Tissue-specific promoter usages of acetyl-CoA carboxylase β gene in human and rodent

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Tissue-specific promoter usages of acetyl-CoA carboxylase β gene in human and rodent

Directed by Professor Kyung-Sup Kim

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학위과정 동안 가장 가슴에 감사함으로 남는 분들이 서울여대 분방 인연으로 만난 선배님들입니다. 수영언니, 혜수언니, 혜진언니, 지윤언니, 언니들의 가르침으로 제가 학위과정을 마칠 수 있었다고 생각합니다. 또한, 과정 동안 늘 곁에서 응원해준 오랜 친구들 채숙, 지애, 지순, 혜정, 같은

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ABSTRACT

Tissue-specific promoter usages of acetyl-CoA carboxylase β gene in human and rodent

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(Directed by Professor **Kyung-Sup Kim**)

Acetyl-CoA carboxylase β (ACCβ) 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-Iβ and P-IIβ, control the transcription of the ACCβ gene. However, the precise mechanism involved in controlling tissue-specific gene expression of ACCβ is largely unknown yet. This study showed that promoter P-Iβ, being active in the skeletal muscle and heart, but not in the liver, could be activated by myogenic regulatory factors (MRFs) and retinoid X receptor α (RXRα) in a synergistic manner. Moreover, P-Iβ was also activated markedly by the cardiac-specific

transcription factors, Csx/Nkx2.5 and GATA4. These results suggest that the proper stimulation of P-Iβ by these tissue-specific transcription factors is important for ACCβ expressions in skeletal muslce and heart. In addition, CpG sites around human exon 1a transcribed by P-Iβ are half-methylated in muscle, but completely methylated in the liver where P-Iβ is absolutely inactive. In humans, the skeletal muscle uses P-IIβ as well as P-Iβ, whereas only P-Iβ is active in rat skeletal muscle. The proximal MRF-binding sites in human P-IIβ, which are not conserved in rat P-IIβ, might contribute to this difference in P-IIβ usage between human and rat skeletal muscle. Hepatoma-derived cell lines primarily use another novel promoter located about 3 kb upstream of P-Iβ, designated as P-Oβ. This study is the first to explain the mechanisms underlying the differential regulation of ACCβ gene expression between tissues in living organisms.

Key words : acetyl-CoA carboxylase β (ACCβ), promoters, myogenic regulatory factors (MRFs), retinoid X receptors (RXR), cardiac-specific transcription factors

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I. INTRODUCTION

In mammals, acetyl-CoA carboxylase β (ACCβ) is a critical enzyme in fatty acid metabolism. ACCβ exists as two isoforms, α and β, which are encoded by the separate genes and show different tissue distribution[1-5]. Because ACCβ 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 palmitoyl transferase-I (CPT-I), which is the

rate-limiting enzyme in the fatty acyl-CoA transport system for fatty acid β-oxidation. Therefore, ACCβ plays a critical role for regulating mitochondrial fatty acid oxidation.

ACCβ is expressed abundantly in the heart, skeletal muscle and liver, all tissue in which fatty acid oxidation actively occurs[2,7,8]. ACCβ transcripts contain two 5'-UTRs, which contain exon 1a or exon 1b via the alternative usage of two promoters, *i.e.* P-Iβ and P-IIβ. Exon 1a and exon 1b are located ~15 kb apart in human genome, but both of them are 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, ACCβ 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 which actively oxidizes fatty acids, although the purpose of fatty acid oxidation in the liver differs from its function 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 ACCβ is regulated by SREBP-1 in response to feeding status, through the P-IIβ[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 ACCβ amounts by transcriptional regulation is important in the liver, while the rapid regulation of enzyme activity by

phosphorylation/dephosphorylation is the major controling mechanism in skeletal and cardiac muscles.

P-IIβ is also active in human skeletal muscle, and is regulated by myogenic regulatory factors (MRFs)[9]. MRFs, including Myf5, MyoD, myogenin and MRF4, are the basic helix-loop-helix transcription factors involved in myogenic differentiation. Although all of these factors 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 retinoic acid receptors, and act as transcriptional activators during differentiation[20-22]. The synergistic action between MFR4 and RXRα, which are the abundant members of their families in fully differentiated myocytes, is most effective in the activation of ACCβ P-II activity in humans[23].

The protein level of ACCβ is higher in the heart than in the skeletal muscle. However, it is currently not clear which promoter directs ACCβ expression in the heart. Cardiomyocyte-specific transcription factors, such as Csx/Nkx2.5, GATA4, MEF2 and eHand, but not MRFs, have been implicated in the 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 alpha actin gene[28-31]. In the present study, we identified the promoter which directs ACCβ expression in the heart and is indeed activated by the cardiac-specific transcription factors, Csx/Nkx2.5 and GATA4.

ACCβ 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 showed that ACCβ levels change drastically in liver, as a response to feeding status, whereas they are maintained at a constant level in both skeletal muscle and heart. This differential regulation of ACCβ gene expression originates from the alternative usages of multiple promoters, such as P-Iβ and P-IIβ. P-Iβ is the sole promoter found in the heart and skeletal muscle of rats, while both P-Iβ and P-IIβ are active in human skeletal muscle. We demonstrated the activation of P-Iβ via synergistic action between MRF4 and retinoid X-receptor α, as well as Csx/Nkx2.5 and GATA4, which explains the tissue- specific activation of P-Iβ 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-Iβ. This study is the first to explain the mechanisms underlying the differential regulation of ACCβ gene expression in human and rodent tissues.

II. MATERIALS AND METHODS

1. 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 fasting for 48 hours, and then refeeding with a fat-free high-carbohydrate diet for 0, 24, or 48 hours. 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, USA).

2. 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 PMSF, 1X protease inhibitor cocktail (Roche, Indianapolis, IN, USA) with glass pestles, and then centrifuged at 5000 rpm at 4ºC for 10 minutes. Supernatants were precipitated in 12.5% polyethylene glycol. Precipitated proteins dissolved in 1/5 initial volume of homogenization buffer and the concentration of soluble protein was determined by Bradford assay (BioRad, Hercules, CA, USA). Extracts were separated in 5% SDS-polyacrylamide gel, and transferred onto Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).

Immunoblot analysis was carried out with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA) and polyclonal anti-ACCβ antibody, and specific bands were visualized using a SuperSignal West Pico Trial Chemiluminescent substrate Kit (Pierce Biotechnology, Rockford, IL, USA).

3. RNase protection assay

Rat cRNA probes were synthesized from the rat ACCβ cDNA sequences of either exon 1a (90 bp) or exon 1b (52 bp), extending to exon 2 (69 bp) subcloned into pCRII plasmids. Human cRNA probes I, II and O were also 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 ug) by *Hind*III, [^{32}P]-labeled cRNA was synthesized by T7 RNA polymerase (Ambion, Austin, TX, USA). Probes were purified by gel elution after electrophoresis with 6% polyacrylamide/6 M urea gel. RNase protection assay with purified probes were performed with the RPAIII kit (Ambion, Austin, TX, USA). The total RNA (20 μg), isolated from rat and human liver, heart, and skeletal muscle, was hybridized with a probe (1.6 x 10^5 cpm) in 30 μl hybridization buffer at 42ºC, for 12-16 hours. The unhybridized RNA was digested by adding 150 μl of the diluted solution (1:100) of RNase A/T1 at 37ºC for 30 minutes. Probes protected from RNase were precipitated by the addition of 225 μl of RNase inactivation/precipitation III solution, followed by 15 minutes of centrifugation at 12,000 rpm. Precipitates were washed with

70% ethanol, and dissolved in 4 µl sequencing gel loading buffer followed by denaturation on 6% polyacrylamide/6 M urea gel. Gels were dried and exposed to Kodak BioMax film at –70ºC with intensifying screens. A sequencing ladder was loaded in the adjacent lane in order to determine the size of the products.

4. Primer extension analysis

Primer extension was performed as described by Kim et al[32]. Antisense oligonucleotides of rat exon 1a and human exon 1o of ACCβ gene, 1a_AS and 1o_AS, were labeled with α-[^{32}P] ATP (NEN Life Science Products, Boston, MA, USA) by T4 polynucleotide kinase. The labeled oligonucleotides (2 X 10^5 cpm) were mixed with 50 µg of RNAs in 100 µl hybridization buffer (40 mM PIPES, pH 6.8, 1 mM EDTA, 0.4 M NaCl, 80% deionized formamide). The mixtures were incubated at 90ºC for 3 minutes and hybridized overnight at 37ºC. Annealed mixtures were precipitated by ethanol, and used for the extension reaction. These mixtures were extended with SuperScript™II (Invitrogen Life Technologies, Carlsbad, CA, USA) at 42ºC for 1 hour under buffer conditions specified by the manufacturer's instructions. After phenol: chloroform: isoamyl alcohol (25:24:1) extraction and ethanol precipitation, the sizes of products were determined by 6% denaturing polyacrylamide gel electrophoresis. The lengths of rat exon 1a and human exon 1o were determined by comparing to sequencing products of the cloned promoter region in both rats and humans.

5. Construction of plasmids

The luciferase constructs of human ACCβ P-II, phP-IIβ(-569/+65) and phP-IIβ(-93/+65), were described by Lee et al.[9]. The oligonucleotides used in promoter construction are shown in Table 1. Constructs of phP-Iβ(-2038/ +387), phP-Iβ(-1735/+100) and phP-Iβ(-616/+387) were generated by amplifying the human ACCβ promoter region, and introducing it into the *Sma*I site of the pGL3-Basic vector. Constructs of prP-Iβ(-1864/ +14), prP-IβI(-485/+65) and prP-IIβ(-90/+65) were generated from the rat ACCβ promoter regions and cloned in the *Sma*I sites of pGL3-Basic, respectively. phP-Oβ(-1143/+191) was constructed by amplifying the upstream region containing human ACCβ exon 1o, and introducing it into the *Sac*I/*Sma*I site of the pGL3-Basic vector. Rat GATA4 cDNA was amplified by RT-PCR, using primers shown in Table 1, and was inserted into the *Hind*III/*Xho*I site of pcDNA3. The plasmid of pcDNA3-mycCSX was a generous gift from Dr. Issei Komuro (Chiba University, Chiba, Japan).

6. Cell cultures and transient transfections

All reagents for cell cultures and Lipofectamine PLUS reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 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 μg/ml antibiotics/antimycotics at 37ºC in an 80% ~ 90% humidified

CO_2 incubator. Rat primary hepatocyte culture and transfection were performed as described by Ahn et al[33]. Cells were prepared for experiments on 6-well plates at 2.5 X 10^5 ~ 1 X 10^6 cells. 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 μl of PLUS reagent were mixed in 100 μl of serum-free media, and then added to 100 μl of serum-free media containing 2 μl of Lipofectamine reagent, then Lipofectamine-DNA mixtures were incubated for 15 min. The amount of DNA was used in transient transfection with 400 ng of reporter plasmid, 100 ng of expression vector, and 100 ng of β-gal. The total amount of DNA were adjusted to the same amount by the addition of mock vector plasmid. The cells were washed with PBS, and supplied with 800 μl of serum-free media during incubation. After 15 minutes, Lipofectamine-DNA mixture was added to the wells. The cells which had been transfected for 3 hours were washed twice with PBS, then grown for 48 hours in media supplemented with 10% FBS and 100 μg/ml antibiotics/antimycotics. For the activation of promoters by myogenic regulatory factors and retinoic acid receptors, RXRα and RARα ligands, 1 μM of 9-*cis*-retinoic acid and all-*trans*-retinoic acid, were treated after 20 hours and cultured further for additional 24 hours. Cells were harvested and lysed with 200 μl of reporter lysis buffer (Promega, Madison, WI, USA), and cell debris was removed by centrifugation. Luciferase activities were measured using 10 μl of cell extract and 50 μl luciferase assay reagent (Promega, Madison, WI, USA). For the

β-galactosidase assay, the color changes of extracts by hydrolysis of o-nitrophenol-β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO, USA) were detected as kinetics at 420 nm, at 37ºC, for 5 minutes.

7. Methylation analysis of CpG islands

The 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 μg/ml RNase A, 1 mg/ml Proteinase K) at 50ºC for 5 hours. After phenol: chloroform: isoamyl alcohol extraction, genomic DNA precipitated by ethanol was picked up and was dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). After 10 μg of genomic DNA was digested by 10 units of *Eco*RI at 37ºC for 5 hours, unmethylated C's were converted into U's via the bisulfite reaction. In brief, 2 μg of linearized DNA were denatured in 0.3 M NaOH at 37ºC for 20 minutes, and treated with 550 μl of converting solution (10 mM hydroxyquinone, 2.8 M sodium bisulfite, pH 5.0), then incubated in 55ºC for 16 hours in darkness. Sulfonated single strand DNA fragments were purified using the Wizard DNA Clean-Up system (Promega). Sulfonated C's were desulfonated and deaminated with 0.3 M NaOH at 37ºC for 15 minutes, and neutralized with 3 M ammonium acetate, pH 7.0. The converted DNA in which C's had been converted to U's was precipitated with ethanol and dissolved in 50 μl of TE buffer. The primers were designed according to C-to-T converted sequence of the region surrounding exon 1a as denoted in Table. 1 as CpG_S and

CpG_AS. The PCR reaction mixture contained 10 μl of converted DNA, 0.2 pmole primers, Gold taq reaction buffer, 1.5 mM $MgCl_2$, 1.25 mM dNTPs, and 1U of Gold taq polymerase (Roche, Basel, Switzerland), amplified as follows: denaturation for 5 minutes at 94ºC, 40 cycles of denaturation for 30 seconds at 94ºC, annealing for 30 seconds at 52ºC, and extension for 30 seconds at 72ºC. Amplified products were directly sequenced using CpG_S primer.

8. *In vitro* methylation assay

Twenty μg of reporter plasmids, phP-Iβ(-2038/+387) and phP-Iβ (-616/+387), were incubated with or without 20 U of *Sss*I methylase (New England BioLabs, Beverly, MA, USA) at 37ºC for 24 hours, supplemented with 160 μM S-adenosyl-methyonine. *Sss*I-treated plasmids were purified with Wizard clean-up system (Promega), and then purified DNA was eluted with 50 μl of distilled water. For transient transfection, C2C12 myoblast cell lines were cultured until 50% ~ 70% confluence for 16 ~ 24 hours. Methylated or non- methylated luciferase reporter plasmids (0.4 μg) were transiently transfected with or without pcDNA3-mycCSX (0.1 μg) and luciferase activities were assayed 48 hours after transfections as described in cell cultures and transient transfection.

9. RT-PCR

Total RNAs were extracted from Alexander, HepG2, Hep3B and PLC/PRF5 hepatoma cell lines using the TRIzol (Invitrogen) according

to the manufacturer's instructions. First-strand cDNAs were synthesized from 5 μg of total RNA in 20 μl of reaction volume using SuperScript II (Invitrogen) reverse transcriptase. Each reverse transcription mixture (1 μl) was used as the template for amplifying ACCβ cDNA. The sense primers for each ACCβ transcript, spanning exon 1o (hexon1o_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.

Table 1. Sequences of oligonucleotides used in the experiments

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

III. RESULTS

1. Changes of ACCβ expression level in the rat liver, heart and skeletal muscle by dietary control

ACCβ is expressed predominantly in the skeletal muscle and heart, where the β-oxidation of fatty acid actively occurs, constituting a major energy source. Sterol regulatory element-binding protein-1 (SREBP-1) was previously reported to induce ACCβ gene expression in the liver as a response to the intake of a high-carbohydrate diet[15]. We attempted to ascertain whether or not ACCβ levels in the heart and skeletal muscle, changed in response to feeding status, as did ACCβ levels in the liver. The levels of pyruvate carboxylase, detected with streptavidin-HRP conjugate as a control, were almost the same between fasted and refed groups in the liver, heart and skeletal muscle extracts. The nutritional control had no significant effect on ACCβ expression in the heart and skeletal muscle, whereas hepatic ACCβ levels were drastically increased by food intake (Fig. 1). This result is consistent with the findings of many previous reports, that the posttranslational regulation of ACCβ was much more important regulatory mechanism in skeletal muscle rather than the control of enzyme expresseion levels[10-12,33]. This also suggests that the expression of ACCβ is differently regulated between cardiac/skeletal muscle and the liver.

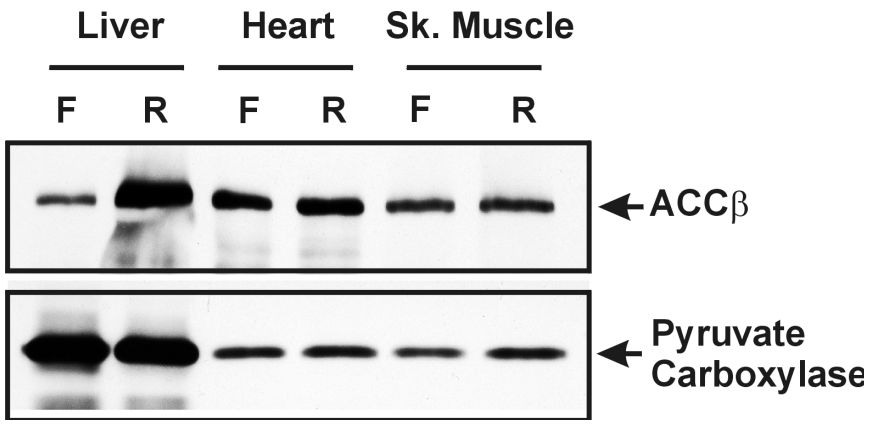

Figure 1. The differential regulation of ACCβ expression in the liver, heart and skeletal muscle. Proteins were extracted, as described in *Materials and Mothods*, from the livers, hearts and skeletal muscles of SD rat groups which were fasted for 48 hours (F) or were fasted and then refed for 48 h (R). Fifty micrograms of proteins were run on 5% SDS-PAGE, and were transferred to a nitrocellulose membrane. The blots were stained, using polyclonal anti-ACCβ antibody and streptavidin-conjugated horseradish peroxidase for the detection for ACCβ and internal control, pyruvate carboxylases, respectively. The bands for ACCβ and pyruvate carboxylase were indicated.

2. Different promoter usages between the rat cardiac/skeletal muscle and liver

It was reported that human and rat ACCβ gene expression could be controlled from two types of promoters, designated as P-Iβ and P-IIβ[9]. Differences in the regulation of ACCβ gene expression between tissues led us to perform RNase protection assay, in order 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 hours, and refed with a fat-free high-carbohydrate diet for 0 or 24 hours. Exon 1a and 2 in probe I were fully protected in the rat skeletal muscle and heart, while 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 ACCβ transcripts (Fig. 2B). These data indicate that rat ACCβ gene expression in heart and skeletal muscle is controlled by P-Iβ, whereas P-IIβ is a major promoter in the liver. The alternative promoter usages would appear to explain the mechanism of differential transcriptional regulations of the ACCβ gene between these organs.

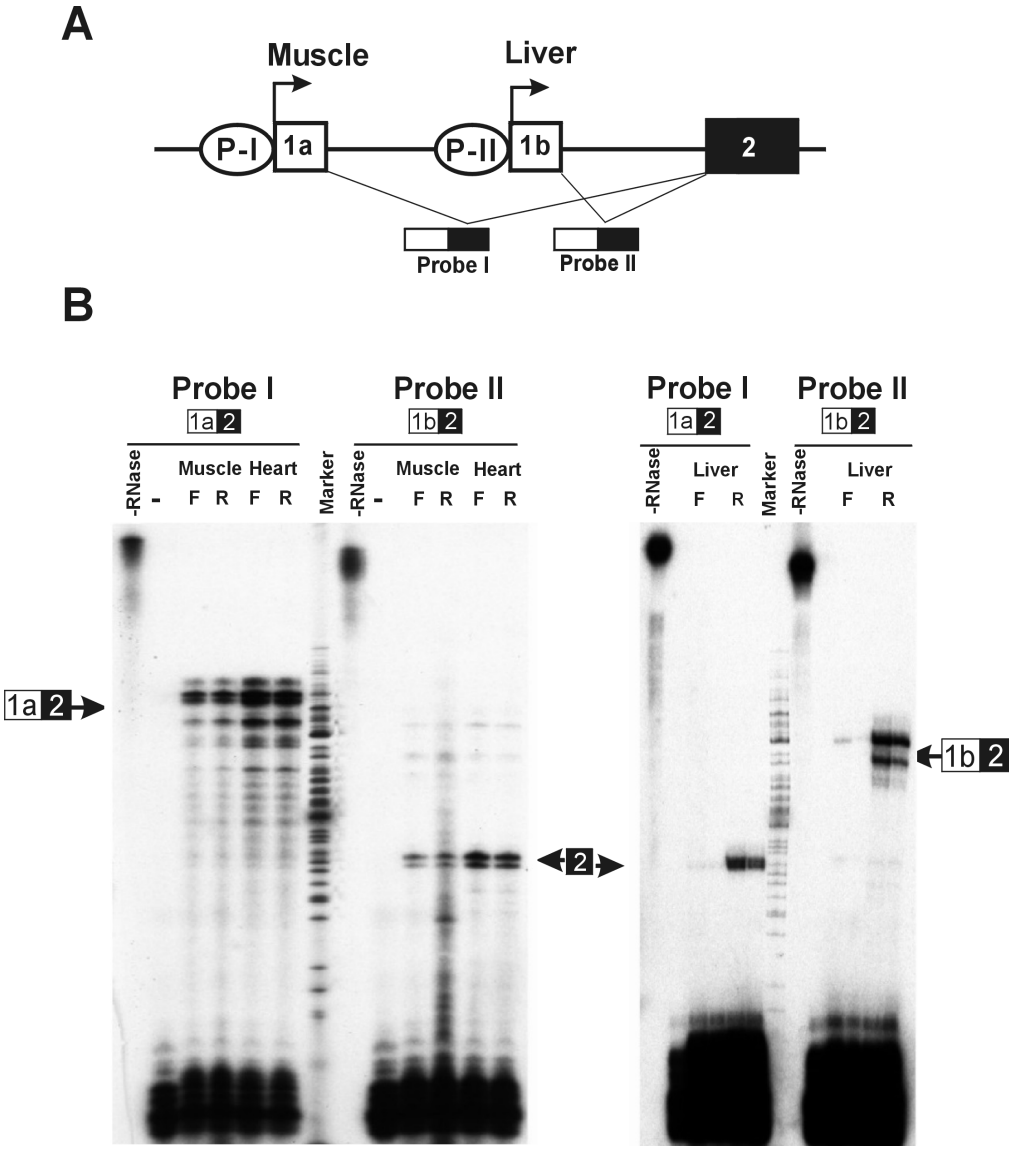

Figure 2. Promoter usages for ACCβ gene expressions in the rat skeletal muscle, heart and liver. RNase protection assays (RPA) were performed, using total RNAs prepared from skeletal muscles, hearts, and livers of rats which had been fasted for 48 h (F), or had been fasted and then refed a high-carbohydrate 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 such as (A). (B) After 50μg of 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 a 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 (-) and (-RNase), respectively.

3. Determination of transcription start site in rat ACCβ promoter I

In the previous report, the size of human exon 1b transcribed by P-IIβ was determined as 67 bp by primer extension analysis using the antisense primer corresponding to exon 2, while 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'RACE (data not shown). In order to determine the precise transcription start site in P-Iβ, A primer extension analysis was performed 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 ACCβ 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 TATA-, CCAAT- or GC-box, was not found in the proximal P-Iβ promoter.

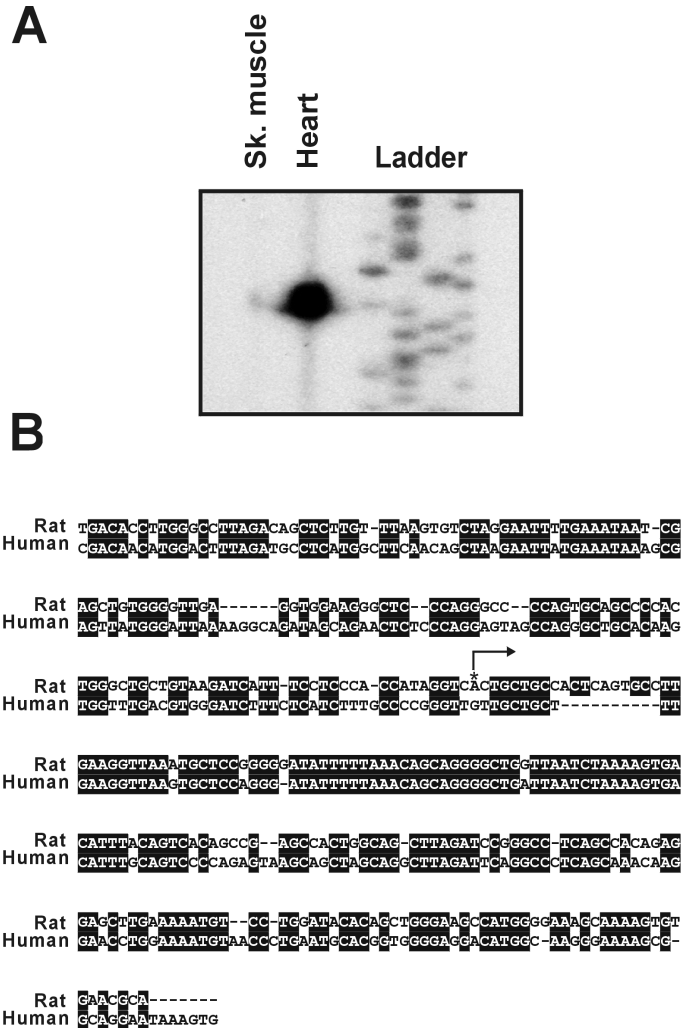

Figure 3. Primer extension analysis for the determination of transcription start site in P-Iβ the length of exon 1a. (**A)** Transcription start site in ACCβ P-I was identified by primer extension analysis, using rat skeletal muscle and heart RNAs. Total RNA was hybridized with antisense oligonucleotide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

site of rat ACCβ P-I.

4. ACCβ promoter I is activated by MRFs and retinoic acid receptors (RARα and RXRα)

The fact that the muscle-specific expression of ACCβ is controlled by promoter P-Iβ as shown in Fig. 2 make us to check the responsiveness of ACCβ 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-Iβ sequence in front of the luciferase gene, was transiently transfected with the expression vectors for MyoD, MRF4, RARα and/or RXRα into NIH3T3 cells. As shown in Fig. 4A, MyoD and RARα rarely affect P-Iβ activities, and MRF4 and RXRα cause a 2- to 3-fold increase in P-Iβ activity. Combined treatment with ligand-activated retinoic acid receptors and MRFs synergistically activates P-Iβ, and a combination of RXRα and MRF4 exhibits the most effective synergism, inducing a 16-fold increase in P-Iβ activity (Fig. 4A). MRF4 and RXRα could also synergistically activate rat P-Iβ, just as in human P-Iβ (Fig. 4B). Because MRF4 and RXRα are the most abundant forms of their families in fully differentiated muscle cells[20,21], the synergistic action of MRF4 and RXRα might play an important role in the muscle-specific expression of ACCβ.

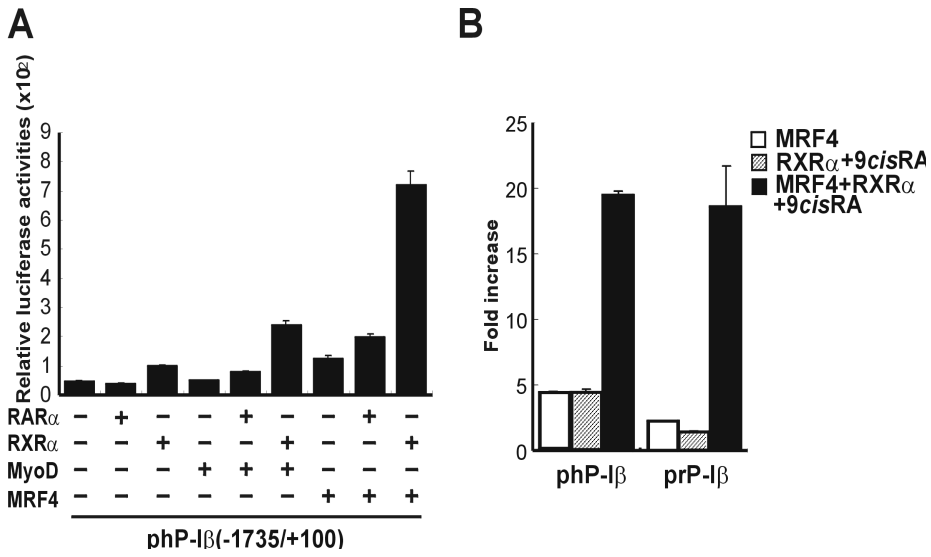

Figure 4. Activation of ACCβ P-I by MRFs and ligand dependent retinoic acid receptors (RARα and RXRα). (**A)** The phP-Iβ(-1735/+100) reporter construct (0.4 µg) and pCMV-*β*-gal reference construct (0.1 µg) were transfected into NIH3T3 cells with overexpression vectors (0.1 µg) of pcDNA3, pcRARα, pcRXRα, pcMyoD or pcMRF4, as indicated. For the activation of retinoic acid receptors, 9-*cis*-retinoic acid (1 µM) and *all-trans*-retinoic acid (1 µM) were used, respectively. (**B)** The responsiveness of human and rat ACCβ P-I to MRF4 and RXRα were assayed, with phP-Iβ(-1735/+100) and prP-Iβ(-1864/+14) reporter constructs. Total amounts of transfected DNA were adjusted to 0.7 µg with pcDNA3. In the groups overexpressing RARα or RXRα, the respective ligand of *all-trans*- or 9-*cis*-retinoic acid was added, respectively 24 hours after transfection, to a final concentration of 1 µM. Luciferase activities were measured 48 hours after transfection, and normalized by *β*-galactosidase activities. The data are represented as the mean +/- S.D. of three independent experiments, each performed three times.

5. ACCβ promoter I is activated by GATA4 and Csx/Nkx2.5

ACCβ is most abundantly expressed in the heart, suggesting the possibility that cardiac transcription activators, such as GATA4 and Csx/Nkx2.5, might activate ACCβ P-I. As expected, human P-Iβ of the ACCβ gene was markedly activated by GATA4 and Csx/Nkx2.5, while P-IIβ was not (Fig. 5). This would appear to explain the mechanism by which the high level of expression of ACCβ in the heart is dependent on the P-Iβ 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 alpha actin gene[28-31]. However, Csx/Nkx2.5 alone had a tremendous effect on ACCβ P-I, to a 42-fold increase in activation, while 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 Csx/ Nkx2.5, inducing a 62-fold increase (Fig. 5).

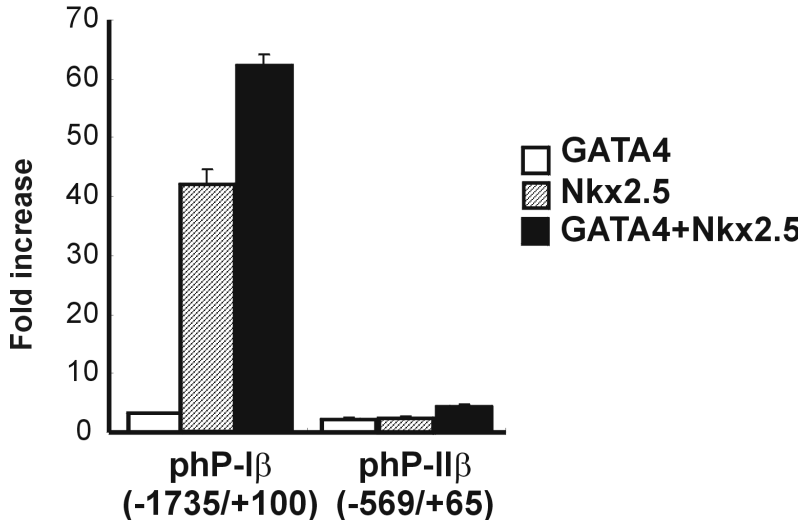

Figure 5. ACCβ P-I is activated by GATA4 and the cardiac-specific homeobox Csx/Nkx2.5. A transient transfection assay was performed to measure the levels of activation of ACCβ P-I and P-II by GATA4 and Csx/Nkx2.5. The reporter construct (0.4 μg) of phP-Iβ(-1735/+100) or phP-IIβ(-569/+65) and pCMV-*β*-gal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

6. Methylation of CpG around the exon 1a of human ACCβ gene

In addition to tissue-specific transcription factors, DNA-methylation is another regulatory mechanism underlying tissue-specific gene expression. This fact that ACCβ 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. 6). All cytosine in CpGs was almost completely protected from deamination in the liver genomic DNA, while 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. To evaluate the effect of CpG methylation of transcription, luciferase reporter constructs of human PIβ was methylated in vitro and its transcriptional activation by Csx/Nkx2.5 was assayed. The methylation of CpG abruptly prevented the activation of human P-Iβ by Csx/Nkx2.5 overexpression (Fig. 7). This result suggest that the status of CpG methylation may be one of the important factor in the tissue-specific activities of ACCβ P-I.

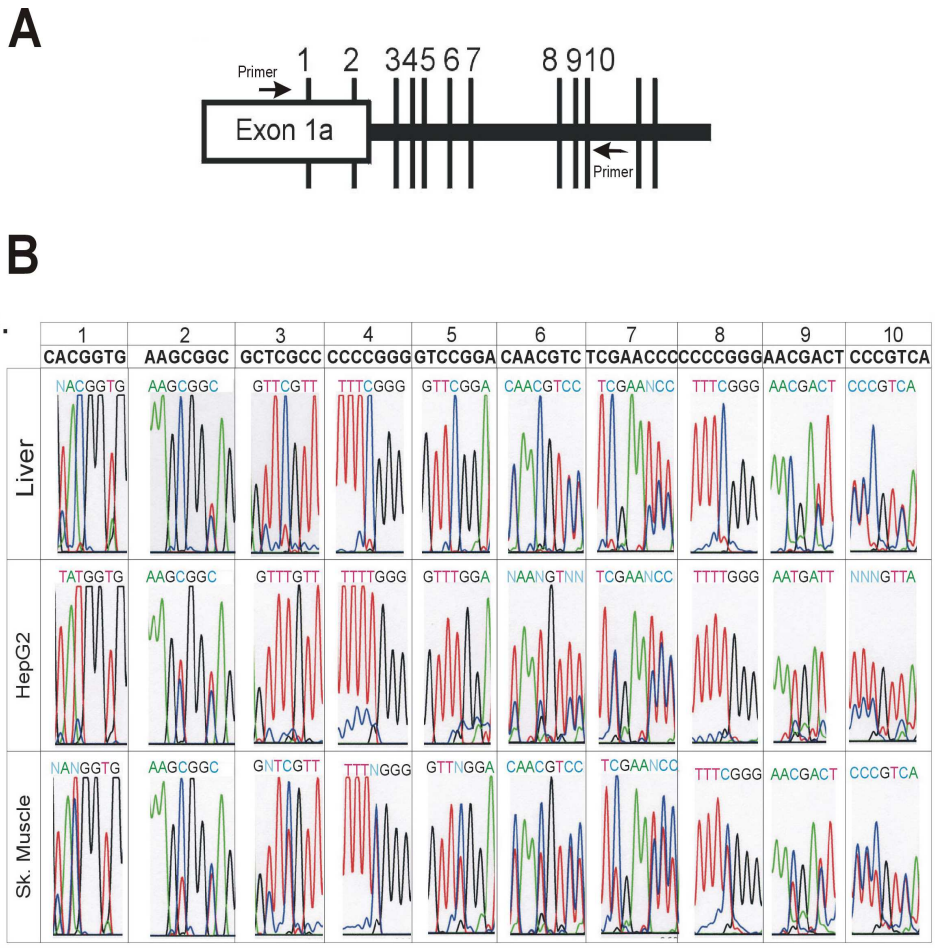

Figure 6. CpG methylation status around the exon 1a of ACCβ gene in the liver, skeletal muscle and HepG2 cells. (**A)** A diagram of the CpG sites around exon 1a were schematically illustrated. (**B)** The deaminations of C's were shown by direct sequencing of PCR products. The C's, except methylated C's, were deaminated into U's by treating genomic DNAs extracted from human liver, HepG2 cells and skeletal muscle with sodium bisulfite. 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 muscle.

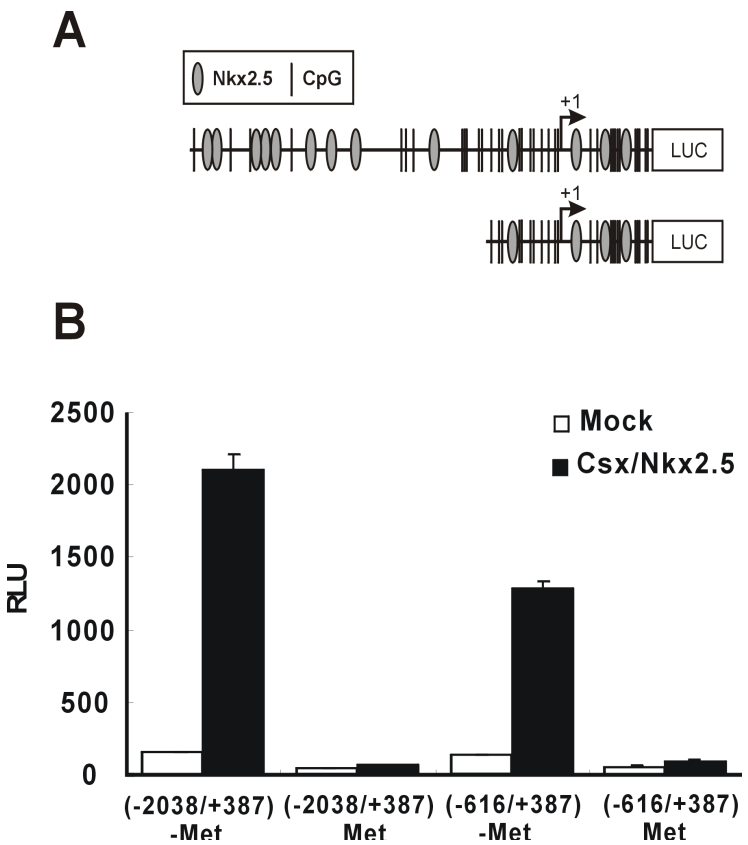

Figure 7. CpG methylation completely blocks Csx/Nkx2.5-mediated activation of ACCβ P-I. (A) Ph-PIβ(-2038/+387) and ph-PIβ(-616/+387) are luciferase reporter constructs containing putative Csx/Nkx2.5 binding sites and CpG sites as described figures. **(B)** Reporter constructs, Ph-PIβ(-2038/+387) and ph-PIβ(-616/+387), were treated with or without *Sss*I methylase as described in *Materials and methods*. Non-methylated (-Met) or methylated (Met) reporter constructs (0.4 μg) were co-transfected into the C2C12 cell in company with overexpression vectors (0.1 μg) of pcDNA3 empty vector and pcDNA3-mycCSX. Luciferase activities were measured 48 hours after transfection, and normalized by β-galactosidase activities. The data are represented as the mean +/- S.D. of three independent experiments, each performed three times.

7. Alternative promoter usages of the ACCβ gene in the human skeletal muscle

It was previously reported that human ACCβ 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-IIβ is active only in the liver, and not in skeletal muscle and the heart. These discrepant results led us speculate that ACCβ promoter usages in skeletal muscle might differ between rats and humans. To demonstrate whether the 5'UTR of ACCβ transcripts contains equences of exon 1a or exon 1b, RNase protection assays were performed, using total RNA isolated from human skeletal muscle and liver (Fig. 8). 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. These data suggest that human skeletal muscle uses both P-Iβ and P-IIβ, in contrast to rat skeletal muscle, in which only P-Iβ is utilized. The sequences around the transcription start site in P-IIβ 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-IIβ play an important role in MRF-mediated activation[9]. These elements were, interestingly, not conserved in rat P-IIβ (Fig. 9A). In order to determine differences in MRF-responsiveness between human and rat promoters, transient transfection assays were performed. Overexpression of MyoD stimulated only human P-IIβ, and not rat P-IIβ (Fig. 9B). Taken together, the difference of these short elements for MRF-binding might suggest P-IIβ

to be active only in human skeletal muscle, and not in rat skeletal muscle.

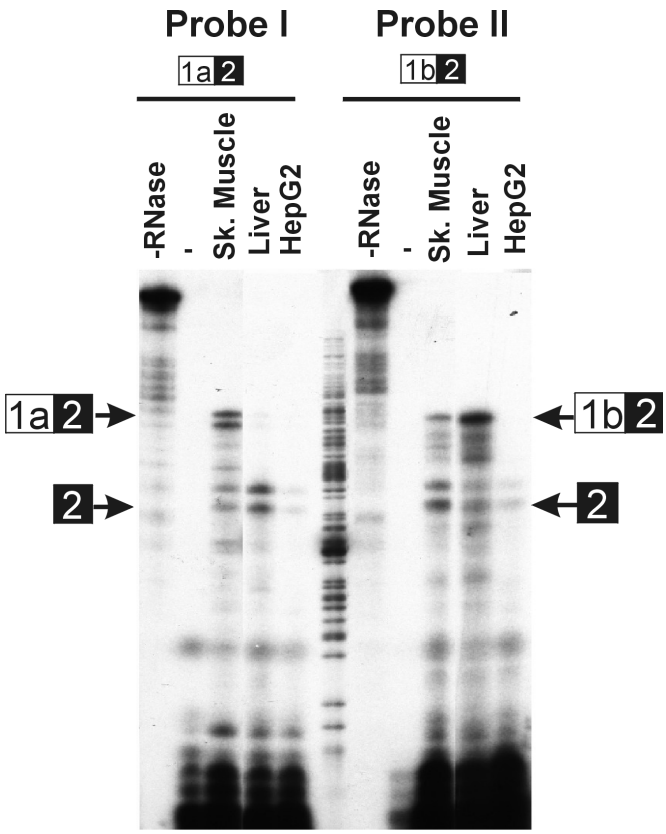

Figure 8. Identification of 5'- UTR of ACCβ transcripts expressed in the human skeletal muscle, liver and HepG2 cells. Total RNAs of human skeletal muscle, liver and HepG2 cells were hybridized with cRNA probes I and II, and then treated with RNase A/T1 mix. Probe I and II, consists of 58 bp of exon 1a or 60 bp of exon 1b, respectively, and a common 100 bp of exon 2. 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 - and -RNase, respectively.

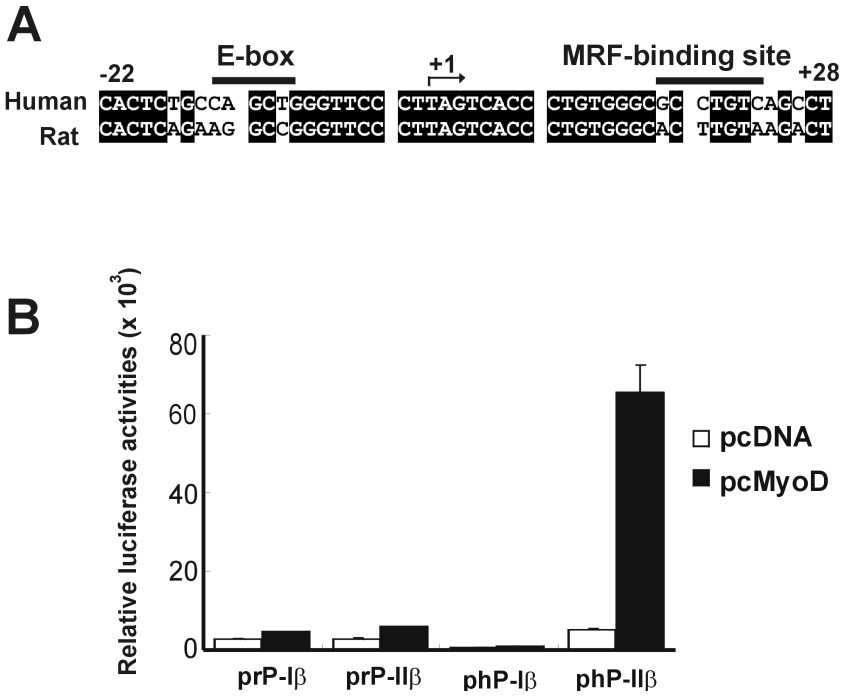

Figure 9. MRF-binding sites in the proximal region of human ACCβ P-II are not conserved in rat. (**A)** The sequences of proximal ACCβ P-II region were compared between human and rat. One E-box (-14 to －9) and novel MRF- binding site (+17 to +24), previously reported in human ACCβ P-II[9], were not conserved in the rat promoter. (**B)** The responsiveness of human and rat ACCβ promoters to MyoD is assayed. Rat reporter constructs, prP-Iβ (-574/+147) and prP-IIβ(-90/+65), and human reporter constructs, phP-Iβ (-961/+100) and phP-IIβ(-93/+65) were used in this assay.

8. Another promoter, P-Oβ, plays a primary role in ACCβ expression in hepatoma cell lines

In HepG2 cells, ACCβ expression was previously reported not to be driven by P-IIβ[9], which is the primary promoter in normal liver[15]. In the present study, RNase protection assay revealed that neither P-Iβ nor P-IIβ appeared to play a role in ACCβ gene transcription (Fig 8). These results led us to study which sequences of ACCβ gene direct the transcription in HepG2 cells. 5'RACE, using the total RNA of HepG2 cells, revealed the sequences of the 5'UTR of the HepG2 ACCβ transcripts. Most clones isolated from 5'RACE contained an identical sequence, corresponding to the region located about 3 kb upstream of exon 1a. These data suggest that another promoter, located at 5' upstream of P-Iβ, controls ACCβ expression in HepG2, and we designated this promoter and exon as P-Oβ and exon 1o, respectively (Fig 10A). Next, an RNase protection assay was performed, using an antisense RNA probe containing the exon 1o joined to exon 2 (Fig. 10B). Almost all of the HepG2 ACCβ mRNA, and the small portion of hepatic ACCβ mRNA, contained the exon 1o sequence. Next, We performed primer extension analysis was performed to determine the transcription start site in P-Oβ-driven transcription. The size of exon 1o was 179 bp (GenBank AY701053). The analysis of the 5'UTR of ACCβ transcripts revealed that the major promoter is P-Oβ in established human hepatoma cell lines such as Alexander, HepG2, Hep3B and PLC/PRF5 (Fig. 10C). The promoter activities of P-Oβ are higher than P-IIβ in HepG2 cells, whereas P-IIβ is much higher than

P-Oβ in the primary hepatocyte (Fig. 10D). These data indicate that the human hepatoma cell lines use the promoter Oβ, which is not active in cardiac/skeletal muscle and the liver in vivo.

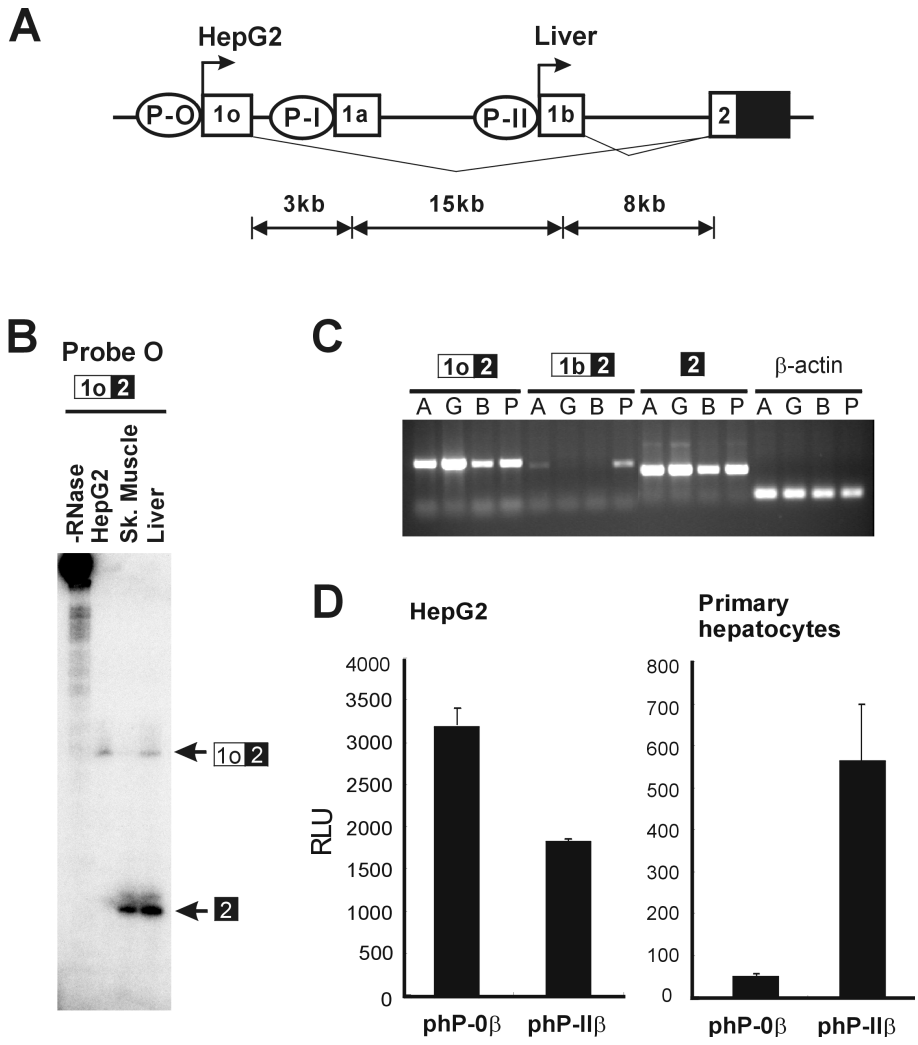

Figure 10. Promoter Oβ plays a primary role in ACCβ expression of hepatoma cell lines. (A) Alternative usages of the promoters and splicing of

ACCβ gene transcription in HepG2 cells and in vivo liver, and genomic distances of each exon were explained. **(B)** RNas protection assay identified the exon 1o sequence in ACCβ 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 1o and 100 bp of exon 2. (**C)** RT-PCR was performed to identify which promoter plays a major role of ACCβ expression in hepatoma cell lines such as Alexander (A), HepG2 (G), Hep3B (B) and PLC/PRF5 (P). ACCβ cDNA fragments, containing exon 1o/2, exon 1b/2 or exon 2, or β-actin cDNA as the internal control were PCR-amplified and the products were visualized on 1% agarose gel. (**D)** phP-Oβ(-1143/+100) and phP-IIβ(-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 μg and 0.1 μg in HepG2 cells, and 1.8 μg and 02 μg in primary hepatocytes, respectively.

IV. DISCUSSION

The regulation of ACCβ activities is important for controlling the rate of fatty acid oxidation in the liver, heart and skeletal muscle. Hepatic ACCβ expression is controlled transcriptionally by feeding status, and SREBP-1 is a key transcription factor in this process. However, fatty acid oxidation increased during exercise is mainly mediated by the inactivation of ACCβ by phosphorylation in skeletal muscle[4,10,11,34]. Thus, it is conceivable that ACCβ 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 ACCβ gene was expected to be quite different between the liver and the cardiac/skeletal muscles (Fig. 1). In the present study, we showed the basic mechanism of the differential transcriptional regulations of ACCβ gene in human and rodent tissues, by showing alternative promoter usages in respective tissues, and summarized it in Fig. 11.

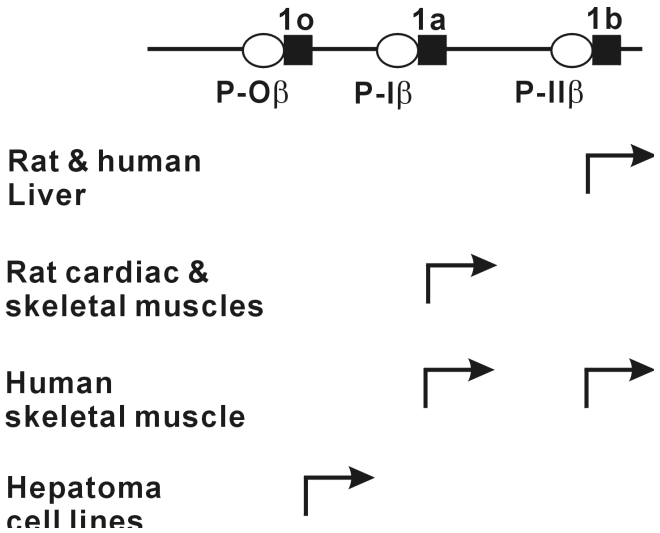

Figure 11. Schematic diagram showing alternative promoter usages for ACCβ gene expression in rat and human tissues. In the livers of rat and human, ACCβ gene expression is directed by the promoter P-IIβ. In rat cardiac and skeletal muscles, the promoter P-Iβ is a sole promoter to maintained the constant levels of ACCβ, whereas both promoters of P-Iβ and P-IIβ are active in human skeletal muscle. Another promoter located 3 kb upstream of P-Iβ cont- rols ACCβ expression in hepatoma cell lines, and we designated this novel promoter as P-Oβ.

In rat cardiac and skeletal muscles, ACCβ gene expression was dependent on the P-Iβ promoter. Luciferase reporter assays revealed that P-Iβ was synergistically activated by MRF4 and RXRα, which were the most abundant transcription factors in terminally differentiated muscle, explaining the role of P-Iβ in muscle-specific expression. ACCβ is expressed at a high level in the heart, in which MRF's 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-Iβ activation, but did not affect P-IIβ, suggesting that P-Iβ is major promoter for ACCβ expression in the heart. The methylation status of the region around exon 1a also helps to explain the tissue-specific expression of the ACCβ gene. All of the tested CpG was completely methylated in the liver, where P-Iβ was absolutely inactive, whereas the CpG was half-methylated in the skeletal muscle, where P-Iβ was active. Taken together, we concluded that P-Iβ is a tissue-specific promoter, which is directed to the constitutive expression of ACCβ in skeletal muscle and in the heart.

In human skeletal muscle, both promoters P-Iβ and P-IIβ were active, while only P-Iβ was operant in rat skeletal muscle. Two elements for MRF-responsiveness around transcription start site of human P-IIβ 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-IIβ. These differences might indicate species-specific P-IIβ usage in skeletal muscle. The fact that P-IIβ is an inducible promoter, which is responsive to feeding status, suggests the possibility that the ACCβ 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'RACE and RPA, the novel promoter (P-Oβ) of ACCβ gene was identified in the human hepatoma cell line, HepG2. All established hepatoma cell lines tested in the present study expressed the ACCβ under the control of P-Oβ promoter but not liver promoter, P-IIβ. Moreover, in HepG2 cell, basal activity of P-Oβ is higher than P-IIβ and, in contrast, in rat primary hepatocyte P-IIβ showed much higher activities than P-Oβ. In this study, We just identify that P-Oβ is located approximately 3 kb upstream of exon 1a, and plays a major role of the ACCβ expression in established hepatoma cell lines. The elucidation of its regulatory characteristics in hepatoma cell lines needs further profound study.

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

V. CONCLUSION

1. The nutritional control had no significant effect on ACCβ expression in heart and skeletal muscle, whereas hepatic ACCβ levels were drastically increased by food intake.
2. Rat ACCβ gene expression in heart and skeletal muscle is controlled by P-Iβ, whereas P-IIβ is a major promoter in the liver.
3. The size of exon 1a was revealed to be 201 bp in the rat ACCβ gene, and the sequence of exon 1a shows higher conservation between human and rat than the 5' flanking region of exon 1a.
4. The synergistic action of MRF4 and RXRα might play an important role in the muscle-specific expression of ACCβ.
5. Cardiac specific transcription factors, Csx/Nkx2.5 and GATA4, induced a drastic upshift in P-Iβ activation, but did not affect P-IIβ, suggesting that P-Iβ is major promoter for ACCβ expression in the heart.
6. CpG sequences around exon 1a are completely methylated in the liver, completely unmethylated in HepG2 cells, and half-methylated in muscle.
7. The analysis of promoter usage of ACCβ transcripts revealed that the major promoter is P-Oβ in established human hepatoma cell lines such as Alexander, HepG2, Hep3B and PLC/PRF5. In addition, promoter Oβ is not active in cardiac/skeletal muscle and the liver in vivo.

REFERENCES

1. Lopez-Casillas F, Bai DH, Luo XC, Kong IS, Hermodson MA, and Kim KH. Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme a carboxylase. *Proc Natl Acad Sci U S A* 1988;85:5784-5788.
2. Abu-Elheiga L, Almarza-Ortega DB, Baldini A, and Wakil SJ. Human acetyl-CoA carboxylase 2. *J Biol Chem* 1997;272:10669-10677.
3. Ha J, Lee JK, Kim KS, Witters LA, and Kim KH. Cloning of human acetyl-CoA carboxylase-b and its unique features. *Proc Natl Acad Sci U S A* 1996;93:11466-11470.
4. Widmer J, Fassihi KS, Schlichter SC, Wheeler KS, Crute BE, King N, et al. Identification of a second human acetyl-CoA carboxylase gene. *Biochem J* 1996;316(Pt 3):915-922.
5. Abu-Elheiga L, Jayakumar A, Baldini A, Chirala SS, and Wakil SJ. Human Acetyl-CoA Carboxylase: Characterization, Molecular Cloning, and Evidence for Two Isoforms. *Proc Natl Acad Sci U S A* 1995;92:4011-4015.
6. Abu-Elheiga L, Brinkley WR, Zhong L, Chirala SS, Woldegiorgis G, and Wakil SJ. The subcellular localization of acetyl-CoA carboxylase 2. *Proc Natl Acad Sci U S A* 2000;97:1444-1449.
7. Kim KH. Regulation of mammalian acetyl-coenzyme A carboxylase. *Annu Rev Nutr* 1997;17:77-99.
8. Zhang S, and Kim KH. Acetyl-CoA carboxylase is essential for nutrient-induced insulin secretion. *Biochem Biophys Res Commun* 1996;229:701-705.
9. Lee JJ, Moon YA, Ha JH, Yoon DJ, Ahn YH, and Kim KS. Cloning of Human Acetyl-CoA Carboxylase Promoter and Its Regulation by Muscle Regulatory Factors. *J Biol Chem* 2001;276:2576-2585.
10. Rasmussen BB, and Winder WW. Effect of exercise intensity on

skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J Appl Physiol* 1997;83:1104-1109.

11. Dean D, Daugaard JR, Young ME, Saha A, Vavvas D, Asp S, et al. Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. *Diabetes* 2000;49:1295-1300.
12. Boone AN, Rodrigues B, and Brownsey RW. Multiple-site phosphorylation of the 280 kDa isoform of acetyl-CoA carboxylase in rat cardiac myocytes: evidence that cAMP-dependent protein kinase mediates effects of beta-adrenergic stimulation. *Biochem J* 1999;341(Pt 2):347-354.
13. Dyck JR, Kudo N, Barr AJ, Davies SP, Hardie DG, and Lopaschuk GD. Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. *Eur J Biochem* 1999;262:184-190.
14. Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang CC, Itani SI, et al. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: Acetyl–CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc Natl Acad Sci U S A* 2002; 99:16309-16313.
15. Oh SY, Park SK, Kim JW, Ahn YH, Park SW, and Kim KS. Acetyl-CoA Carboxylase b Gene Is Regulated by Sterol Regulatory Element-binding Protein-1 in Liver. *J Biol Chem* 2003;278(31):28410 -28417.
16. Smith TH, Block NE, Rhodes SJ, Konieczny SF, and Miller JB. A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. *Development* 1993;117:1125-1133.
17. Rudnicki MA, and Jaenisch R. The MyoD family of transcription factors and skeletal myogenesis. *Bioessays* 1995;17:203-209.
18. Sassoon DA. Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. *Dev Biol* 1993;156:11-23.
19. Sabourin LA, and Rudnicki MA. The molecular regulation of myogenesis. *Clin Genet* 62000;57:1-25.

20. Froeschle A, Alric S, Kitzmann M, Carnac G, Aurade F, Rochette-Egly C, et al. Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid-induced myogenesis. *Oncogene* 1998;16: 3369-3378.
21. Downes M, Mynett-Johnson L, and Muscat GE. The retinoic acid and retinoid X receptors are differentially expressed during myoblast differentiation. *Endocrinology* 1994;134:2658-2661.
22. Alric S, Froeschle A, Piquemal D, Carnac G, and Bonnieu A. Functional specificity of the two retinoic acid receptor RAR and RXR families in myogenesis. *Oncogene* 1998;16:273-282.
23. Kim JY, Lee, JJ, and Kim KS. Acetyl-CoA carboxylase beta expression mediated by MyoD and muscle regulatory factor 4 is differentially affected by retinoic acid receptor and retinoid X receptor. *Exp Mol Med* 2003;35:23-29.
24. Olson EN, and Srivastava D. Molecular Pathways Controlling Heart Development. *Science* 1996;272:671-676.
25. Kasahara H, Usheva A, Ueyama T, Aoki H, Horikoshi N, and Izumo S. Characterization of Homo- and Heterodimerization of Cardiac Csx/Nkx2.5 Homeoprotein. *J Biol Chem* 2001;276: 4570-4580.
26. Komuro I, and Izumo S. Csx: A Murine Homeobox-Containing Gene Specifically Expressed in the Developing Heart. *Proc Natl Acad Sci U S A* 1993;90: 8145-8149.
27. Molkentin JD, Lin Q, Duncan SA, and Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 1997;11:1061-1072.
28. Durocher D, Charron F, Warren R, Schwartz RJ, and Nemer M. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J* 1997;16:5687-5696.
29. Lee Y, Shioi T, Kasahara H, Jobe SM, Wiese RJ, Markham BE, and Izumo S. The Cardiac Tissue-Restricted Homeobox Protein Csx/Nkx2.5 Physically Associates with the Zinc Finger Protein GATA4 and Cooperatively Activates Atrial Natriuretic Factor Gene Expression. *Mol Cell Biol* 1998;18:3120-3129.

30. Dentice M, Morisco C, Vitale M, Rossi G, Fenzi G, and Salvatore D. The Different Cardiac Expression of the Type 2 Iodothyronine Deiodinase Gene between Human and Rat Is Related to the Differential Response of the dio2 Genes to Nkx-2.5 and GATA-4 Transcription Factors. *Mol Endocrinol* 2003;17:1508-1521.
31. Sepulveda JL, Belaguli N, Nigam V, Chen CY, Nemer M, and Schwartz RJ. GATA-4 and Nkx-2.5 CoActivate Nkx-2 DNA Binding Targets: Role for Regulating Early Cardiac Gene *Mol Cell Biol* 1998;18:3405-3415.
32. Kim KS, Park SW, Moon YA, and Kim YS. Expression. Organization of the 5' region of the rat ATP citrate lyase gene. *Biochem J* 1994; 302(Pt 3):759-764.
33. Ahn YH, Kim JW, Han GS, Lee BG, and Kim YS. Cloning and characterization of rat pancreatic beta-cell/liver type glucose transporter gene: a unique exon/intron organization. *Arch Biochem Biophy*s 1995;323(2):387-396.
34. Vavvas D, Apazidis A, Saha AK., Gamble J, Patel A, Kemp BE, et al. Contraction-induced Changes in Acetyl-CoA Carboxylase and 5-AMP-activated Kinase in Skeletal Muscle. *J Biol Chem* 1997;272: 13255-13261.

ABSTRACT(IN KOREAN)

Acetyl-CoA carboxylase β 유전자의 조직 특이적인 프로모터 이용

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Acetyl-CoA carboxylase β (ACCβ)는 지방산 산화를 조절하는 중요한 효소로서 근육과 심장, 간에서 고 발현 된다. ACCβ 유전자 발현은 두 종류의 프로모터들, P-Iβ과 P-IIβ, 에 의해 조절되는 것으로 알려져 있다. 그러나 아직까지 조직 특이적인 ACCβ 유전자 발현이 어떻게 조절되는 지에 관한 명확한 기작은 알려진 바가 없다. 본 연구를 통해, 근육과 심장에서의 ACCβ 유전자 발현은 P-Iβ를 통해 조절되고 있음을 확인하였고 P-Iβ는 근육 특이적인 단백질의 전사를 조절하는 전사인자인 myogenic regulatory factor 4 (MRF4)와 retinoid X receptor α (RXRα)에 의해서, 심장 특이적 발현 단백질을 조절하는 전사인자들로 알려진 cardiac-specific homeobox protein (Csx)과 GATA4에 의해서 발현이 조절됨을 확인하였다. 이는 골격근과 심장에서의 ACCβ 발현이 조직 특이적인 전사인자에 의한 전사 조절 결과임을 나타낸다. 또다른 조절 기작을 DNA 염기서열상 메틸화 (CpG methylation)를 분석함으로써 규명하고자 하였다. 각 조직에서 ACCβ 유전자의 exon 1a 주변의 CpG 메틸화를 분석한 결과, 간 조직에서 추출한 DNA는 P-Iβ 프로모터는 완전히 메틸화되어 있으나, 골격근에서 추출한 DNA는 절반 정도 메틸화가 되어 있었다. 이를 통해, 근육에서의 P-Iβ 프로모터를 이용한 전사 조절은 조직 특이적인 전사인자 뿐만 아니라 DNA 염기 서열상 디메틸화에

의한 조절을 포함한다는 결론을 얻을 수 있었다. ACCβ의 프로모터 이용은 사람과 쥐에서 다른 양상을 보였다. 사람의 골격근에서는 P-Iβ 프로모터 뿐만 아니라 P-IIβ 프로모터에 의해서도 전사가 활발히 일어나지만, 쥐의 골격근에서는 P-Iβ 프로모터만이 전사에 기여한다. 종간에 ACCβ 프로모터 이용의 차이가 P-IIβ 프로모터의 염기서열 차이에 기인할 가능성을 나타내는 결과로, 사람의 P-IIβ 프로모터에는 전사 시작 부위 주변에 MRF에 의한 전사 활성화에 중요한 역할을 담당하는 2개의 MRF 결합 부위가 존재하는데 쥐에서는 이러한 결합자리가 보존되지 않았다. 마지막으로, 본 논문에서는 간암 세포주들에서 특이적으로 ACCβ 전사를 담당하는 새로운 프로모터(Promoter Oβ)가 P-Iβ 보다 3Kb 위쪽에 존재함을 확인하였다.

이상의 연구 결과들을 통해 조직에 따라 ACCβ 유전자 발현이 다르게 나타나는 기전을 밝히었다.

핵심되는 말 : acetyl-CoA carboxylase β, 프로모터, 조직 특이적 전사인자, DNA 메틸화, 전사 조절