J. Biochem. **133**, 523–532 (2003)
DOI: 10.1093/jb/mvg069

The Gene Encoding Muscle-Type Carnitine Palmitoyltransferase I: Comparison of the 5′-Upstream Region of Human and Rodent Genes

Naoshi Yamazaki*[,1], Yasuhisa Yamanaka[1], Yoshiko Hashimoto[1], Takayuki Hiramatsu[1], Yasuo Shinohara[1,2,3] and Hiroshi Terada[4]

[1]Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770-8505; [2]Institute for Genome Research, University of Tokushima, Kuramotocho 3-18, Tokushima 770-8503; [3]Single-Molecule Bioanalysis Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Hayashicho 2217-14, Takamatsu 761-0395; and [4]Faculty of Pharmaceutical Sciences, Tokyo University of Science, Shinjuku-ku, Tokyo 162-0826

Received December 9, 2002; accepted January 31, 2003

Muscle-type carnitine palmitoyltransferase I (M-CPTI) is the key enzyme for fatty acid β-oxidation in heart and skeletal muscles and in adipose tissue. So far, M-CPTI mRNA has been detected in white adipocytes from epididymal fat pads of rats and humans, but not in mouse adipocytes. To characterize the gene expression of M-CPTI in mice, we isolated the genomic DNA encoding mouse M-CPTI and determined its transcription initiation site. As a result, the mouse M-CPTI gene seemed to have multiple initiation sites, as in the case of the rat and human genes. Furthermore, the conserved nucleotide sequence of the response element for peroxisome proliferators was shown to exist in the upstream of the mouse gene as in that of the rat and human genes. From these observations, we suggest that the anomalous expression of M-CPTI in mouse adipocytes reported previously may be regulated by factors other than peroxisome proliferators. Previously, we reported that there were transcripts containing regions of both CK/EK-β and M-CPTI genes in humans. In this study, we found that such transcripts also exist in rodents and that the amounts of the transcripts containing regions of both of these genes did not depend on the expression level of CK/EK-β.

Key words: β-oxidation, carnitine, fatty acid, gene structure, overlapping transcript.

Abbreviations: BAT, brown adipose tissue; CK/EK-β, choline/ethanolamine kinase–β; CPTI, carnitine palmitoyltransferase I; L-CPTI, liver-type CPTI; M-CPTI, muscle-type CPTI; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SLIC-PCR, single-strand ligation to ss-cDNA-PCR; ss-cDNA, single-strand cDNA.

Mitochondrial carnitine palmitoyltransferase I (CPTI), located on the outer membrane of the mitochondrion, mediates the transfer of the acyl-chain of the cytosolic long-chain acyl-CoA to carnitine. The acylcarnitine thus formed crosses to the inner mitochondrial membrane *via* the carnitine-acylcarnitine carrier, and is reconverted to acyl-CoA and carnitine by carnitine palmitoyltransferase II located on the inner surface of the inner membrane. Through these steps, the acyl moiety of long-chain acyl-CoA can move into the matrix space and undergo β-oxidation to produce acetyl-CoA. In addition, the catalytic activity of CPTI is inhibited by malonyl-CoA synthesized from excess acetyl-CoA. Therefore, the conversion of acyl-CoA to acylcarnitine by CPTI plays an important role in regulation of mitochondrial β-oxidation (*1*, *2*).

Two isoforms of CPTI are presently known: the liver type (L-CPTI) and the muscle type (M-CPTI) (*3*). Although their primary structures are similar, their affinities for carnitine and susceptibilities to malonyl-CoA are quite different. In addition, they are encoded by different genes, and their tissue distributions are completely different. L-CPTI is dominant in liver, kidney, and intestine; whereas M-CPTI is more abundant in heart and skeletal muscles and in brown adipose tissue (BAT). It is noteworthy that M-CPTI is expressed in cells that consume bioenergy extensively by using fatty acids as their major respiratory substrate.

The structures of the human (*4–6*) and rat (*7*) genes encoding M-CPTI have been characterized. The 5′-upstream region of the human gene contains two transcription initiation sites, which were reported to be utilized in a tissue-dependent manner (*6*). In the rat M-CPTI gene, only one initiation site has been identified (*7*). Accordingly, it would seem that transcription of the M-CPTI gene is regulated by different mechanisms in humans and rats. In addition, although M-CPTI mRNA was detected in white adipocytes from epididymal fat pad of rats, hamsters, and humans, it was not detected in mouse adipocytes (*8*). This anomalous expression of mouse M-CPTI has been attributed to a lack of induction of this gene during the differentiation of preadipocytes to mature adipocytes. Therefore, it has been suggested that the transcriptional regulation of the M-CPTI gene in adipocytes is different between the mouse and other species.

The gene encoding mouse M-CPTI was reported to be located on chromosome 15 (*9*); however, its structure has not been characterized. Since characterization of the mouse M-CPTI gene is expected to provide valuable

*To whom correspondence should be addressed. Tel: +81-88-633-7278, Fax: +81-88-633-9512, E-mail: yamazaki@ph.tokushima-u.ac.jp

information on the differences in expression of M-CPTI genes between species, in this study, we determined the gene structure of mouse M-CPTI. Furthermore, as we earlier reported that the gene encoding human choline/ethanolamine kinase-β (CK/EK-β) was located just 300 bp 5′-upstream of the M-CPTI gene in the same strand direction and that there were transcripts containing regions of both human CK/EK-β and M-CPTI genes (*10*, *11*), in this study, we also analyzed whether there are such transcripts in the mouse and rat.

MATERIALS AND METHODS

Materials and General Methods—λFIXII/*Xho*I Partial Fill-in Vector Kit and Gigapack III XL Packaging Extract were obtained from Stratagene (La Jolla). Total RNAs of male ddY mouse and male Wistar rat tissues were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (*12*) or by centrifugation through $CsCl_2$ (*13*). Poly(A)$^+$ RNAs of mouse and rat tissues were extracted from total RNAs by oligo(dT) cellulose column chromatography or by using Oligotex™-dT30 (Roche Diagnostics, Tokyo). Poly(A)$^+$ RNAs of human tissues were obtained from Clontech (Palo Alto). Cap-site cDNA™ of mouse, rat, and human heart were obtained from Nippon Gene (Tokyo). Other enzymes and reagents were of the highest grade commercially available. Recombinant DNA experiments were employed according to the standard protocols (*14*) and/or the supplier's recommendations.

Isolation of the cDNA Fragments Encoding Mouse M-CPTI—cDNA fragments encoding mouse M-CPTI were isolated from ddY mouse BAT by use of the reverse transcription-polymerase chain reaction (RT-PCR). To obtain single-strand cDNA (ss-cDNA) of mouse BAT, we reverse-transcribed poly(A)$^+$ RNA with random hexamer or oligo(dT) primer [T_{17} Adp (*4*)] as described previously (*10*), and amplified the ss-cDNAs obtained by use of the polymerase chain reaction (PCR) with appropriate primer pairs. Oligonucleotide primers for PCR were designed from the nucleotide sequence of the rat M-CPTI cDNA (*15*). By RT-PCR, cDNA fragments containing nucleotides –1 to +265, –1 to +466, –1 to +1826, +183 to +466, +183 to +1826, +425 to +1826, +1724 to +2403, and +1783 to +2403 were isolated (in which the adenine base of the translation initiation codon of mouse M-CPTI cDNA is taken as +1). These cDNA fragments were ligated with a plasmid vector, and their nucleotide sequences were determined. To avoid sequence errors by PCR, we sequenced at least three clones obtained by independent reactions. The nucleotide sequence of mouse M-CPTI cDNA has been submitted to the DDBJ with the accession number AB010826.

Characterization of the 3′-Region of M-CPTI and CK/EK-β Genes—The nucleotide sequence of the 3′-region of mouse M-CPTI cDNA was determined by 3′-rapid amplification of cDNA ends (3′-RACE), as described previously (*10*). Two specific sense primers (nucleotide sequences +2079 to +2096 and +2110 to +2125) were designed and used with Adp primer for isolation of the cDNA containing the 3′-region of M-CPTI by PCR (*4*). cDNAs obtained by 3′-RACE were inserted into a plasmid vector, and their nucleotide sequences were determined.

Similarly, to determine the 3′-ends of mouse and rat CK/EK-β genes, 3′-RACE was carried out. In the case of the CK/EK-β gene, the two specific primers that had been used for 3′-RACE of human CK/EK-β were used (*10*). The 3′-ends of mouse and rat CK/EK-β genes were determined by comparing the nucleotide sequences of cDNAs obtained by 3′-RACE with the genomic DNA sequences (*7*, *16*).

Isolation and Characterization of the Gene Encoding Mouse M-CPTI—The genomic DNA library of a male ddY mouse was constructed and screened as described previously (*16*). Briefly, mouse genomic DNA was prepared from the liver of a 25-week-old mouse. High molecular weight genomic DNA isolated from the liver was partially digested by using *Sau*3AI to obtain DNA fragments of approx. 20 kbp, and these fragments were partially filled-in with deoxyadenine and deoxyguanine by use of Klenow DNA polymerase. The DNA fragments obtained were ligated to λFIXII partially filled-in vector, and concatenated DNAs were packaged into phage particles. The number of original phages was 5.4×10^5 plaque-forming units, and the phage library was used without amplification. To isolate a genomic DNA clone of mouse M-CPTI, we radiolabeled a mouse cDNA fragment obtained by PCR (–1 to +466) by the multipriming method with [α-^{32}P]dCTP and used it as a probe. Screening procedures were carried out as described previously (*17*). After three rounds of screening, clone MG3 was selected and its phage DNA was isolated. Then, the mouse genomic DNA inserted into phage DNA was digested with appropriate restriction enzymes, and DNA fragments containing the 5′-upstream portion of the M-CPTI gene were subcloned and sequenced. The nucleotide sequence of the 5′-upstream region of mouse M-CPTI gene has been submitted to the DDBJ with the accession number AB085661.

Determination of the transcription initiation sites of M-CPTI genes by the oligo-capping method—The transcription initiation site of the mouse M-CPTI gene was determined by the oligo-capping method (*18*) using Cap-site cDNA™ from mouse heart. The Cap-site cDNA™ used in this study was prepared by reverse-transcription of the modified mRNA, which was obtained by ligation of the RNA oligonucleotide to the 5′-end of the de-capped mRNA, with random primer. For amplifying the Cap-site cDNA™ by PCR, the following oligonucleotide primers were used: 1RC and 2RC, both attached to the Cap-site cDNA™; P1 (5′-GGGAGGCATCTCTGGATGC, antisense), corresponding to exon 3 of human and rat M-CPTI genes; and P2 (5′(-ATAAGGCGTTTCTTCCAGG, antisense), corresponding to exon 2. Cap-site cDNA™ was diluted 5-fold and used as a template for the 1st PCR with 1RC and P1. After the 1st PCR, the reaction mixture was diluted 10^5-fold and used as a template for the 2nd PCR with 2RC and P2. cDNA fragments obtained by the 2nd PCR were ligated to a plasmid vector, and their nucleotide sequences were analyzed. The transcription initiation site(s) was determined by comparison of the nucleotide sequences of cDNA clones with the corresponding sequence of genomic DNA and that of RNA oligonucleotide. Similarly, rat and human heart Cap-site cDNA™ were also used to confirm the transcription initiation sites of M-CPTI genes reported previously.

RT-PCR for the 5′-Upstream Region of M-CPTI Genes—To characterize the transcripts containing the 5′-upstream region of the M-CPTI gene, we designed oligonucleotides P3, P4, P5, P6, and P7 and used them as sense primers for RT-PCR in combination with the antisense primer P1. The primers P3, P4, and P5 corresponded to the 5′-flanking region of exon 1 determined by the oligo-capping method; primer P6, to exon 1 of the M-CPTI gene; and primer P7, to the last exon of the mouse CK/EK-β gene (*16*). For amplification of cDNA, poly(A)$^+$ RNAs of human, mouse, and rat tissues were reverse-transcribed with T_{17} Adp primer, and 1 μl of ss-cDNAs obtained were used as templates for PCR. Amplifications were performed in 25 μl reaction mixtures. After RT-PCR, 5 μl of the reaction mixtures were subjected to agarose gel electrophoresis and stained with ethidium bromide. To confirm the correctness of the RT-PCR products, we subcloned the cDNA fragments into a plasmid vector and sequenced them. In some cases, the nucleotide sequences of RT-PCR products were determined by direct sequencing without subcloning.

Northern Blot Analysis of CK/EK-β—Samples of poly(A)$^+$ RNA (1 μg) of rat, human, and mouse kidney and heart were loaded onto denatured agarose gel, electrophoresed, and then transferred to nitrocellulose membrane. The cDNA fragment of human CK/EK-β was prepared by digestion of human cDNA with *Pvu*II (position 400–1164 according to Ref. *10*), and the cDNA fragments of rat (position 503–1391 according to Ref. *19*) and mouse (position 524–1412 according to Ref. *20*) were prepared by RT-PCR. cDNA fragments obtained were radiolabeled by multipriming with [α-^{32}P]dCTP and mixed, each with almost the same radioactivity. Using these mixed radiolabeled probes, we carried out the hybridization procedure as described previously (*10*). Before Northern analysis, Southern analysis was carried out to compare the signal intensities obtained by using the mixed probes. cDNA fragments used as probes were loaded onto agarose gel without radiolabeling, transferred to a nitrocellulose membrane, and then hybridized with the mixed probe. The probe for β-actin (rat) was prepared as described previously (*15*).

RESULTS

Isolation of the cDNA Fragments Encoding Mouse M-CPTI—For analysis of the gene structure of mouse M-CPTI, we first isolated the cDNA fragments encoding mouse M-CPTI by RT-PCR and sequenced them (data not shown). From the nucleotide sequence, we found that mouse M-CPTI consisted of 772 amino acids, like the human and rat enzymes, and had a predicted size of 88.2 kDa, similar to that of rat (88.2 kDa) and human M-CPTI (87.8 kDa). The amino acid sequence of mouse M-CPTI was highly homologous with those of the rat and human proteins (about 95% and 87%, respectively).

Determination of the Transcription Initiation Site of Mouse and Rat M-CPTI Genes—The structure of the gene encoding M-CPTI has hitherto been reported for human (*4–6*) and rat (*7*), as schematically shown in Fig. 1A. The 5′-upstream region of the human gene contains two transcription initiation sites (*6*), here referred to as "PEU" and "PEM." In rat M-CPTI gene, only one initiation site was identified (*7*), here referred to as "PER." To explore species differences in the gene structure, we isolated the genomic DNA encoding mouse M-CPTI and sequenced it. To determine the transcription initiation site(s), cDNA clones containing the 5′-upstream region of the message were obtained by the oligo-capping method and their nucleotide sequences were compared with the genomic DNA sequence. As shown in Fig. 1B, we found only one transcription initiation site in the M-CPTI gene in mouse heart (shown by the arrow "OCM"). Its position was slightly different from that of the initiation site in the rat M-CPTI gene reported previously (*7*) (shown by the arrow "PER").

We next tried to confirm the transcription initiation site of the rat M-CPTI gene by using rat heart Cap-site cDNA™. As a result, we found one transcription initiation site in the rat gene, "OCR," as shown in Fig. 1B. Its position was identical to that of "OCM" determined for the mouse gene. In the rodent M-CPTI genes, we could not identify other initiation sites located farther upstream that would correspond to the human "PEU." Therefore, we considered the transcription initiation site determined here to be the sole initiation site of mouse and rat M-CPTI genes in heart muscle.

Confirmation of the Transcription Initiation Sites of the Human M-CPTI Gene—The transcription initiation sites of the human M-CPTI gene, referred to as "PEU" and "PEM" in Fig. 1A, were determined by primer extension (*6*). To confirm the initiation sites of the human M-CPTI gene, we carried out oligo-capping with human heart Cap-site cDNA™. As shown in Fig. 1C, in the region of exon U (referred to as exon 1A in Ref. *4*), we identified three transcription initiation sites. In these sites, the one shown by arrow "OCH1" was located upstream of the initiation site reported in Ref. *6* (shown by arrow "PEU" in Fig. 1C). On the other hand, the other two sites, "OCH2" and "OCH3," were slightly downstream of "PEU." In the region of exon M (referred to as exon 1B in Ref. *4*), we identified one transcription initiation site, "OCH4", located slightly downstream of the initiation site reported in Ref. *6* (shown by arrow "PEM"). As a result, we identified four transcription initiation sites in the human M-CPTI gene by the oligo-capping method, in contrast to the one site in the mouse and rat genes.

Comparison of the Nucleotide Sequences of Peroxisome ProLiferator Response Element of Mammalian M-CPTI Genes—The 5′-flanking region of human (*6*, *21*, *22*) and rat (*7*) M-CPTI genes has been reported to contain a response element for peroxisome proliferators and fatty acids, as depicted in Fig. 1A. As peroxisome proliferators and fatty acids are known to induce the transcription of many genes in adipocytes, the anomalous expression of mouse M-CPTI in adipocytes reported (*8*) might be due to a difference in the nucleotide sequence of the response element for peroxisome proliferators and fatty acids. Therefore, we next characterized the nucleotide sequence of the peroxisome proliferator response element in the mouse gene. As a result, we found conserved nucleotides of this element at essentially the same positions as those in the human and rat genes (Fig. 1D). In fact, the nucleotide sequence of mouse element was completely the same as that of the rat gene. This observation suggests

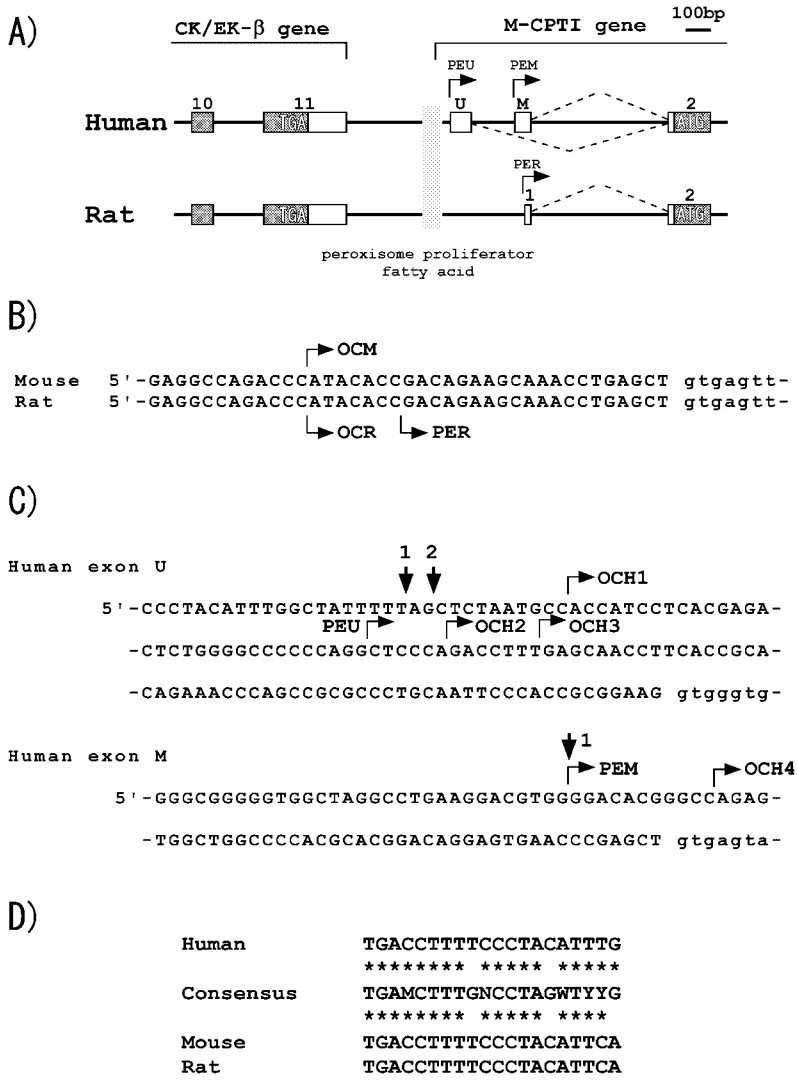

Fig. 1. **The structures of the 5′-region of M-CPTI genes.** (A) Comparison of the 5′-region of M-CPTI genes of human and rat reported previously. The gene structures are schematically shown. Boxes represent exons of CK/EK-β and M-CPTI genes, and amino acid-coding and noncoding regions are shown by closed and open boxes, respectively. Bent arrows "PEU," "PEM," and "PER" represent the transcription initiation sites of M-CPTI genes (for details, see following sections B and C), and broken lines represent splicing on the 5′-region of M-CPTI genes. The shadowed box represents the peroxisome proliferator and fatty acid response element. For the CK/EK-β region, the structure of the human gene is shown according to Ref. *10*. As the gene structure of rat CK/EK-β has not been reported, it was deduced by comparison of the nucleotide sequence of rat CK/EK-β cDNA (*19*) with that of the 5′-flanking region of rat M-CPTI gene (*7*); and the 3′-end of rat CK/EK-β was determined by 3′-RACE (data not shown). For the M-CPTI region, there are three papers on the structure of the human M-CPTI gene (*4–6*). To avoid confusion, in this figure, the gene structure of human M-CPTI is shown according to Ref. *6*; and that of rat M-CPTI, according to Ref. *7*. (B) The transcription initiation sites of mouse and rat M-CPTI genes. The nucleotide sequences of exon 1 and its upstream and downstream regions are shown. Lower case letters represent the intron between exons 1 and 2. Bent arrows "OCM" and "OCR" represent the transcription initiation site of the mouse and rat M-CPTI gene, respectively, as determined by the oligo-capping method in this study. The bent arrow "PER" represents the transcription initiation site of the rat M-CPTI gene as determined by primer extension (*7*). (C) The transcription initiation sites of the human M-CPTI gene. The nucleotide sequences of exons U and M and of their upstream and downstream regions are shown. In the region of exon U, bent arrows "OCH1," "OCH2," and "OCH3" represent the transcription initiation sites determined by the oligo-capping method in this study. Bent arrow "PEU" represents the transcription initiation site as determined by primer extension (*6*). Downward arrow 1 represents the 5′-end of exon 1A reported previously (*4*); and downward arrow 2, the splice acceptor site observed in the transcripts containing exons of both CK/EK-β and M-CPTI genes (*10*). Lower case letters represent the intron between exons U and M. In the region of exon M, bent arrow "OCH4" represents the transcription initiation site determined by the oligo-capping method in this study; and arrow "PEM", the transcription initiation site as determined by primer extension (*6*). Downward arrow 1 represents the 5′-end of exon 1B reported previously (*4*). Small letters represent the intron between exons M and 2. (D) Comparison of the nucleotide sequences of the peroxisome proliferator response element. Asterisks indicate the nucleotides identical with those of the consensus sequence (*25*).

that the transcription of the mouse M-CPTI gene is also regulated by peroxisome proliferators and fatty acids.

Isolation and Characterization of the cDNA Containing the 5′-Upstream Region of the M-CPTI Gene—As described above, we determined the transcription initiation site of rodent M-CPTI genes by the oligo-capping method. However, when we carried out single-strand ligation to ss-cDNA-PCR (SLIC-PCR) of mouse and rat M-CPTI messages, cDNA clones containing a region 5′-upstream from the transcription initiation site determined were isolated (data not shown). Hence, to confirm whether such transcripts containing a region farther upstream actually exist, we performed RT-PCR for the region 5′-upstream from the initiation site. Reverse-transcribed ss-cDNAs of mouse and rat heart were amplified by oligonucleotides P3, P4, P5, and P6 in combination with P1, as shown in Fig. 2A. Of these primers, primer P3, located in the most upstream region, was designed from the nucleotide sequence of the most extended cDNA obtained by SLIC-PCR. As shown in Fig. 2B, for both mouse and rat hearts, specific products were observed with all primer pairs when the reverse-transcribed ss-cDNAs (non-diluted) was used as a template. From the results of sequence analyses, the organizations of these PCR products were determined as schematically shown in Fig. 2C. The DNA fragments obtained by PCR using P3, P4, and P5 contained regions 5′-upstream from the initiation site, but they were not derived from genomic DNA. In the case of the RT-PCR product obtained with primer P3 in combination with P1, for example, the amplified DNA of mouse contained the nucleotides from P3 to P1 of the mouse M-CPTI gene without introns between exons 1 and 2 and between exons 2 and 3. This result suggests that DNA fragments obtained by PCR were derived from messages that had been spliced correctly. On the other hand, when diluted ss-cDNAs were used as templates for RT-PCR,

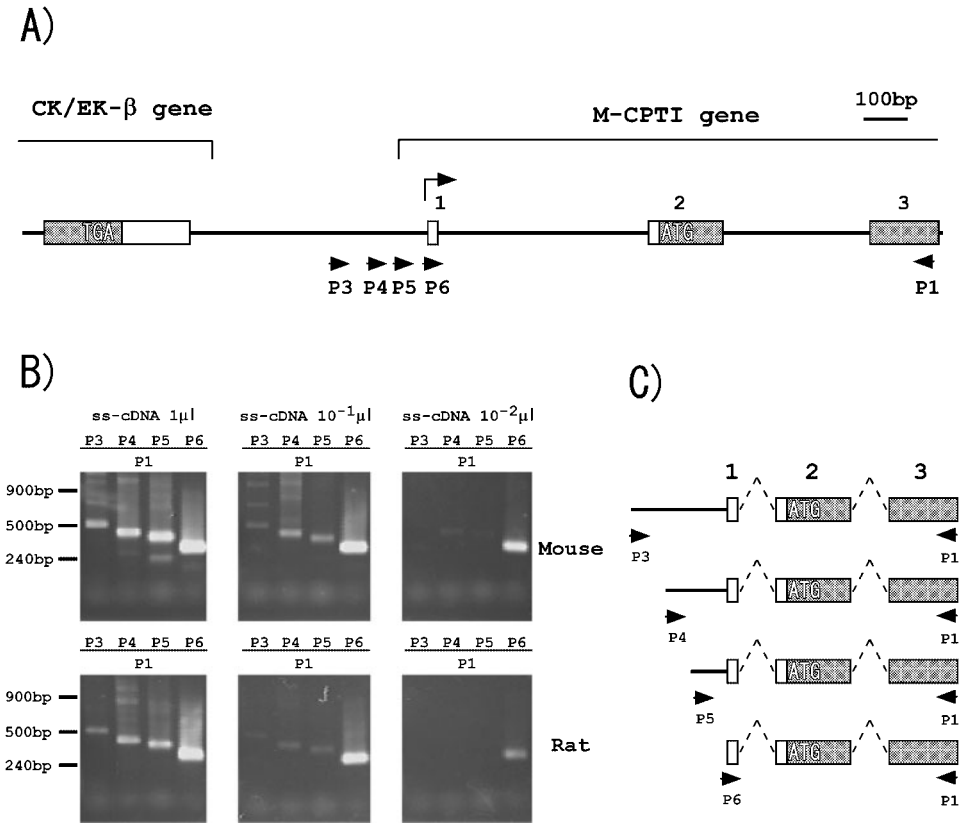

Fig. 2. **RT-PCR of the transcripts containing 5′-upstream region of M-CPTI gene.** (A) The gene structures of rodent M-CPTI. Open and closed boxes are defined in Fig. 1A. The transcription initiation site of the M-CPTI gene determined by the oligo-capping method is shown by the bent arrow. The loci of oligonucleotides P1, P3, P4, P5, and P6 used for PCR are shown by arrowheads. (B) RT-PCR of the 5′-upstream region of the M-CPTI gene. ss-cDNAs of mouse and rat heart were obtained by reverse-transcription with T_{17} Adp primer, and 1 μl of ss-cDNA or diluted ss-cDNA was used as a template for PCR. For details, see text. C) The organization of the cDNAs obtained by RT-PCR. Broken lines represent splicing determined from the nucleotide sequences of the products obtained by RT-PCR.

the intensities of the products obtained by using P3, P4, and P5 were remarkably decreased (in Fig. 1B, ss-cDNA 10^{-1} μl and 10^{-2} μl). Similar results were obtained by using ss-cDNAs of skeletal muscle and BAT, although the intensities of the electrophoretic bands were slightly different (data not shown). From these observations, we conclude that the transcript starting from the initiation site determined by the oligo-capping method was a major transcript. However, there were transcripts containing regions farther upstream from the transcription initiation site determined by the oligo-capping method, although their amounts were much lower than the amount of the major transcript.

Characterization of the Transcripts Containing both Regions of the CK/EK-β and M-CPTI in Rodents—As described above, in mouse and rat M-CPTI genes, we obtained RT-PCR products containing regions farther upstream from the transcription initiation site determined by the oligo-capping method. Accordingly, there was a possibility that another transcription initiation site(s) was present farther upstream or that the transcripts containing the 5′-upstream region were synthesized by a mechanism giving rise to transcripts of more than just that of the M-CPTI gene. Concerning the latter case, we have reported that transcripts containing exons of both CK/EK-β and M-CPTI genes were detectable in human heart and skeletal muscle and that one of these transcripts contained exon U of M-CPTI (*10*), as schematically shown in Fig. 3A. Therefore, the transcripts containing the 5′-upstream region of the initiation site of mouse and rat M-CPTI genes, as shown in Fig. 2, may have been derived from such a transcript like the one observed in humans.

To determine whether such overlapping transcripts also exist in rodents, we next carried out RT-PCR between exons of CK/EK-β and M-CPTI genes of mouse and rat. As shown in Fig. 3A, by using primer P7, located at the last exon of the CK/EK-β gene, in combination with P1, ss-cDNAs of mouse and rat heart were amplified. As a control, ss-cDNA of human heart was also analyzed. The results of electrophoresis of the reaction mixtures are shown in Fig. 3B. When 5 μl of the reaction mixture (total reaction volume was 25 μl) of human heart was subjected to agarose gel electrophoresis, two products, with sizes of approx. 500 bp and 600 bp (referred to as products 1 and 2, respectively), were detected. From the nucleotide sequences, the organizations of PCR products of human heart were confirmed to be as depicted in Fig. 3C. The structures of these two products were in agreement with those reported previously (*10*).

On the other hand, in mouse heart, amplified DNA could not be detected by loading 5 μl of total reaction mixture (25 μl). By loading all the reaction mixtures of two PCR tubes onto agarose gel, two products, with approximate sizes of 450 bp and 600 bp, were detected, as shown in Fig. 3B. From their nucleotide sequences, the product from the faster migrating band, referred to as product 1, was found to contain the nucleotides of exon 11 of the CK/EK-β gene and exon 2 and exon 3 of the M-CPTI gene without exon 1, as depicted in Fig. 3C. The structure of mouse product 1 was similar to that of human product 1, although the position of the splice donor site located in the CK/EK-β gene was slightly different. The product

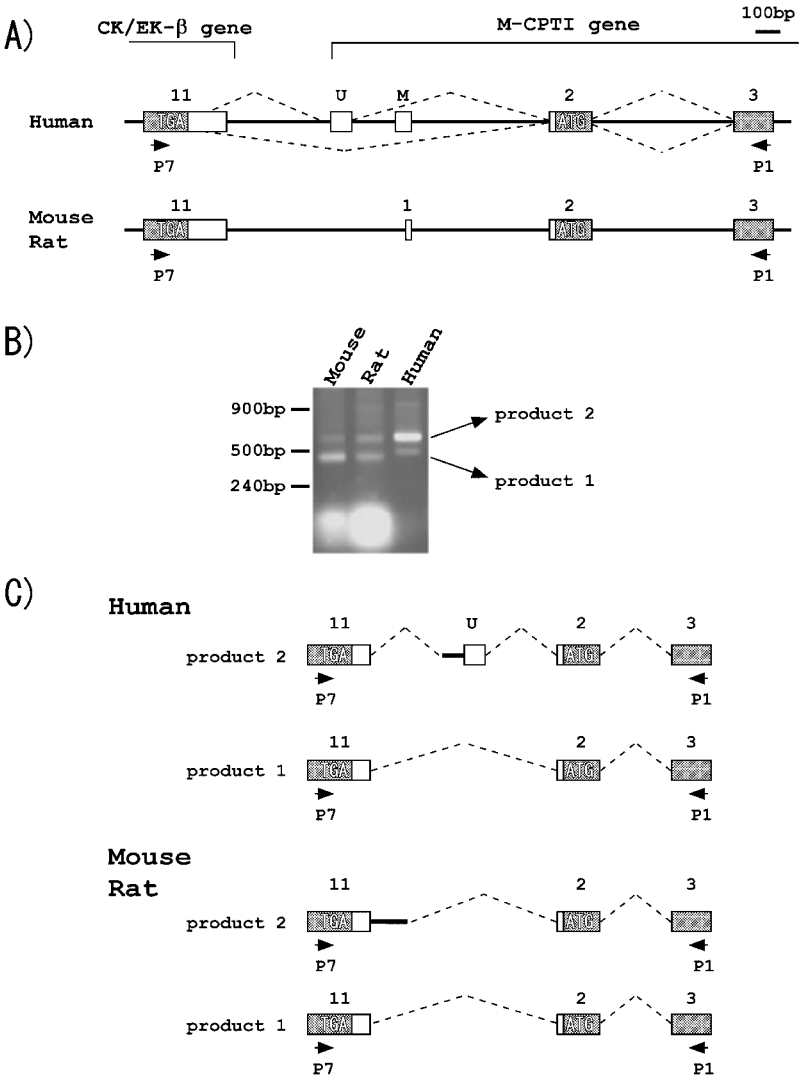

Fig. 3. **RT-PCR between the regions of CK/EK-β and M-CPTI genes.** (A) The gene structures of human and rodent CK/EK-β and M-CPTI. Open and closed boxes are defined in Fig. 1A. In the human gene, the broken lines represent splicing of the transcripts containing exons of both CK/EK-β and M-CPTI genes previously reported (*10*). The locus of oligonucleotides P1 and P7, used for PCR, are shown by arrowheads. (B) RT-PCR of the transcripts containing the region of CK/EK-β and M-CPTI genes. ss-cDNAs of mouse, rat, and human heart were obtained by reverse-transcription with T_{17} Adp primer, and 1 μl of ss-cDNAs was used as a template for PCR. For details, see text. (C) The organization of the cDNAs obtained by RT-PCR. Broken lines represent splicing determined from the nucleotide sequences of the products obtained by RT-PCR.

from the slower migrating band, referred to as product 2, contained more of the downstream region of the CK/EK-β gene than did product 1. In other words, the splice donor site of product 2 was located downstream of the 3′-end of the mouse CK/EK-β gene, *i.e.*, in the spacer region between the two genes. In these two mouse RT-PCR products, the boundaries of the CK/EK-β region and M-CPTI region always followed the GT-AG rule, and the 5′-end of exon 2 of the M-CPTI gene was the same as that of the ordinary transcript of M-CPTI, suggesting that these PCR products came from spliced transcripts. Similarly, in the rat heart, we detected two amplified DNA products by loading all of the reaction mixtures of eight PCR tubes (Fig. 3B). From the nucleotide sequences, the organization of these two products was essentially the same as that observed in the mouse products, except for their nucleotide sequences. These products were also obtained by RT-PCR when ss-cDNAs of mouse and rat skeletal muscle and BAT were utilized as templates, although their amounts differed (data not shown).

The Structure of the 5′-Region of the Mouse M-CPTI Gene—Figure 3 showed that transcripts containing exons of both CK/EK-β and M-CPTI genes also existed in rodents, although in much lower amounts than in humans. Furthermore, in the mouse and rat, we could not obtain PCR products containing the 5′-upstream region of exon 1 of the M-CPTI gene, like product 2 of human, as shown in Fig. 3B. To confirm the possible existence of such transcripts, we further amplified RT-PCR products of mouse and rat heart obtained with P7 and P1 by using primers P3, P4, P5, and P6 with P2. However, appropriate products could not be detected by agarose gel electrophoresis (data not shown). On the other hand, transcripts containing just the upstream region of exon 1 were easily detected by RT-PCR using primers P3, P4, and P5 with P1, as was shown in Fig. 2. Therefore, it seemed that RT-PCR products obtained by using P3, P4, and P5 were not derived from the overlapping transcripts containing exons of both CK/EK-β and M-CPTI genes, but from transcripts that started at another initiation site(s) located 5′-upstream of "OCM" and "OCR."

From these observations, we determined the structure of the 5′-region of the mouse M-CPTI gene, and refined the structure of the rat M-CPTI gene, as schematically shown in Fig. 4A. The nucleotide sequence of the 5′-

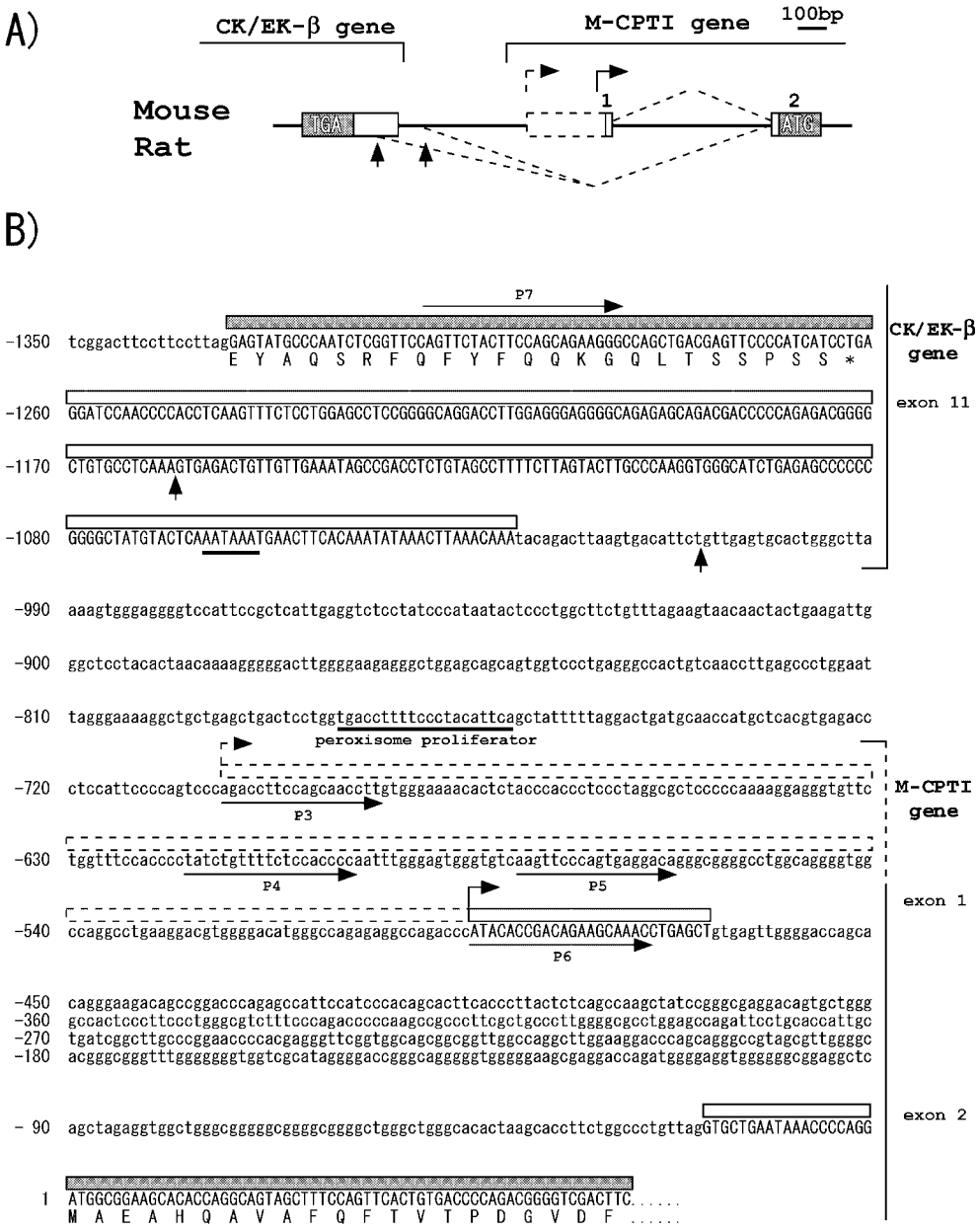

Fig. 4. **Detailed structure of the 5′-region of the mouse M-CPTI gene.** (A) Gene structure of the 5′-region of mouse and rat M-CPTIs. Boxes represent exons as in Fig. 1A, and the solid bent arrow represents the transcription initiation sites of mouse and rat M-CPTI genes as determined by the oligo-capping method. The broken box and broken bent arrow represent the putative exon and putative transcription initiation site, respectively, of the M-CPTI gene. Broken lines represent splicing of M-CPTI messages and the overlapping transcripts. The splice donor sites for the overlapping transcripts located in the region of CK/EK-β gene are shown by upward arrowheads. (B) Nucleotide sequence of the 5′-region of the mouse M-CPTI gene. Nucleotides in exons and introns are shown by capital and lowercase letters, respectively, and the deduced amino acids are shown by the 1-letter abbreviation codes under the nucleotides. Nucleotides are numbered taking the adenine base in the translation initiation codon of mouse M-CPTI as +1, and their numbers are shown in the left margin. Primers used for RT-PCR are shown by long arrows. Boxes, bent arrows, and arrowheads are the same as those shown in "A." In the CK/EK-β gene, the translation termination codon is marked by an asterisk, the nucleotide sequence of the underlined AATAAA represents a poly(A) additional signal, and the 3′-end was determined by 3′-RACE (data not shown).

upstream region of the mouse M-CPTI gene and the loci of oligonucleotides used for RT-PCR are shown in Fig. 4B. In mouse and rat M-CPTI genes, there are at least two transcription initiation sites, the one determined by the oligo-capping method, and other(s) not yet defined, though located at least upstream of primer P3.

Steady-State Transcript Level of Mammalian CK/EK-β—Previously, we reported that the overlapping transcripts observed in humans contained the entire open reading frame of M-CPTI (*10*). This observation suggested that these transcripts were started from the region of the CK/EK-β gene (possibly from the transcription initiation site of CK/EK-β gene) and synthesized by splicing between the regions of the two genes without transcriptional termination of CK/EK-β (more exactly, without the addition of the poly(A) tail to the primary transcript).

This suggested a possible relationship between the amount of the CK/EK-β message and that of the overlapping transcripts; and so we next analyzed the steady-state transcript level of CK/EK-β. However, as the nucleotide sequence of CK/EK-β cDNA is not highly conserved among species (the nucleotide sequence of human CK/EK-β cDNA used as a probe was about 86% homologous with those sequences of rodents, and the nucleotide sequence of rat CK/EK-β cDNA was about 92% homologous with that of the mouse), we prepared probes for each species and mixed them before use. To examine whether the probe thus made is effective to detect equally the transcripts of CK/EK-β over species differences, we carried out Southern analysis. As a result, we found that the transcript levels of CK/EK-β could be compared among species by using the mixed probe (Fig. 5A).

Using this mixed probe, we analyzed the transcript level of CK/EK-β in the kidney and heart of all three spe-

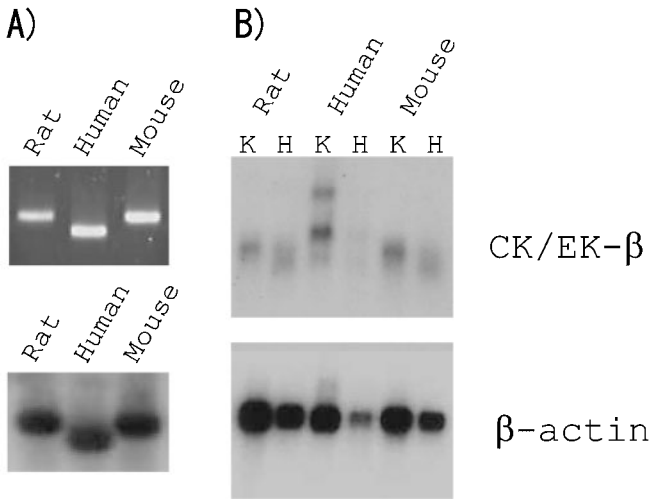

Fig. 5. **Steady-state level of CK/EK-β transcripts.** (A) cDNA fragments of rat, human, and mouse CK/EK-β used as probes were subjected to agarose gel electrophoresis without radiolabeling and stained with ethidium bromide (Upper photograph), then transferred to a nitrocellulose membrane and hybridized with the mixed radiolabeled probes of CK/EK-β (Lower autoradiogram). (B) Samples of poly(A)$^+$ RNA (1 μg) of mammalian kidney (K) and heart (H) were subjected to agarose gel electrophoresis and hybridized with the mixed probe of CK/EK-β (Upper autoradiogram). After exposure for the detection of the transcripts of CK/EK-β, the membrane was washed to strip off the probe for CK/EK-β, then rehybridized with the probe for β-actin (Lower autoradiogram).

cies by Northern analysis. As shown in Fig. 5B, the signal intensities of CK/EK-β did not differ greatly between species, as was also the case for β-actin. Therefore, we concluded that the level of the overlapping transcripts containing exons of both genes does not depend on the transcript level of CK/EK-β.

DISCUSSION

It was previously reported that the message of M-CPTI was detected in rat and human adipocytes, but not in mouse adipocytes (*8*). Expecting that comparison of the gene structure of mouse M-CPTI with that of other species would provide information on the expression mechanism of M-CPTI gene, in this study, we characterized the gene encoding mouse M-CPTI.

First, we isolated cDNA fragments of ddY mouse M-CPTI and determined their nucleotide sequence. On the other hand, Kox *et al.* have reported the nucleotide sequence of a partial cDNA fragment encoding ICR mouse M-CPTI (*9*) and registered that of full-length cDNA in the DNA database with the accession number AF017174.2. Comparison of the nucleotide sequence determined by us with that of AF017174.2 revealed that three nucleotides were different in the open reading frame; and two of them caused a change in the translated amino acids (K528E and I761V). The difference between amino acid sequences of ddY and ICR mouse may have some physiological meaning.

To determine the transcription initiation site of M-CPTI gene, we carried out the oligo-capping method. By using heart Cap-site cDNA™, we found only one transcription initiation site in the mouse M-CPTI gene. Its position was identical to that of the rat gene determined by us (in Fig. 1B, "OCM" and "OCR," respectively). Previously, Wang *et al.* reported the transcription initiation site of rat M-CPTI gene by reverse-transcription of the message of neonatal rat cardiac myocytes (*7*) ("PER" in Fig. 1B). Although the position of "PER" was slightly different from that of "OCR", only a single reverse-transcribed product was observed by primer extension of rat mRNA, and "PER" had been deduced from the electrophoretic mobility of this product (*7*). Therefore, the observed differences in the transcription initiation site of rat M-CPTI gene ("PER" and "OCR") would seem to be due to the poor resolution of the primer extension product in the previous study, and "OCR" would be the actual initiation site.

However, during the preparation of the manuscript for this paper, the transcription initiation sites of mouse and rat M-CPTI genes were also reported by another group (*23*). In Ref. *23*, van der Leij and coworkers carried out oligo-capping and determined several initiation sites of the mouse and rat M-CPTI genes, near to "OCM" and "OCR" determined by us, but "OCM" and "OCR" were not listed. We cannot explain why the initiation sites determined by us were different from those determined by van der Leij *et al.*, even though the same experimental method was carried out. However, these heterogeneous initiation sites surrounding "OCM" or "OCR" (including "OCM" and "OCR") might be properly utilized in rodents *via* some mechanism.

Furthermore, other transcription initiation sites of mouse and rat M-CPTI genes, located at about 200 bp upstream of "OCM" and "OCR," were also identified by van der Leij and coworkers (*23*). We could not determine such initiation sites by the oligo-capping method; however, the presence of other transcription initiation site(s) upstream of "OCM" and "OCR" was suggested by comparison of the results of RT-PCR shown in Figs. 2 and 3. The positions of the initiation sites determined by van der Leij *et al.* were almost the same as the 5′-end of the primer P3 that we used for RT-PCR. Furthermore, we could not obtain appropriate cDNA by RT-PCR using primers located upstream of P3 with P1 (in Fig. 2, data not shown). Therefore, in addition to "OCM" and "OCR," nucleotides corresponding to the 5′-end of primer P3 would seem to act as another transcription initiation site in the M-CPTI gene of the mouse and rat. As the position of the 5′-region of primer P3 is far from "OCM" ("OCR"), at least two proximal promoters may exist, one for transcription from the 5′-region of primer P3 and the other for transcription from "OCM" ("OCR").

To determine the transcription initiation sites of the human M-CPTI gene, we previously carried out 5′-RACE (*4*) and SLIC-PCR (*10*), and others carried out primer extension (*6*). Although primer extension is the usual method used for identification of the transcription initiation site of a gene, it cannot identify minor initiation sites. On the contrary, by 5′-RACE and SLIC-PCR, minor transcription initiation sites can be determined, because reverse-transcribed ss-cDNAs are amplified. However, insufficiently reverse-transcribed products could be isolated by 5′-RACE and SLIC-PCR based on primer extension. For these reasons, the transcription initiation sites

of the human M-CPTI gene reported previously may not reflect the exact initiation sites. Therefore, we further examined the transcription initiation sites of the human M-CPTI gene by the oligo-capping method. As a result, although all of the initiation sites determined by us were different from those reported previously (*6*), the positions of "OCH2" and "OCH3" were near that of "PEU", and the position of "OCH4" was near that of "PEM" (Fig. 1C). In addition, it has been suggested that there might be several minor initiation sites upstream of exon U (*6*), and "OCH1", which is located upstream of the 5′-end of exon U (PEU), could be one of these sites. In the region of exon U of the human M-CPTI gene, the position of "OCH1" is far from that of "OCH2" or "OCH3". Therefore, it seems that there are at least three proximal promoters for transcription initiation of the human M-CPTI gene, namely, "OCH1," "OCH2(3)," and "OCH4." In this study, the 5′-ends of exon 1A and exon 1B determined by 5′-RACE (*4*) could not be detected as initiation sites by the oligo-capping method. As these 5′-ends were both located upstream of "OCH1" and "OCH4," cDNAs isolated by 5′-RACE previously might have been derived from the minor transcripts that started from a site upstream of the initiation sites determined in this study. From these observations, not only the human M-CPTI gene, but also the mouse and rat ones, would appear to possess multiple promoters; however, the number of proximal promoters appears to differ between humans (at least three) and rodents (at least two). As the multiple promoters could be used alternatively in a tissue and/or hormone-dependent manner, the transcriptional regulation of the human M-CPTI gene is undoubtedly more complicated than that of the mouse or rat one. In the 5′-upstream region of the determined or the predicted transcription initiation sites of mouse, rat, and human M-CPTI genes, no TATA box was observed, as reported previously in the human and rat genes (*6*, *7*).

One of the objectives of this study is to explain the anomalous expression of M-CPTI gene in mouse adipocytes (*8*), and we thought that the transcription of the mouse M-CPTI gene might be not regulated by peroxisome proliferators and fatty acids during the period of adipocytes differentiation. However, from the nucleotide sequence, mouse M-CPTI gene was found to have a peroxisome proliferator response element. Therefore, the anomalous expression of the M-CPTI gene in mouse adipocytes reported previously (*8*) must be the result of regulation *via* unidentified elements other than peroxisome proliferator response element, or caused by some difference in the mouse adipocyte itself. For example, the mouse cells may lack some transcription factor(s) that act on the M-CPTI gene during adipocyte differentiation. To explore species differences in the M-CPTI expression in adipocytes, further detailed analysis of the promoter region is necessary.

Previously, we reported that there were overlapping transcripts containing regions of both human CK/EK-β and M-CPTI genes (*10*). In this study, we found similar transcripts in rodents, but in much smaller amounts than those in humans. By Northern analysis, we found that the transcript level of rodent CK/EK-β was not so much lower than that of the human enzyme (Fig. 5B). This result is in good accord with that reported recently (*24*). There are at least two possible explanations for the species differences in the amount of overlapping transcripts between humans and rodents. First, the overlapping transcripts containing both regions start from inside the CK/EK-β gene *via* an unidentified promoter, and the promoter activity of the human gene is much higher than that of the rodent genes. Second, the overlapping transcripts start from the initiation site of the CK/EK-β gene, but the addition of the poly(A) tail to the primary transcript of CK/EK-β occurs more easily in rodents than in humans, or the splicing between the regions of transcripts of the two genes is more difficult in rodents than in humans. In addition, transcripts showing slower migration than the ordinary message of CK/EK-β but hybridized with the CK/EK-β probe were observed in human kidney, as was shown in Fig. 5B. Although we could not confirm whether these transcripts contained CK/EK-β and M-CPTI regions, they might be overlapping transcripts. From these observations, the primary transcript of human CK/EK-β would seem to be more flexible than that of rodents. The production mechanism and the physiological role of these overlapping transcripts should be analyzed.

This work was supported in part by a research grant from a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and a research grant from the Faculty of Pharmaceutical Sciences, The University of Tokushima. Nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB010826 and AB085661.

REFERENCES

1. McGarry, J.D. and Foster, D.W. (1980) Regulation of hepatic fatty acid oxidation and ketone body production. *Annu. Rev. Biochem.* **49**, 395–420
2. McGarry, J.D., Woeltje, K.F., Kuwajima, M., and Foster, D.W. (1989) Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. *Diabetes Metab. Rev.* **5**, 271–284
3. McGarry, J.D. and Brown, N.F. (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244**, 1–14
4. Yamazaki, N., Yamanaka, Y., Hashimoto, Y., Shinohara, Y., Shima, A., and Terada, H. (1997) Structure features of the gene encoding human muscle type carnitine palmitoyltransferase I. *FEBS Lett.* **409**, 401–406
5. van der Leij, F.R., Takens, J., van der Veen, A.Y., Terpstra, P., and Kuipers, J.R. (1997) Localization and intron usage analysis of the human *CPTIB* gene for muscle type carnitine palmitoyltransferase I. *Biochim. Biophys Acta* **1352**, 123–128
6. Yu, G.-S., Lu, Y.C., and Gulick, T. (1998) Co-regulation of tissue-specific alternative human carnitine palmitoyltransferase Iβ gene promoters by fatty acid enzyme substrate. *J. Biol. Chem.* **273**, 32901–32909
7. Wang, D., Harrison, W., Buja, L.M., Elder, F.F., and McMillin, J.B. (1998) Genomic DNA sequence, promoter expression, and chromosomal mapping of rat muscle carnitine palmitoyltransferase I. *Genomics* **48**, 314–323
8. Brown, N.F., Hill, J.K., Esser, V., Kirkland, J.L., Corkey, B.E., Foster, D.W., and McGarry, J.D. (1997) Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation. Inter-tissue and inter-species expression of CPT I and CPT II enzymes. *Biochem. J.* **327**, 225–231
9. Cox, K.B., Johnson, K.R., and Wood, P.A. (1998) Chromosomal locations of the mouse fatty acid oxidation genes *Cpt1a*, *Cpt1b*,

*Cpt*2, *Acadvl*, and metabolically related *Crat* gene. *Mamm. Genome* **9**, 608–610
10. Yamazaki, N., Shinohara, Y., Kajimoto, K., Shindo, M., and Terada, H. (2000) Novel expression of equivocal messages containing both regions of choline/ethanolamine kinase and muscle type carnitine palmitoyltransferase I. *J. Biol. Chem.* **275**, 31739–31746
11. Hirosawa, M., Nagase, T., Murahashi, Y., Kikuno, R., and Ohara, O. (2001) Identification of novel transcribed sequences on human chromosome 22 by expressed sequence tag mapping. *DNA Res.* **8**, 1–9
12. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
13. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299
14. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp. 1.21–1.39, Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY.
15. Yamazaki, N., Shinohara, Y., Shima, A., and Terada, H. (1995) High expression of a novel carnitine palmitoyltransferase I like protein in rat brown adipose tissue and heart: Isolation and characterization of its cDNA clone. *FEBS Lett.* **363**, 41–45
16. Aoyama, C., Yamazaki, N., Terada, H., and Ishidate, K. (2000) Structure and characterization of the genes for murine choline/ethanolamine kinase isozymes α and β. *J. Lipid Res.* **41**, 452–464
17. Yamazaki, N., Shinohara, Y., Shima, A., Yamanaka, Y., and Terada, H. (1996) Isolation and characterization of cDNA and genomic clones encoding human muscle type carnitine palmitoyltransferase I. *Biochim. Biophys Acta* **1307**, 157–161
18. Maruyama, K. and Sugano, S. (1994) Oligo-capping: A simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138**, 171–174
19. Aoyama, C., Nakashima, K., Matsui, M., and Ishidate, K. (1998) Complementary DNA sequence for a 42 kDa rat kidney choline/ethanolamine kinase. *Biochim. Biophys Acta* **1390**, 1–7
20. Aoyama, C., Nakashima, K., and Ishidate, K. (1998) Molecular cloning of mouse choline kinase and choline/ethanolamine kinase: their sequence comparison to the respective rat homologs. *Biochim. Biophys Acta* **1393**, 179–185
21. Mascaro, C., Acosta, E., Ortiz, J.A., Marrero, P.F., Hegardt, F.G., and Haro, D. (1998) Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor. *J. Biol. Chem.* **273**, 8560–8563
22. Brandt, J.M., Djouadi, F., and Kelly, D.P. (1998) Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor α. *J. Biol. Chem.* **273**, 23786–23792
23. van der Leij, F.R., Cox, K.B., Jackson, V.N., Huijkman, N.C., Bartelds, B., Kuipers, J.R., Dijkhuizen, T., Terpstra, P., Wood, P.A., Zammit, V.A., and Price, N.T. (2002) Structural and functional genomics of the *CPT1B* gene for muscle-type carnitine palmitoyltransferase I in mammals. *J. Biol. Chem.* **277**, 26994–27005
24. Aoyama, C., Ohtani, A., and Ishidate, K. (2002) Expression and characterization of the active molecular forms of choline/ethanolamine kinase-α and -β in mouse tissues, including carbon tetrachloride-induced liver. *Biochem. J.* **363**, 777–784
25. Palmer, C.N., Hsu, M.H., Griffin, H.J., and Johnson, E.F. (1995) Novel sequence determinants in peroxisome proliferator signaling. *J. Biol. Chem.* **270**, 16114–16121