Novel Expression of Equivocal Messages Containing Both Regions of Choline/Ethanolamine Kinase and Muscle Type Carnitine Palmitoyltransferase I*

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For characterization of the detailed gene structure of human muscle type carnitine palmitoyltransferase I (M-CPTI), we analyzed the 5′-upstream region of the M-CPTI transcripts. As a result, we found a cDNA clone containing a nucleotide sequence unexpected from the reported M-CPTI gene structure in the upstream region of its 5′ end. Comparison of this nucleotide sequence with that of genomic DNA showed that this sequence was derived from the 3′-untranslated region of the gene encoding choline/ethanolamine kinase-β (CK/EK-β) located upstream of the M-CPTI gene. Southern blot analysis showed that there was no other region homologous to the CK/EK-β gene in the whole human genome. Thus, the overlapping transcript was concluded to be produced from the functional genes of CK/EK-β and M-CPTI. Furthermore, cDNAs containing both exons of these genes were detected by the polymerase chain reaction using the cDNA of human heart M-CPTI obtained by specific reverse transcription from its 3′-untranslated region as a template. From these results, the production and organization of these overlapping transcripts are discussed.

For fatty acid β-oxidation in mitochondria, long chain fatty acids are transported into the mitochondrial matrix space across the inner membrane in forms of acyl-CoAs. However, as the inner membrane is not permeable to long chain fatty acyl-CoAs, they enter the mitochondrial matrix space via a "carnitine system" consisting of three steps (1, 2): 1) formation of acylcarnitines from long chain fatty acyl-CoAs catalyzed by carnitine palmitoyltransferase I (CPTI) § located in the mitochondrial outer membrane, 2) import of acylcarnitines in exchange with carnitine in the matrix space mediated by carnitine-acylcarnitine carrier located in the inner membrane, and 3) formation of long chain fatty acyl-CoAs from the imported acylcarnitines catalyzed by carnitine palmitoyltransferase II located on the inner side of the inner membrane.

Because CPTII is responsible for the first rate-limiting step in oxidation of fatty acids in mitochondria, considerable attention has been paid to its structural and functional features (for recent review, see Ref. 3). Two isoforms of CPTII, liver type and muscle type (M-CPTII), are expressed in mammals. Although the primary structures of these CPTII isoforms are similar, their K_m values for carnitine and susceptibilities to malonyl-CoA inhibition are different. Furthermore, their tissue distributions are quite different (3). M-CPTII is dominantly expressed in major energy consuming cells, such as heart, skeletal muscle, brown adipose tissue, and testis. In contrast, liver type CPTII is expressed in cells that do not show extensive energy metabolism, such as liver, kidney, lung, intestine, and pancreas.

We first isolated cDNA clones of M-CPTII from rat brown adipose tissue and human heart (4, 5). Later, Wang et al. (6) determined the gene structure of rat M-CPTII, in which transcription and translation are initiated at exon 1 and exon 2, respectively. In contrast, we (7) and van der Leij et al. (8) reported that, in the human M-CPTII gene, there are two additional noncoding exons 1A and 1B, which are used alternatively, in the 5′-upstream region of exon 2 containing the transcription initiation codon. We suggested that the 5′ end of exon 1A and/or its upstream region as well as the 5′ end of exon 1B function are transcription initiation sites (7). Subsequently, Yu et al. (9) found that transcription is initiated alternatively at two noncoding exons U and M located upstream of exon 2 in the human M-CPTII gene. Exon M corresponds exactly to exon 1B, whereas the 5′ end of exon U is located about 40 base pairs downstream of the 5′ end of exon 1A. These results suggested that the transcription initiation mechanism(s) of M-CPTII is not simple.

In addition, a gene encoding a choline kinase-like protein was found to be located only about 300 base pairs upstream of exon 1A of the human M-CPTII gene with the same strand direction (7). Quite recently, the gene of a mouse choline kinase like protein was reported to encode choline/ethanolamine kinase-β (CK/EK-β) (10). Therefore, the gene encoding the human choline kinase-like protein should be the human CK/EK-β gene. Because this gene is located in close proximity to the M-CPTII gene, it is possible that there is a regulatory element(s) that controls transcription of M-CPTII in the CK/EK-β gene. For understanding the transcription mechanism of the human M-CPTII gene, we studied its gene structure and found novel transcripts containing exons of both the CK/EK-β and M-CPTII gene.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Human poly(A)+ RNAs, genomic DNA and a placental genomic DNA library were obtained from CLONTECH (Palo Alto, CA), and T4 RNA ligase and EX Taq polymerase were from TaKaRa Shuzo (Tokyo, Japan). All other reagents and enzymes were obtained as described previously (7). Gene-specific oligonucleotides were prepared and used as primers. The nucleotide sequences and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB029885 and AB029886.

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§ The abbreviations used are: CPTI, carnitine palmitoyltransferase I; M-CPTI, muscle type CPTI; CK/EK-β, choline/ethanolamine kinase-β; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SLIC-PCR, single strand ligation to ss-cDNA-PCR; ss-cDNA, single-strand cDNA.
Heterogeneous Transcripts of Human Muscle Type CPTI

RESULTS

SLIC-PCR Analysis of the 5’-Upstream Region of the M-CPTI Gene

The structure of the upstream region of the human M-CPTI gene reported to date (7, 9) is schematically summarized in Fig. 1A. In the upstream of exon 2 containing the translation initiation codon, there are two additional exons, which are used alternatively, i.e. exon 1A and 1B determined from the results of 5’ RACE by Yu et al. (9). Exon M corresponds to exon 1B, whereas the nucleotide of the 5’ end of exon U is the cytosine base shown by the symbol (#) located inside exon 1A. We tried to determine the exact 5’ end of the human M-CPTI message for understanding its transcription mechanism.

First, we carried out SLIC-PCR. Amplified cDNAs derived from heart and skeletal muscle were obtained by reverse transcription with random hexamer and T17 Adp. These four cDNA samples showed almost the same electrophoretic patterns, and their nucleotide sequences were determined after subcloning. Comparison of the nucleotide sequences of the SLIC-PCR clones obtained with that of the human M-CPTI gene (7) showed that most cDNA clones contained either exon 1A or 1B to various extents, both being linked to exon 2, as we reported previously (7). All clones containing exon 1BM contained its 5’ terminus or more downstream region, whereas clones containing exon 1A were diverse, starting at more upstream and

loci of these oligonucleotides are shown in Table I. Recombinant experiments were carried out essentially according to standard methods (11) or as recommend by the supplier.

Reverse Transcription of Human poly(A)+ RNAs—Poly(A)+ RNAs of human tissues were reverse transcribed with random hexamer, oligo(dT) primer (T7 Adp) or the human M-CPTI-specific primers. The nucleotide sequences of human M-CPTI-specific primers P1, P2, and P3 were based on that of the human M-CPTI gene (7). After reverse transcription, the reaction mixtures were treated with NaOH to hydrolyze mRNA. The single-stranded cDNAs (ss-cDNAs) obtained were used as templates in the polymerase chain reaction (PCR), single strand ligation to ss-cDNA-PCR (SLIC-PCR) and 3’ rapid amplification of cDNA ends (5’ RACE).

Isolation of cDNA Clones of Human M-CPTI Containing Its 5’-Upstream Region by SLIC-PCR—The cDNA clones corresponding to the 5’-upstream region of mRNA of human M-CPTI were isolated by SLIC-PCR (12, 13) with synthetic oligonucleotides of the adapter primer A

Southern Blot Analysis of the Human Genome—Human genomic DNA (10 μg) was digested with restriction enzymes, and reaction mixtures were subjected to agarose gel electrophoresis. Then the separated DNA fragments were transferred to nitrocellulose membranes and hybridized with the probe. Human genomic DNA clone HG1 containing parts of the CK/EK-β gene (5, 7) was digested with BamHI and HindIII and used as a probe after radiolabeling by the multiprimer method. Determination of the Gene Structure of Human CK/EK-β—The genomic DNA clone HG21 containing the human CK/EK-β gene was isolated from a human placental genomic DNA library by screening with the DNA probe used for Southern blot analysis of the human genome. The clone HG21 was digested with appropriate restriction enzymes, and DNA fragments were subcloned into plasmid vectors and sequenced. Three types of PCR were employed to isolate and characterize the cDNA encoding human CK/EK-β: 1) RT-PCR using ss-cDNA of human heart with a primer pair of P13/P14 prepared by comparison of the nucleotide sequence of genomic DNA of human CK/EK-β with those of cDNAs of rat and mouse CK/EK-β (14, 15); 2) for isolation of cDNA in the 3’-downstream region of P14, 3’ RACE using ss-cDNA obtained by reverse transcription with T7 Adp; and a subsequent second reaction using the diluted first PCR mixture with a primer pair of P15/Adp; and 3) for isolation of cDNA in the 5’-upstream region of P13, SLIC-PCR with primer pairs of the adapter primers B/P16 and C/P17. cDNAs obtained by RT-PCR, 3’ RACE, and SLIC-PCR were subcloned into plasmid vectors and sequenced. From the nucleotide sequences of cDNA and genomic DNA, the intron-exon junctions of the human CK/EK-β gene were determined. The nucleotide sequences of genomic DNA and cDNA of human CK/EK-β have been submitted to the DDBJ Nucleotide Sequence Data Base under accession numbers AB029885 and AB029886, respectively.

RESULTS

SLIC-PCR Analysis of the 5’-Upstream Region of the M-CPTI Gene

FIG. 1. Organization of the 3’ region of the CK/EK-β gene and the 5’ region of the M-CPTI gene (A) and the nucleotide sequence of the clone HHR-A10 obtained by SLIC-PCR (B). The genomic DNA structures of human M-CPTI and CK/EK-β are as reported (7). Boxes represent exons, and amino acid coding and noncoding regions are shown by closed and open boxes, respectively. There are two noncoding exons in the M-CPTI gene, exons 1A/1B (7) and U/M (9). Exon M corresponds to exon 1B, whereas exon U (shown by a cross-hatched box) exists inside exon 1A. The 5’ end of exon U reported as a transcription initiation site (9) is marked by #. Parts of nucleotides of exon 11 of the CKEK-β gene and exon 1A/U of M-CPTI are shown by capital letters, and those of the spacer region between the two genes are shown by lowercase letters. The nucleotides observed in the 5’ region of HHR-A10 are marked by asterisks.
downstream regions of exon U. Interestingly, the clone HHR-A10 from heart mRNA was found to contain a sequence unexpected from the human M-CPTI gene.

Characterization of Clone HHR-A10—The determined nucleotide sequence of the cDNA clone HHR-A10 is shown in Fig. 1B. It contained the sequence of exon 1A from the third cytosine, and there were nucleotides unexpected from the sequence of the upstream region of exon 1A, as marked by asterisks in Fig. 1, in its 5’ region. Homology analysis showed that the unexpected sequence was derived from the 3’-untranslated region of the CK/EK-β gene located upstream of human M-CPTI, and this nucleotide chain was linked to exon 1A, conforming to the GT-AG rule of intron/exon boundary. These results suggested that HHR-A10 was derived from the mRNA, in which the 3’-untranslated region of the CK/EK-β gene is directly linked to the third cytosine base of exon 1A of the human M-CPTI gene by splicing (Fig. 1A).

To confirm the existence of a transcript(s) containing exons of both the CK/EK-β gene and M-CPTI gene, we carried out RT-PCR of mRNA of human heart and skeletal muscle using the primer pairs P6/P10 and P7/P10, as shown in Fig. 2A. The primer P6 corresponds to the 3’-untranslated region of the CK/EK-β gene containing the nucleotide sequence of the HHR-A10 clone marked by an asterisk, P7 corresponds to a more upstream region, and P10 corresponds to exon 3 of the M-CPTI gene. On agarose gel electrophoresis of these RT-PCR products stained by ethidium bromide, the organization of cDNAs are also shown. For details, see text.

To confirm the existence of a transcript(s) containing both exons of the CK/EK-β gene and M-CPTI gene, we performed RT-PCR of mRNA between the regions of the CK/EK-β and M-CPTI genes of human heart and skeletal muscle. ss-cDNAs of human heart and skeletal muscle obtained by reverse transcription with either random hexamers or oligo (dT) Adp were mixed and used as templates for PCR. PCR was performed using the primer pairs P6/P10 and P7/P10. A Coding (closed boxes) and noncoding (open boxes) regions of both genes. The loci of P6 and P7 in the 3’-untranslated region of the CK/EK-β gene are shown by a hatched box. The nucleotides marked by asterisks are those found in the 5’ region of the CK/EK-β gene, as shown in Fig. 1B.

Characterization of the CK/EK-β Gene Located 5’-Upstream of the M-CPTI Gene—As described above, we found transcripts containing exons of both the CK/EK-β gene and M-CPTI gene in heart and skeletal muscle. The gene structure of human M-CPTI has been well studied (7–9), and its localization is reported to be on chromosome 22q13.3 (16). On the other hand, the human CK/EK-β gene has not been studied extensively, but it is registered as a choline kinase isolog (384D8_3) on human chromosome 22q13 BAC clone CIT987SK-384D8 (data base accession number U62317). Therefore, before performing detailed analysis of overlapping transcripts, we characterized the human CK/EK-β gene.

First, we performed Southern blot analysis of the CK/EK-β gene. A genomic DNA fragment of the 3’ region of the human CK/EK-β gene, the organization of which was estimated from the structure of the mouse gene (10), was prepared as a probe (Fig. 3A). This was hybridized with samples of human genomic DNA digested with various restriction enzymes. As shown in Fig. 3B, the probe DNA was hybridized with only one DNA fragment in all samples. Furthermore, the lengths of all the hybridized DNA fragments were in agreement with those expected from the nucleotide sequence of the registered sequence in the human chromosome 22q13 BAC clone CIT987SK-384D8, as shown in Fig. 3C. The results of Southern blot analysis showed that there is no other gene having a similar nucleotide sequence to that of the 3’ region of CK/EK-β used as a probe and that CK/EK-β is encoded only by the gene located in the upstream region of the M-CPTI gene in the human genome. Thus, we concluded that the possible formation of overlapping transcripts from the other genomic region was excluded and that the overlapping transcripts were derived from functional genes of CK/EK-β and M-CPTI located on chromosome 22q13.
Comparison of the registered nucleotide sequence of the 22q13 BAC clone CIT987SK-384D8 with those of rat and mouse CK/EK-b cDNAs (14, 15) suggested that there was a misreading in the registered nucleotide sequence. To determine the exact gene structure, we isolated the genomic DNA and cDNA of human CK/EK-b. Because the genomic DNA clone FIG. 3. Southern blot analysis of the human CK/EK-b gene. Human genomic DNA fragments obtained by digestion with various restriction enzymes were subjected to agarose gel electrophoresis and then transferred to nitrocellulose membranes and hybridized with the probe, which was prepared by digestion of the 3' region of the CK/EK-b gene with BamHI and HindIII. A, locus of the probe DNA of the CK/EK-b gene, the organization of which is shown in Fig. 2. B, autoradiogram of Southern blot analysis. Bars beside the autoradiogram represent the electrophoretic bands of size markers (λ/styI). C, lengths and loci of DNA fragments hybridized with the probe estimated from the nucleotide sequence of human chromosome 22q13 BAC clone CIT987SK-384D8. For instance, in the case of digestion with ApaI, the probe was expected to hybridize with a 3115-base pair DNA fragment. kbp, kilobase pairs.

FIG. 4. Structure of the human CK/EK-b gene. A, organization of the human CK/EK-b gene and its downstream regions. Exons shown by closed, open, and hatched boxes are as for Fig. 2. Primers P13 and P14 were used for RT-PCR to obtain partial CK/EK-b cDNA, primers P8 and P15 were used for 3' RACE to characterize the 3' region of CK/EK-b cDNA, and primers P16 and P17 were used for SLIC-PCR to characterize the 5' region of CK/EK-b cDNA. B, nucleotide sequence of cDNA encoding human CK/EK-b. Numbers of nucleotides are shown on the left margin, the adenine base in the translation initiation codon being numbered +1. The translation termination codon is shown by an asterisk. The nucleotide sequence of the underlined AATAAA represents a poly(A) additional signal. The deduced amino acids are shown by the one-letter abbreviation code under the nucleotides. Arrowheads represent intron insertion sites.
part of this study, we used a mixture of reverse transcription and the 3' end was determined by 3' RACE. The transcription initiation site of exon U of the M-CPTI gene (9) is marked by #.

HG11 isolated by us (5, 7) did not contain the whole region of the CK/EK-β gene, a human genomic DNA library was screened again and HG21 clone was isolated. By comparison of the nucleotide sequence of genomic DNA with that of cDNA obtained by RT-PCR, 3' RACE, and SLIC-PCR, we determined the structure of the human CK/EK-β gene. The gene organization and nucleotide sequence of cDNA of human CK/EK-β determined are shown in Fig. 4 (A and B, respectively). In addition, the detailed structures of the 3' region of the CK/EK-β gene and 5' region of M-CPTI are shown in Fig. 5.

The gene encoding human CK/EK-β was found to consist of at least 11 exons and was suggested to encode a protein of about 45 kDa consisting of 395 amino acids, like those of rat and mouse, which encode a protein of 45 kDa having 394 amino acids (14, 15). Furthermore, the amino acid sequence of human CK/EK-β was about 86% homologous with those of rat and mouse. A comparison of the nucleotide sequence determined by us with that of the 22q13 BAC clone showed that one guanine base at position +43 in exon 1 (here the nucleotide sequence was numbered taking the adenine base in the putative translation initiation codon of human M-CPTI as +1, and their numbers are shown on the left margin (7). Arrows represent splice sites in the overlapping transcript. Closed, open, and hatched boxes are as for Fig. 2. In exon 11 of 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. The transcription initiation site of exon U of the M-CPTI gene (9) is marked by #.

Therefore, we prepared ss-cDNAs of human M-CPTI by reverse transcription with oligonucleotides P1, P2, and P3, which correspond to exons 4, 15, and 20 of M-CPTI, respectively (Table I), and performed PCR with these ss-cDNAs as specific templates. Namely, the messages expressed in human heart were reverse transcribed with the primer P1, P2, or P3, all of which specifically annealed the message of M-CPTI, and PCRs were performed using these ss-cDNAs as templates with various primer pairs shown in Fig. 7A and Table I. The results of agarose gel electrophoresis of PCR products stained with ethidium bromide are shown in Fig. 7B. When ss-cDNAs obtained by reverse transcription of mRNAs with primers P1 and P2, referred to as P1 and P2 ss-cDNAs, respectively, were used as templates, only a significant band of the expected size from the gene structure of M-CPTI was observed in each amplification by primer pairs of P11/P10 and P12/P10. Nucleotide sequence analyses of these products showed that they contained exons 1A, 2, and 3 of the M-CPTI gene and exons 1B, 2, and 3, respectively. In contrast, two bands, a significant band with lower electrophoretic mobility and a weak band with higher mobility, were detected with the primer pairs P7/P10, P8/P10, and P9/P10. After subcloning, sequencing of the products giving the strong band with P8/P10 using P1 and P2 ss-cDNAs showed that both products contained exons 9, 10, and 11 of the CK/EK-β gene and exons 1A, 2, and 3 of the M-CPTI gene. In addition, both products gave the weak band containing the same exons of both genes, but exon 1A was deleted. In these products, the 3' end of the CK/EK-β region was connected by proper splicing to the 5' end of the M-CPTI region in similar manners to those shown in Fig. 2B. From the electrophoretic mobilities, the products of P7/P10 and P8/P10 with P1 and P2 ss-cDNAs were expected to have similar overlapping structures.

When ss-cDNA prepared by reverse transcription with P3 (P3 ss-cDNA) was used as a template, a product derived from the M-CPTI message was detected with both the primer pairs P11/P10 and P12/P10 as in RT-PCR with P1 and P2 ss-cDNA,
but no clear electrophoretic bands were observed with primer pairs P7/P10, P8/P10, and P9/P10 (Fig. 7B). However, as shown in Fig. 7C, two products having expected sizes in all PCRs with P3 ss-cDNA were detected by Southern blot analysis. The nucleotide sequences of the products of P9/P10 with P3 ss-cDNA showed that they contained the same sequences as those of corresponding products of P1 and P2 ss-cDNAs. Because primer P3 corresponds to exon 20 of the M-CPTI gene, the products of P9/P10 with P3 ss-cDNA should be derived from messages containing the sequence between exon 9 of the CK/EK-β gene and exon 20 of the M-CPTI gene, although their amounts were low (Fig. 7A). These messages were the most possible extended transcripts covering the entire region of the RT-PCR performed in this study.

The amounts of PCR products obtained with the primer pairs P11/P10 and P12/P10 using P1, P2, and P3 ss-cDNAs were always greater than those obtained with the primer pairs P7/ P10, P8/P10, and P9/P10 (Fig. 7B). Therefore, the structural gene of human M-CPTI starts mainly at either exon 1A/U or 1B/M, as reported (7, 9). However, PCR products were distinctly observed with the primer pair P9/P10 with P1, P2, and P3 ss-cDNAs, showing that there were definite amounts of overlapping transcripts of the CK/EK-β gene and M-CPTI gene. In addition, the band intensities of RT-PCR products with P2 ss-cDNA were lower than those with P1 ss-cDNA, and those with P3 ss-cDNA were significantly lower than those with P1 and P2 ss-cDNAs. This could be due, at least in part, to the fact that reverse transcription became inefficient as its range became wider.

**DISCUSSION**

Previously, we isolated a cDNA clone containing exon 1A of the human M-CPTI gene by 5′ RACE (7). Subsequently, Yu et al. (9) reported that transcription starts at the 5′ end of exon U located inside exon 1A. To determine the exact gene structure of human M-CPTI, in this study we isolated various cDNA clones containing the 5′-upstream region of M-CPTI by SLIC-PCR instead of 5′ RACE. Unexpectedly, we isolated a clone HHR-A10 from heart mRNA contained the 3′-untranslated region of the CK/EK-β gene. RT-PCR (Fig. 2) and Southern blot analysis of the human genome (Fig. 3) showed that there were distinct transcripts containing both exons of the functional CK/EK-β and M-CPTI genes in human heart and skeletal muscle. In the overlapping transcripts containing exon 1A of M-CPTI, the first two nucleotides adenine and guanine of exon 1A were always excluded because of splicing according to the GT-AG rule. Therefore, it is possible that the reported cDNA, which started at the 5′ end of exon 1A obtained by 5′ RACE (7), was not derived from overlapping transcripts and could have been derived from another transcript initiated at exon 1A or a more upstream region. These results suggested that the transcription of M-CPTI is “loosely” controlled and is initiated at multiple sites. It should be noted that because of the existence of definite amounts of the overlapping transcripts, the amount of mRNA of human M-CPTI will be estimated as higher than it really is, when the amount is determined from a part of its message by such methods as RT-PCR and RNase protection assay.

Overlapping genes are commonly observed in prokaryotes, bacteriophages, and viruses (17). However, they are quite rare in mammals. In 1986, Williams and Fried (18) first found the overlapping of two unknown mouse genes with opposite directions. In this case, both strands of a particular genomic region of 133 bases were utilized as templates for transcription. Overlapping transcription using the same DNA strand in mammals, like those of the human CK/EK-β and M-CPTI genes, was first reported for genes of the bovine myosin I heavy chain-like protein and preprotachykinin B by Hoshimaru and Nakanishi (19). Subsequently, overlapping genes with the same strand direction were reported for the mouse RNA polymerase II promoter motif and the first intron of the β-glucuronidase gene (20) and the vesicular acetylcholine transporter gene and choline acetyltransferase gene in humans and rats (21, 22). Of these, the genes of the myosin I heavy chain-like protein and preprotachykinin B are located close together, and the overlapping region is transcribed in both messages. However, unlike the overlapping transcripts of CK/EK-β and M-CPTI genes, the splice sites in this overlapping region are not completely the
same, resulting in the formation of exons with different nucleotide sequences in both genes.

There should be at least four possibilities for formation of such overlapping transcripts between CK/EK-β and M-CPTI genes, as shown in Fig. 8. Namely, 1) incomplete termination of the CK/EK-β message, in which transcription does not terminate at the 3’ end of CK/EK-β gene but proceeds to certain regions of the M-CPTI gene such as to a cluster of poly(A) additional signals in the intron between exon 7 and exon 8 (possibility A); 2) transcription of the M-CPTI gene from certain regions of CK/EK-β through the 3’ end of CK/EK-β (possibility B); 3) transcription initiated at certain regions of the CK/EK-β gene and termination in a certain region of the M-CPTI gene such as at its intron between exons 7 and 8 (possibility C); and 4) transcription initiated from the transcription initiation site of the CK/EK-β gene to the 3’ end of the M-CPTI gene (possibility D). In the case of possibility D, a message containing both the open reading frames of CK/EK-β and M-CPTI should be produced.

The results of PCRs in various regions between exon 9 of the CK/EK-β gene and exon 3 of the M-CPTI gene using specific

| Nucleotide sequence | Strand | Gene | Exon | Experiment |
|---------------------|--------|------|------|------------|
| P1                  | AS     | CPTI | 4    | RT         |
| P2                  | AS     | CPTI | 15   | RT         |
| P3                  | AS     | CPTI | 20   | RT         |
| P4                  | AS     | CPTI | 4    | SLIC       |
| P5                  | AS     | CPTI | 4    | SLIC       |
| P6                  | S      | CK/EK| 11   | PCR        |
| P7                  | S      | CK/EK| 11   | PCR        |
| P8                  | S      | CK/EK| 10   | 3’ RACE/PCR|
| P9                  | S      | CK/EK| 9    | PCR        |
| P10                 | AS     | CPTI | 3    | PCR        |
| P11                 | S      | CPTI | 1AU  | PCR        |
| P12                 | S      | CPTI | 1BM  | PCR        |
| P13                 | S      | CK/EK| 1    | PCR        |
| P14                 | S      | CK/EK| 11   | PCR        |
| P15                 | S      | CK/EK| 4    | SLIC       |
| P16                 | S      | CK/EK| 3    | SLIC       |

a Shown from the 5’ end to the 3’ end.

b S and AS represent sense and antisense, respectively.

c CPTI and CK/EK are the genes encoding M-CPTI and CK/EK-β, respectively.

d Exons of the M-CPTI gene are numbered as in Ref. 7. Exons of the CK/EK-β gene are numbered with the exon containing the translation initiation codon as exon 1.

FIG. 7. Results of RT-PCR of the overlapping region of the CK/EK-β and M-CPTI genes. A, RT-PCR specific to M-CPTI messages was performed with the primers shown by arrowheads with reference to the CK/EK-β and M-CPTI genes, in which boxes represent exons according to Fig. 2. B, PCR products were subjected to agarose gel electrophoresis, and gels were stained with ethidium bromide. C, RT-PCR products using P3 ss-cDNA were transferred to a nitrocellulose membrane, and Southern blot analysis was performed by hybridization of the products with the probe.

FIG. 8. Possible manners of overlapping transcript production between the CK/EK-β gene and M-CPTI gene. Ordinarily, messages consisting solely of CK/EK-β and M-CPTI are transcribed. Overlapping transcripts will be formed by transcription from the transcription initiation site of the CK/EK-β to certain regions of the M-CPTI gene (A), from certain regions of the CK/EK-β gene to the 3’ end of the M-CPTI gene (B), from certain regions of the CK/EK-β gene to certain regions of M-CPTI (C), and from the transcription initiation site of the CK/EK-β gene to the 3’ end of the M-CPTI gene (D).
ss-cDNAs of M-CPT as templates showed that most messages of human M-CPTI started at exon 1A/U and exon 1B/M. In addition, we obtained cDNAs containing the region between exon 9 of the CK/EK-β gene and exon 3 of the M-CPTI gene in PCR using P3 ss-cDNA (Fig. 7). Because primer P3 corresponds to exon 20 located downstream of the translation termination codon in exon 19 of the M-CPTI gene, we concluded that there are overlapping transcripts containing the region between exon 9 of the CK/EK-β gene and exon 20 of the M-CPTI gene, which is the most possible extended transcript of RT-PCR in this study. These overlapping transcripts contain the open reading frame of M-CPTI (possibilities B and D). The amounts of RT-PCR products with P3 ss-cDNA were apparently much lower than those with P1 and P2 ss-cDNAs corresponding to exon 4 and exon 15 of the M-CPTI gene, respectively. This could be due to either or both the facts that: 1) there is a 3′-splice variant lacking exon 20 of the M-CPTI gene (8) that cannot be used as a template of reverse transcription with primer P3 and 2) the production of ss-cDNA from the 3′ region of the M-CPTI transcript using P3 primer is more difficult than those using P1 and P2 primers because of a wide range of reverse transcription of more than 2 kilobases of its entire message. In addition to these overlapping transcripts, there could be overlapping transcripts that terminated between primers P1 and P2 (possibilities A and C). However, this seems unlikely from the fact that the intensities of electrophoretic bands of the products using P2 ss-cDNA were not significantly lower than those using P1 ss-cDNA. The lower amounts of the products with P2 ss-cDNA could be a result of inefficient reverse transcription covering a wider range from exon 15.

Because the nucleotide chains of overlapping transcripts between exon 9 of the CK/EK-β gene and exon 3 of the M-CPTI gene were spliced as observed with “ordinary messages” of both genes with the exception of their boundary region, it is possible that the message transcribed from the transcription initiation site of the CK/EK-β gene proceeds to the region of the M-CPTI gene. Accordingly, there could be an overlapping transcript containing the region from the 5′ end of the CK/EK-β gene to the 3′ end of the M-CPTI gene (possibility D). Then how are these overlapping transcripts produced? The 3′ end of the human CK/EK-β gene is located about 300 and 1000 base pairs upstream of the 5′ ends of exon 1A and exon 2 of the M-CPTI gene, respectively. With such a proximate organization of the two genes, the transcription of the CK/EK-β gene may proceed to the M-CPTI gene, and splicing may take place between the last exon 11 of CK/EK-β and exon 1A or exon 2 of M-CPTI before addition of the poly(A) tail or without termination of transcription. Therefore, the human CK/EK-β gene and the proximate M-CPTI gene should be useful for understanding the mechanisms of transcription termination and splicing of a transcribed message.

The messages of the M-CPTI region were concluded to be heterogeneous having multiple transcription initiation sites and loose transcription termination of the CK/EK-β gene. Studies on the control mechanism of gene expression of M-CPTI as well as the physiological roles of these overlapping transcripts are underway.

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