR reaction mixture contained 10 μ l of converted DNA, 0.2 pmol of primers, Gold Taq reaction buffer, 1.5 mM MgCl₂, 1.25 mM dNTPs, and 1 unit of Gold Taq polymerase (Roche Applied Science) amplified as follows: denaturation for 5 min at 94 $^{\circ}$ C, 40 cycles of denaturation for 30 s at 94 $^{\circ}$ C, annealing for 30 s at 52 $^{\circ}$ C, and extension for 30 s at 72 $^{\circ}$ C. Amplified products were directly sequenced using CpG_S primer.

Reverse Transcription-PCR—Total RNAs were extracted from Alexander, HepG2, Hep3B, and PLC/PRF5 hepatoma cells using the TRIzol according to the manufacturer's instructions. First-strand cDNAs were synthesized from 5 μ g of total RNA in 20 μ l of reaction volume using SuperScript II reverse transcriptase. Each reverse transcription mixture (1 μ l) was used as the template for amplifying ACC β cDNA. The sense primers for each ACC β transcript, spanning exon 1a (hexon1a_S), exon 1b (hexon1b_S), and exon 2 (hexon2_S), and antisense primer-containing sequence of exon 2 (hexon2_AS) were used in this experiment. The sizes of the PCR products were determined on 1% agarose gel.

RESULTS

Changes of ACC β Expression Levels in Rat Liver, Heart, and Skeletal Muscle by Diet—ACC β is expressed predominantly in skeletal muscle and in the heart, where the β -oxidation of fatty acid actively occurs, constituting a major energy source. Sterol regulatory element-binding protein-1 was previously reported to induce ACC β gene expression in the liver as a response to the intake of a high carbohydrate diet. We attempted to ascertain whether or not ACC β levels in the heart and skeletal muscle changed in response to feeding status, as did ACC β levels in the liver. The levels of pyruvate carboxylase, detected with streptavidin-horseradish peroxidase conjugate as a control, were almost the same between fasted and refed groups in liver, heart, and skeletal muscle extracts. The nutritional control had no significant effect on ACC β expression in heart and skeletal muscle, whereas hepatic ACC β levels were drastically increased by food intake (Fig. 1). This result is consistent with the findings of many previous reports, in that the posttranslational regulation of ACC β was a much more important regulatory mechanism in skeletal muscle rather than were changes in enzyme levels (10–12, 33). This also suggests that the transcriptional controls for the expression of ACC β are quite different between cardiac/skeletal muscle and the liver.

Differences of Promoter Usage in Rat Cardiac/Skeletal Muscles and Livers—It was reported that human and rat ACC β gene expression could be derived from two types of promoters, designated as P-I and P-II (9). Differences in the regulation of ACC β gene expression between tissues led us to perform RNase protection assay to determine which promoter is active in the respective organs. Antisense RNA probes used in RNase protection assays contained either exon 1a or exon 1b joined to the exon 2 sequence and were designated probes I and II, respectively (Fig. 2). The total RNA was isolated from the relevant tissues of rats that had fasted for 48 h and refed with a fat-free high carbohydrate diet for 0 or 24 h. Exon 1a and 2 in probe I were fully protected in the rat skeletal muscle and heart, al-

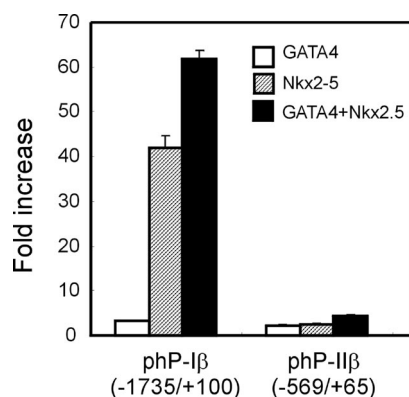


FIG. 5. **ACC β P-I is activated by GATA4 and the cardiac-specific homeobox Csx/Nkx2.5.** A transient transfection assay was performed to measure the levels of activation of ACC β P-I and P-II by GATA4 and Csx/Nkx2.5. The reporter construct (0.4 μ g) of phP-I β (-1735/+100) or phP-II β (-569/+65) and pCMV- β -gal (0.1 μ g) was co-transfected into the C2C12 cell in company with overexpression vectors (0.1 μ g) of pcDNA3 empty vector, pcDNA3-GATA4, and pcDNA3-mycCsx, as indicated in the figure. Luciferase activities were measured 48 h after transfection and normalized by β -galactosidase activities. The data are represented as the mean \pm S.D. of three independent experiments, each performed three times.

though the exon 1b sequences in probe II were almost digested by RNase, resulting in a band consistent in size with exon 2. Moreover, the intensities of the RNase protected bands were not affected by feeding conditions (Fig. 2A). In contrast, hepatic RNA protected only the exon 1b sequence in probe II from RNase digestion and not the exon 1a sequence of probe I. Food intake caused a marked increase in the level of hepatic ACC β transcripts (Fig. 2B). These data indicate that rat ACC β gene expression in heart and skeletal muscle is controlled by P-I, whereas P-II is a major promoter in the liver. The alternative promoter usages would appear to explain the mechanism of different transcriptional regulations of the ACC β gene between these organs.

Determination of Transcription Start Site in Rat ACC β P-I—In the previous report the size of human exon 1b transcribed by P-II was determined as 67 bp by primer extension analysis using the antisense primer corresponding to exon 2, although the size of exon 1a was not determined due to its large size (9). The length of exon 1a was also expected to be much greater than that of exon 1b, judging from sequences of clones obtained from 5' rapid amplification of cDNA ends (data not shown). To determine the precise transcription start site in P-I, we performed a primer extension analysis using total RNA isolated from rat skeletal and cardiac muscle as the templates and antisense primers corresponding to exon 1a. The size of exon 1a was revealed to be 201 bp in the rat ACC β gene according to the size of the primer-extended product (Fig. 3A). Interestingly, the sequence of exon 1a shows higher conservation between human and rat than the 5'-flanking region of exon 1a (Fig. 3B). However, we cannot find any conserved promoter element, such as the TATA-, CCAAT-, or GC-box, in the proximal P-I promoter.

ACC β Promoter I Is Activated by MRFs and Retinoic Acid Receptors (RAR and RXR)—The fact that the muscle-specific expression of ACC β is controlled by promoter P-I, as shown in Fig. 2, led us to check the responsiveness of ACC β P-I to MRFs and retinoic acid receptors, which are important transcription factors mediating the expression of muscle-specific genes. The luciferase reporter construct, containing the human P-I sequence in front of the luciferase gene, was transiently transfected in company with the expression vectors for MyoD, MRF4, RAR α , and/or RXR α into NIH3T3 cells. As shown in Fig. 4A, MyoD and RAR rarely affect P-I activities, and MRF4

and RXR α cause a 2–3-fold upshift in P-I activity. Combined treatment with ligand-activated retinoic acid receptors and MRFs synergistically activates P-I, and a combination of RXR α and MRF4 exhibits the most effective synergism, inducing a 16-fold increase in P-I activity (Fig. 4A). MRF4 and RXR α could also synergistically activate rat P-I, just as in human P-I (Fig. 4B). Because MRF4 and RXR are the most abundant forms of their families in fully differentiated muscle cells (20, 21), the synergistic action of MRF4 and RXR might play an important role in the muscle-specific expression of ACC β .

ACC β Promoter I Is Activated by GATA4 and Csx/Nkx2.5—ACC β is most abundantly expressed in the heart, suggesting the possibility that cardiac transcription activators, such as GATA4 and Csx/Nkx2.5, might activate ACC β P-I. As expected, human P-I of the ACC β gene was markedly activated by GATA4 and Csx/Nkx2.5, although P-II was not (Fig. 5). This would appear to explain the mechanism by which the high level of expression of ACC β in the heart is dependent on the P-I promoter. It was reported that GATA4 and Csx/Nkx2.5 exhibit synergy in a number of heart genes, such as the atrial natriuretic factor gene, the iodothyronine deiodinase gene, and the α -actin gene (28–31). However, Csx/Nkx2.5 alone had a tremendous effect on ACC β P-I, amounting to a 42-fold increase in activation, although GATA4 caused a less drastic upshift, inducing a 3-fold increase in activation. Although the activation by GATA4 alone was much less than by Csx2.5/Nkx, GATA4 significantly augments the activation of Csx2.5, inducing a 62-fold increase (Fig. 5).

Methylation of CpG around Exon 1a in Human—In addition to tissue-specific transcription factors, DNA methylation is another regulatory mechanism underlying tissue-specific gene expression. This fact that ACC β P-I is active exclusively in skeletal muscle and the heart, but not at all in the liver, prompted us to analyze the methylation status around exon 1a in each organ. Sodium bisulfite causes the deamination of intact cytosine to uracil, with the exception of methylated cytosine. After deaminating genomic DNAs, the sequence around exon 1a was amplified and directly sequenced (Fig. 6A). All cytosine in CpGs was almost completely protected from deamination in the liver genomic DNA, whereas cytosine in the HepG2 genomic DNA was changed to thymine. Interestingly, the C/T conversion ratio in CpG sequences in muscle genomic DNA was about 50%. These results clearly indicate that the CpG sequences around exon 1a are completely methylated in the liver, completely unmethylated in HepG2 cells, and half-methylated in muscle. The *in vitro* methylation of reporter construct abruptly prevented activation of human P-I by Csx/Nkx2.5 (data not shown), suggesting that the degree of CpG methylation may be an important factor in the tissue-specific activities of ACC β P-I.

Alternative Promoter Usage of the ACC β Gene in Human Skeletal Muscle—It was previously reported that human ACC β P-II is a muscle-specific promoter and exhibits no activity in hepatoma cell lines, such as HepG2 (9). However, the present study revealed that rat P-II is active only in the liver and not in skeletal muscle and the heart. These discrepant results led us to suspect that ACC β promoter usages in skeletal muscle might differ between rats and humans. To demonstrate whether the 5'-UTR of ACC β transcripts contains sequences of exon 1a or exon 1b, RNase protection assays were performed using total RNA isolated from human skeletal muscle and liver (Fig. 7). As expected, total RNA isolated from human skeletal muscle protected both exon 1a and exon 1b from RNase digestion, whereas liver RNA protected only exon 1b. This result indicated that human skeletal muscle used both P-I and P-II, in contrast to rat skeletal muscle, in which only P-I is utilized. The sequences

FIG. 6. CpG methylation status around exon 1a in liver, skeletal muscle, and HepG2 cells. In *A* the CpG sites around exon 1a were schematically illustrated. In *B* the deaminations of C residues were shown by direct sequencing of PCR products. The C residues, except methylated C residues, were deaminated into U residues by treating genomic DNAs extracted from human liver, HepG2 cells, and skeletal muscle with sodium bisulfite, as described under "Experimental Procedures." After the deamination reaction, the DNAs around exon 1a were amplified by PCR and directly sequenced. The results of sequencing around the 10 CpG sites were compared in the liver, HepG2, and skeletal (*Sk.*) muscle.

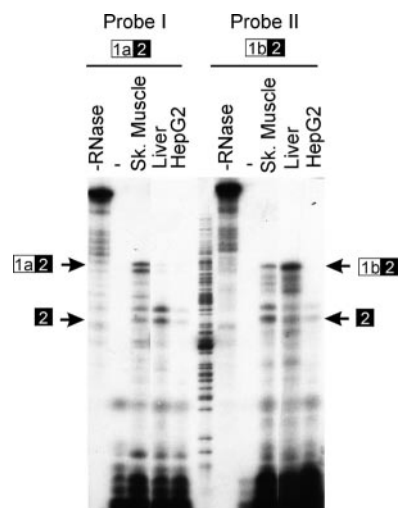
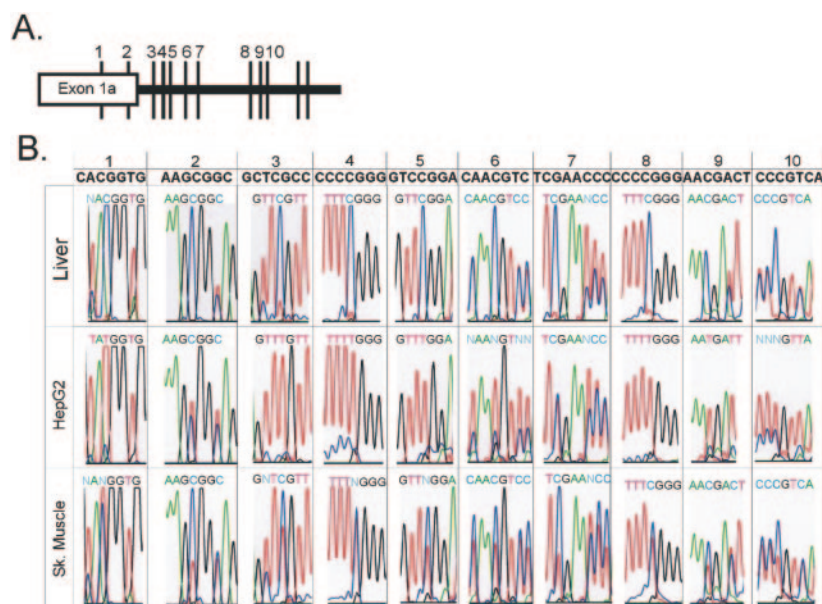


FIG. 7. Identification of 5'-UTR of ACC β transcripts expressed in human skeletal muscle, liver, and HepG2 cells. Total RNAs of human skeletal (*Sk.*) muscle, liver, and HepG2 cells were hybridized with cRNA probes I and II and then treated with RNase A/T1 mix. Probes I and II consist of 58 bp of exon 1a or 60 bp of exon 1b, respectively, and a common 100 bp of exon 2. The RNase protection assay procedures are described under "Experimental Procedures." A negative control using yeast tRNA instead of total RNA and the full length of probes by omission of RNase A/T1 addition are indicated by a - and -RNase, respectively.

around the transcription start site in P-II were well conserved between rats and humans. Previous reports revealed that the E-box and the novel MRF binding element on the proximal region of human P-II play an important role in MRF-mediated activation (9). These elements were, interestingly, not conserved in rat P-II (Fig. 8A). To determine differences in MRF responsiveness between human and rat promoters, transient transfection assays were performed. Overexpression of MyoD stimulated only human P-II, and not rat P-II (Fig. 8B). Taken together, the difference of these short elements for MRF binding might prove P-II to be active only in human skeletal muscle and not in rat skeletal muscle.

Another Promoter, P-O, Plays a Primary Role in ACC β Expression in Hepatoma Cell Lines—In HepG2 cells, ACC β expression was previously reported not to be driven by P-II (9), which is primary promoter in normal liver (15). In the present

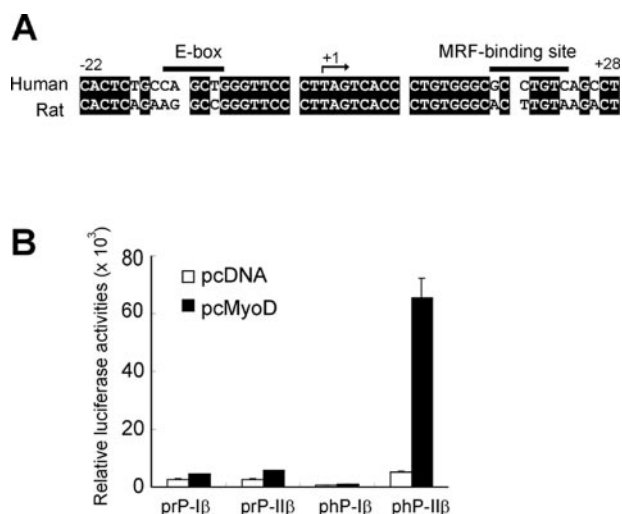


FIG. 8. MRF-binding sites in the region proximal to human ACC β P-II are not conserved in rat. In *A* the sequences of proximal ACC β P-II region were compared between human and rat. One E-box (-14 to -9) and a novel MRF-binding site (+17 to +24), previously reported in human ACC β P-II (9), were not conserved in the rat promoter. In *B*, the responsiveness of human and rat ACC β promoters to MyoD is assayed. Rat reporter constructs prP-I β (-574/+147) and prP-II β (-90/+65) and human reporter constructs pHP-I β (-961/+100) and pHP-II β (-93/+65) were used in this assay.

study, RNase protection assay revealed that neither P-I nor P-II appeared to play a role in ACC β gene transcription (Fig. 7). These results led us to study which sequences of ACC β gene direct the transcription in HepG2 cells. 5' rapid amplification of cDNA ends using the total RNA of HepG2 cells revealed the sequence of the 5'-UTR of the HepG2 ACC β transcript. Most clones isolated from 5' rapid amplification of cDNA ends contained an identical sequence corresponding to the region located about 3 kilobases upstream of exon 1a. This result indicated that another promoter, located 5' upstream of P-I, controls ACC β expression in HepG2, and we designated this promoter and exon as P-O and exon 1o (Fig. 9A). Next, an RNase protection assay was performed using an antisense RNA probe containing the exon 1o joined to exon 2 (Fig. 9B). Almost all of the HepG2 ACC β mRNA and the small portion of hepatic ACC β mRNA contained the exon 1o sequence. Next, we performed primer extension analysis and determined the tran-

FIG. 9. Promoter O plays a primary role in ACC β expression of hepatoma cell lines. A describes alternative usages of the promoters and splicing of ACC β gene transcription in HepG2 cells and *in vivo* liver and genomic distances of each exon. In B RNase protection assays identified the exon 1 α sequence in ACC β transcripts of HepG2 cells. Total RNA of HepG2 cells were hybridized with cRNA probe O, and then unhybridized single-strand RNAs were digested with RNase A/T mix. Probe O consists of 56 bp of exon 1 α and 100 bp of exon 2. *Sk*, skeletal. In C reverse transcription-PCR was performed to identify which promoter plays a major role of ACC β expression in hepatoma cell lines such as Alexander (A), HepG2 (G), Hep3B (B), and PLC/PRF5 (P). ACC β cDNA fragments containing exon 1 α /2, exon 1 β /2, or exon 2 or β -actin cDNA as the internal control were PCR-amplified, and the products were visualized on 1% agarose gel. In D, pH-P-O β (-1143/+100) and pH-P-II β (-569/+65) were transiently transfected into HepG2 cells and rat primary hepatocytes, respectively, and then luciferase activities were measured. Reporters and pCMV- β -gal were transfected 0.4 and 0.1 μ g in HepG2 cells and 1.8 and 0.2 μ g in primary hepatocytes, respectively. *kb*, kilobases.

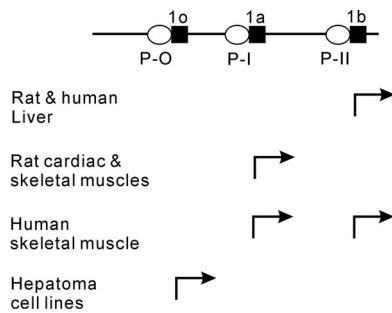
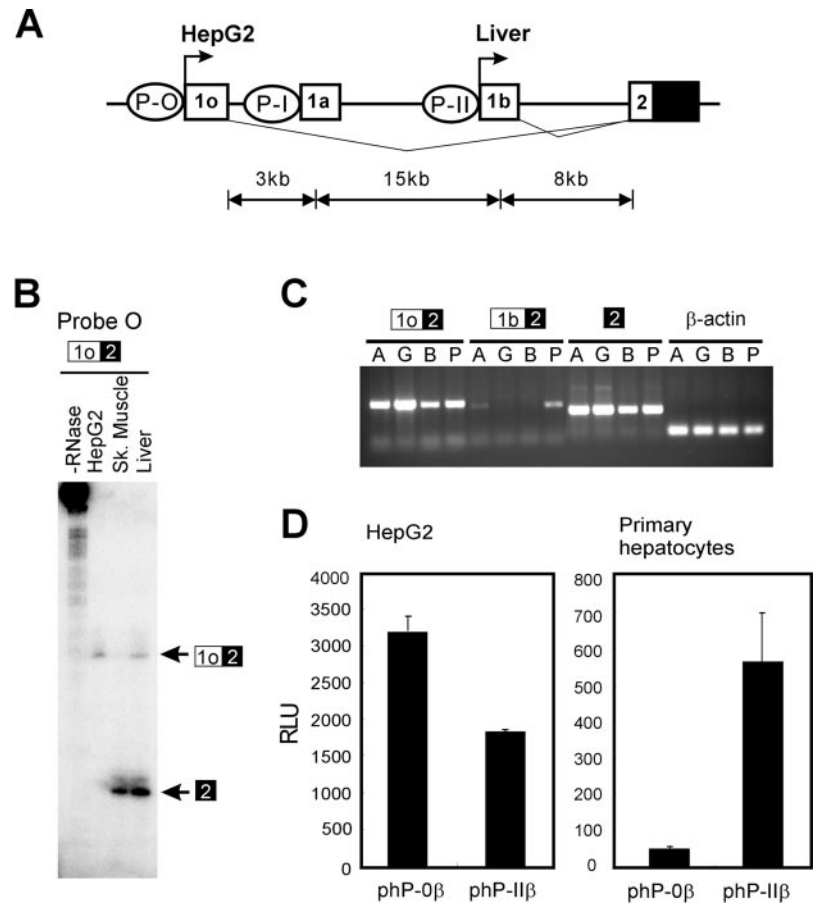


FIG. 10. Schematic diagram explaining alternative promoter usages for ACC β gene expression in rat and human tissues. In the livers of rat and human, ACC β gene expression is directed by the promoter P-II. In rat cardiac and skeletal muscles, the promoter P-I is a sole promoter to maintain the constant levels of ACC β , whereas both promoters of P-I and P-II are active in human skeletal muscle. Another promoter located 3 kilobases upstream of P-I controls ACC β expression in hepatoma cell lines, and we designated this novel promoter as P-O.

scription start site in P-O-driven transcription. The size of exon 1 α was 179 bp (GenBankTM AY701053). The analysis of the 5'-UTR of ACC β transcripts revealed that the major promoter is P-O in established human hepatoma cell lines such as Alexander, HepG2, Hep3B, and PLC/PRF5 (Fig. 9C). The promoter activities of P-O are higher than P-II in HepG2 cells, whereas P-II is much higher than P-O in the primary hepatocyte (Fig. 9D). These data indicate that the human hepatoma cell lines use the promoter O, which is not active in cardiac/skeletal muscle and the liver *in vivo*.

DISCUSSION

The regulation of ACC β activities is important for controlling the rate of fatty acid oxidation in the liver, heart, and skeletal

muscle. Hepatic ACC β expression is controlled transcriptionally by feeding status, and sterol regulatory element-binding protein-1 is a key transcription factor in this process. However, fatty acid oxidation increased during exercise is mainly mediated by the inactivation of ACC β by phosphorylation in skeletal muscle (4, 10, 11, 34). Thus, it is conceivable that ACC β activities are regulated slowly at the transcriptional level in the liver and immediately by phosphorylation/dephosphorylation of this enzyme in the heart and skeletal muscle. In this vein the transcriptional control of ACC β gene was expected to be quite different between the liver and the cardiac/skeletal muscles (Fig. 1). In the present study we explained the basic mechanism of the differential transcriptional regulations of ACC β gene in human and rodent tissues by showing alternative promoter usages in respective tissues and summarized it in Fig. 10.

In rat cardiac and skeletal muscles, ACC β gene expression was dependent on the P-I promoter. Luciferase reporter assays revealed that P-I was synergistically activated by MRF4 and RXR, which were the most abundant transcription factors in terminally differentiated muscle, explaining the role of P-I in muscle-specific expression. ACC β is expressed at a high level in the heart, in which MRFs are not expressed. Many heart-specific genes are known to be activated by heart-specific transcription factors, such as GATA4 and Csx/Nkx2.5. In the transient transfection assay, these transcription factors induced a drastic upshift in P-I activation but did not affect P-II, suggesting that P-I is major promoter for ACC β expression in the heart. The methylation status of the region around exon 1 α also helps to explain the tissue-specific expression of the ACC β gene. All of the tested CpG was completely methylated in the liver, where P-I was absolutely inactive, whereas the CpG was half-methylated in the skeletal muscle, where P-I was active. Taken together, we concluded that P-I is a tissue-specific pro-

motor that is directed to the constitutive expression of ACC β in skeletal muscle and in the heart.

In human skeletal muscle both promoters P-I and P-II were active, although only P-I was operant in rat skeletal muscle. Two elements for MRF responsiveness around the transcription start site of human P-II were previously discovered to play a critical role in MRF-mediated activation (9). This property was not conserved in rats, even though the sequence homology between rat and human was high at the proximal region of P-II. These differences might indicate species-specific P-II usage in skeletal muscle. The fact that P-II is an inducible promoter, which is responsive to feeding status, suggests the possibility that the ACC β gene expression might be also controlled by feeding status in human skeletal muscle, although this was not proved in this study. This difference in transcriptional regulation between species might reflect a slightly different means of control of fatty acid oxidation, resulting in different susceptibilities to metabolic disorders, such as obesity and diabetes.

From the results of 5' rapid amplification of cDNA ends and RNase protection assays, the novel promoter (P-O) of ACC β gene was identified in the human hepatoma cell line, HepG2. All established hepatoma cell lines tested in present study expressed the ACC β under the control of P-O promoter but not liver promoter, P-II. Moreover, in HepG2 cell, basal activity of P-O is higher than P-II, and in contrast, in rat primary hepatocyte P-II showed much higher activities than P-O. In this study, we just identify that P-O is located ~3 kilobases upstream of exon 1a and plays a major role of the ACC β expression in established hepatoma cell lines. The elucidation of its regulatory characteristics in hepatoma cell lines needs further profound study.

ACC β is a key enzyme in determining basal metabolic rate due to its regulation of fatty acid oxidation. We explained the basic mechanisms underlying the different transcriptional regulation of ACC β gene in the liver and cardiac/skeletal muscles, outlining their different roles in metabolic aspects.

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