Cloning, Sequencing, and Expression of a cDNA Encoding Rat Liver Mitochondrial Carnitine Palmitoyltransferase II*

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We report the isolation and characterization of a full-length cDNA encoding rat liver carnitine palmitoyltransferase II (CPT II). Beginning with the purified protein CNBr fragments were generated and sequenced. Corresponding oligonucleotides were used to screen a rat liver cDNA library constructed in the plasmid cloning vector, pcDV. The clone ultimately obtained consisted of a 62 nucleotide 5'-untranslated region, a single open reading frame of 1,974 bases predicting a protein of 658 amino acids (Mr = 74,119), and a 3'-untranslated segment of 260 nucleotides followed by the poly (A) tail.

The identity of the cDNA was confirmed by the findings that (a) the open reading frame encoded all three peptides found in the original protein; (b) a fourth peptide synthesized from a portion of the deduced amino acid sequence and used to immunize a rabbit resulted in the generation of an antibody that recognized pure CPT II on a Western blot; (c) in vitro transcription and translation of the cDNA (ligated into pBlue-script KS (+)) generated a protein that was specifically immunoprecipitated by anti-CPT II antibody and having a Mr slightly greater than that of mature CPT II; (d) transfection of COS cells with the cDNA subcloned into the expression vector, pCMV4, resulted in a 6-fold induction of mitochondrial CPT II catalytic activity.

It seems likely that the de novo synthesized enzyme gains entry into the mitochondrion via a targeting peptide that is subsequently cleaved. The mature protein probably associates (relatively loosely) with the inner membrane through a limited number of membrane spanning domains.

The predicted amino acid sequence of CPT II shows strong identity with those of two other acyltransferases, namely, rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase.

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The nucleotide sequences reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05470.

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The abbreviations used are: CPT, carnitine palmitoyltransferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CoA, coenzyme A.

EXPERIMENTAL PROCEDURES

Animals-Male Sprague-Dawley rats (~150 g) maintained on a regular chow diet were used in the fed state. In some cases di-(2-ethylhexyl)phthalate was added to the diet at 0.2% for 2 weeks prior to killing.

General Methods—Standard molecular biological techniques were employed. cDNA clones were sequenced on both strands using the dideoxy chain-termination method (4) with either the M13 universal sequencing primer or specific internal oligonucleotide primers after subcloning into bacteriophage M13 vectors. Sequencing reactions were performed with the Klenow fragment of Escherichia coli DNA polymerase I (4) or a modified bacteriophage T7 DNA polymerase (Sequenase) (5). Total cellular RNA was isolated by guanidinium thiocyanate extraction followed by centrifugation through a CsCl cushion (6).

Protein Purification and Peptide Sequencing Rat liver CPT II, purified as described previously (2), was digested with cyanogen bromide in 70% foromic acid for 15 h in the dark and lyophilized. The resulting peptide mixture was subjected to reverse-phase high pressure liquid chromatography using a system from the Waters Chromatography Division of Millipore (Milford, MA), equipped with an Applied Biosystems Inc. (Santa Clara, CA) RP-300 column. Chromatography was performed in 0.02% (v/v) trifluoroacetic acid with a gradient of 0-50% acetonitrile. Amino acid sequencing was carried out on three of the peptides using an Applied Biosystems Inc. (Foster City, CA) model 470A automated sequencer equipped with a model 120A PTH amino acid analyzer according to the manufacturer's standard programming and chemicals.

Screening of a cDNA Library.—The 3-6 kilobase fraction of a rat liver cDNA library in the plasmid cloning vector pcDV (7) was used. It was sequenced by colony hybridization (3) using two 32P-labeled oligonucleotides derived from the amino acid sequence obtained from two of the isolated CPT II peptides (see below).

Construction of a Primer-extended cDNA Library—Poly(A)+ RNA from livers of rats treated with di-(2-ethylhexyl)phthalate (a known inducer of CPT II synthesis (8)) was used to construct a primer-extended cDNA library, employing a kit from Promega and a synthesized primer with the sequence 5'-TTGGGTCCGGATTGAAT-3' (complementary to base 498 424 in Fig. 3). The double-stranded cDNA generated was ligated into Agt10 arms (Promega) using EcoRI linkers. The ligation mixture was packaged with a two extract system.
cDNA for Carnitine Palmitoyltransferase II

Molecular Cloning of a Rat Liver CPT II cDNA—Purified rat liver CPT II was subjected to N-terminal amino acid sequence without success, suggesting that the amino terminus of the protein was blocked. Three cyanogen bromide cleavage peptides were, however, successfully sequenced (underlined in Fig. 3). With this information two degenerate oligonucleotide probes were synthesized, one to a region within peptide 1 (5'-NNATCTGTC-GG-TGGAATCAATGCA-3'), the other to a region within peptide 3 (5'-CCCGTGACATGCGC-3'). The probes were complementary to all possible coding sequences for the amino acids represented. Approximately 10<sup>6</sup> colonies of a rat liver cDNA library constructed in the vector pCD-DV were screened with the first oligonucleotide probe, yielding a single positive clone. Its identity was confirmed by hybridization with the second probe. This cDNA clone, ~2.2 kilobases in length, was designated pCD-CPT II.1 (Fig. 1). Initial nucleotide analysis established that all three peptide sequences derived from the original protein could be predicted from the cDNA sequence. However, it seemed certain that the clone was not full length, for several reasons. First, the reading frame 5' to the first potential ATG start codon contained no in frame termination codon. Second, based on analysis of Northern blots, the cDNA was ~200-300 bases shorter than the CPT II mRNA length (see preceding paper). Third, and most important, the first potential start ATG coded for a methionine immediately preceding the first peptide sequenced (Met 43 in the complete sequence, Fig. 3). Miyazawa et al (8) had presented evidence that nascent CPT II has a mitochondrial targeting sequence that is cleaved in formation of the mature protein. Since Met 43 was presumably present in the purified mature enzyme, it could not have been the start of the targeting sequence. To obtain the remaining sequence, a primer-extended cDNA library was constructed in the vector λgt10. 10<sup>6</sup> plaques were screened using a radiolabeled PstI to XhoI fragment (near the 5' end of the first cDNA) as a probe. A single clone, λL4-CPT II.1 (Fig. 1), was obtained. This 0.5-kilobase cDNA contained an additional 78 bases 5' to the 5' terminus of the first cDNA clone. Nucleotide sequence analysis revealed that the reading frame of the original cDNA was now extended to...

Materials—Enzymes were from Boehringer Mannheim, New England Diabs (Beverly, MA), Stratagene (LaJolla, CA), Promega (Madison, WI), and U. S. Biochemical (Cleveland, OH). "S- and "P-labeled nucleotides were from Amersham Corp. and Du Pont-New England Nuclear.

RESULTS

DNA Transfection—Stock cultures of simian COS-M6 cells were grown in monolayer at 37 °C under a 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 44 mM Na<sub>2</sub>HCO<sub>3</sub>, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded on day 0 at a density of 3 x 10<sup>6</sup>/150-mm dish in the above medium. On day 1, the medium was removed, and the cells were washed with buffer A (140 mM NaCl, 3 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.9 mM sodium phosphate, and 25 mM Tris-HCl, pH 7.4). The cells were then exposed to 15 µg of plasmid DNA in 7.5 ml of buffer A containing 0.5 µg/ml DEAE-dextran (Pharmacia LKB Biotechnology Inc., M<sub>n</sub> = 3 x 10<sup>6</sup>) (11). After 30 min at 37 °C, the DNA containing solution was aspirated, and the cells were incubated for 3 h at 37 °C in 15 ml of fresh medium containing 100 µM chloroquine. The cells were washed with buffer A and then incubated for 4 min at room temperature with 5 µl of supernatant Dulbecco's modified Eagle's medium containing 20% (v/v) glycerol. After the glycerol shock, cells were washed with buffer A and placed in 30 ml of fresh supplemented Dulbecco's modified Eagle's medium. On day 4 the cells were harvested in 4 ml of ice-cold phosphate-buffered saline. They were collected by centrifugation at 2,000 x g for 10 min and washed once in phosphate-buffered saline. The final pellet was resuspended in 0.15 M KCl, 5 mM Tris-HCl, pH 7.2, in 5 µl of DNA in 7.5 µl of buffer A containing 50 µM malonyl-CoA, the difference between the two rates representing CPT I (1, 2, 10). A second aliquot was assayed after treatment with octyl glucoside (1% w/v) for 30 min at 0 °C to destroy CPT I activity and allow complete exposure of CPT II (1, 2, 10). A third sample was treated with Tween-20 (1% v/v), which also release CPT II selectively from the membranes (1, 2). This extract was used for immunoprecipitation studies by the methods described in the preceding paper (1).

Software—Alignment and analysis of the DNA and protein sequences were performed using IG-Suite and PC-Gene (Intelli-Genetics, Palo Alto, CA) and the University of Wisconsin Genetic Computer Group Programs (12).
include both an in frame initiation codon and an upstream, in frame termination codon.

The restriction map of the full-length cDNA, together with the sequencing strategies employed, are diagrammed in Fig. 2. The complete cDNA sequence is shown in Fig. 3. It contains a 5′-untranslated stretch of 62 nucleotides, an open reading frame of 1,974 bases, predicting a 658 amino acid protein of 74,119 daltons, and the entire 3′-untranslated region of 260 nucleotides preceding the poly(A) tail. Although no strict consensus motif for a mitochondrial targeting peptide has yet been established, the first 30 amino acids have properties common to many mitochondrial leader sequences. There are no acidic residues, four hydroxyl and four basic amino acids, and the remaining amino acids are mainly hydrophobic. A helical wheel plot (not shown) indicates that the serines and basic residues form one face of a putative helix, while the hydrophobic residues form the other face. (It is unlikely, however, that a continuous helical structure is formed, based on the frequency of proline residues within this stretch of amino acids).

Confirmation of the cDNA Identity—In order to confirm the identity of the cDNA clone, the first cDNA (pCD-CPT II.1) was subcloned into the vector pBluescriptKS(+) to form pBKS-CPT II.3 (Fig. 1). In addition, a full-length cDNA was constructed in the same vector from the two other clones (pCD-CPT II.1 and AL4-CPT II.1); this cDNA was designated pBKS-CPT II.4 (Fig. 1). The vector, pBluescript, contains both a T3 and a T7 promoter, which allows RNA transcription of a cDNA to be synthesized. If a methylgonasine cap structure is incorporated into the RNA, the result is a pseudo-mRNA that is capable of being translated. After in vitro transcription, both pBKS-CPT II.3 and pBKS-CPT II.4 yielded transcripts of the appropriate length (data not shown). When translated, each transcript produced an 35S-labeled protein that was specifically immunoprecipitated by anti-CPT II antibody, RK40. As seen from Fig. 4, the two products were of slightly different size. The shorter (lane 4), generated from

![Fig. 2. Restriction endonuclease map and sequencing strategy for rat liver CPT II cDNA. The stippled bar represents the coding region. Arrows under the solid bars indicate the extent and direction of the sequencing reactions.](http://www.jbc.org/content/journal/jbc/272/24/11789.full)

![Fig. 3. The complete sequence of rat liver CPT II cDNA. Nucleotides are numbered on the left, amino acids on the right. Squares and asterisks denote the stop codons in the 5′-non-coding region and at the 3′ end of the coding sequence, respectively. The arrow at nucleotide 17 shows the 5′-limit of the first clone, pCD-CPT II.1. The amino acids underlined are those identified by automated Edman degradation of CNBr peptides derived from the original protein. They are referred to in the text by number beginning with the closest to the N terminus.](http://www.jbc.org/content/journal/jbc/272/24/11789.full)
pBKS-CPT II.3, migrated to a position fractionally below that of pure CPT II, suggesting that translation began at the first available AUG codon, presumably Met 43 (Fig. 3). By contrast, the product from pBKS-CPT II.4 (lane 6) was slightly larger than the pure enzyme, consistent with translation having begun at Met 1. It seemed likely that this constituted the same protein as that derived from pBKS-CPT II.3 but containing, in addition, a mitochondrial targeting sequence. (Note, however, that both products must have lacked the carboxyl-terminal 7 amino acid residues of mature CPT II since the constructs used for transcription had been linearized with restriction endonuclease, XbaI (Fig. 2).)

As a second approach to establishing the clone's identity, a peptide corresponding to the predicted residues 223 to 242 in Fig. 3 was synthesized and, after conjugation to purified protein derivative of tuberculin, was used to raise an antibody in a rabbit. In experiments not shown the immune serum clearly reacted with authentic CPT II on Western blot analysis. Preimmune serum from the same animal gave no signal.

The most direct evidence that the cDNA of pBKS-CPT II.4 encodes CPT II came from DNA transfection experiments in which the full-length cDNA was introduced into COS cells. A typical result is shown in Table I which illustrates three points. First, mitochondria from these cells contain both CPT I and II activity (the former was completely inhibitable by malonyl-CoA, the latter insensitive). Second, the level of CPT I was unaffected by any of the DNA species used to transfect the cells. Third, CPT II activity was uniquely induced, by a factor of almost 50-fold, in cells transfected with pCMV4-CPT II.4.

Note that the ATG triplets corresponding to Met 1 and Met 43 in Fig. 3 are both preceded by nucleotide sequences that conform to the consensus motif for an initiator codon as defined by Kozak (13).

Table I

<table>
<thead>
<tr>
<th>DNA used for</th>
<th>CPT I activity</th>
<th>CPT II activity</th>
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<tbody>
<tr>
<td>transfection</td>
<td>(nmol.min^-1.mg protein^-1)</td>
<td>(nmol.min^-1.mg protein^-1)</td>
</tr>
<tr>
<td>None</td>
<td>0.24</td>
<td>1.38</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>0.24</td>
<td>1.41</td>
</tr>
<tr>
<td>pCMV4 vector</td>
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<td>1.35</td>
</tr>
<tr>
<td>pCMV4-CPT II.4</td>
<td>0.20</td>
<td>7.72</td>
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</table>

TABLE II

<table>
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<tr>
<th>DNA used for transfection</th>
<th>% of mitochondrial CPT II activity remaining after</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibody</td>
<td>RK39</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>87</td>
</tr>
<tr>
<td>pCMV4 vector</td>
<td>98</td>
</tr>
<tr>
<td>pCMV4-CPT II.4</td>
<td>91</td>
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</tbody>
</table>

FIG. 5. Hydropathy plot for rat liver CPT II. The profile was generated using the method of Kyte and Doolittle (14) with an interval of nine amino acids.

FIG. 6. Alignment of predicted amino acid sequences for three acyltransferases. The deduced amino acid sequence for rat liver mitochondrial CPT II has been aligned with those for rat liver peroxisomal carnitine octanoyltransferase (COT) and porcine choline acetyltransferase (ChA77 using computer program GAP (12). Shaded boxes indicate sequence identity between CPT and one or both of the other proteins. Sequence identity between rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase is not highlighted.
The full-length cDNA ligated into the vector, pCMV4, resulted in proteins that were slightly larger and smaller, respectively, than the original protein. Based on SDS-PAGE analysis, these differences were consistent with the notion that the mature CPT II is less tightly associated with its inner membrane environment. Although no strong predictions were available, the hydrophilic nature of the predicted amino acid sequence for rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase suggested that these molecules were predicted to be membrane spanning. However, it is recognized that in such programs the criteria used for a positive prediction are somewhat rigid and frequently miss known transmembrane domains.

The predicted sequences of two other acyl-CoA utilizing enzymes, rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase have recently been published. Computer alignment of the deduced amino acid sequence of CPT II with those of rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase (Fig. 6) revealed that CPT II possessed 27 and 25% identity with rat liver peroxisomal carnitine octanoyltransferase and porcine choline acetyltransferase, respectively. When allowance is made for conservative changes of amino acids, these values increase to 51 and 53% similarity, respectively.

**DISCUSSION**

To begin to explore the mitochondrial CPT system at a structural level, we set out to determine the primary amino acid sequence of one of its component enzymes, CPT II. This protein was chosen because we already had sufficient purified material from rat liver (2) with which to obtain partial amino acid sequences to initiate cloning procedures. Using classical strategies we succeeded in isolating and sequencing what appeared to be a cDNA corresponding to the entire coding region of the protein. Confidence in its identity comes from the following considerations. First, the nucleotide sequence, in a single open reading frame, predicted all three of the peptides found in the original protein. Second, an oligonucleotide raised against a fourth peptide composed of a stretch of 20 amino acids predicted by the cDNA sequence recognized pure CPT II on a Western blot. Third, the cDNA, pBKS-CPT II.4, as well as a shorter construct lacking 78 base pairs at the 5′ end (pBKS-CPT II.3), were transcribed in vitro, yielding products that were selectively precipitated by anti-CPT II antibody. Based on SDS-PAGE analysis, these proteins were slightly larger and smaller, respectively, than mature CPT II. This would be consistent with the notion that in the case of pBKS-CPT II.4 translation of the message began at the AUG codon representing Met-1 in Fig. 3, whereas Met-43 was the probable start site on the shorter transcript. Fourth, and most importantly, transfection of COS cells with the full-length cDNA ligated into the vector, pCMV4, resulted in a 4-fold increase in mitochondrial CPT II activity that was also immunoprecipitated by the anti-CPT II antibody.

Inspection of the predicted amino acid sequence of CPT II (Fig. 3) reveals that the N terminal segment preceding Aep-32 has the general characteristics of a mitochondrial leader peptide (20). Based on the data of Miyazawa et al (8) and Fig. 4, it seems likely that a significant portion of the targeting sequence is cleaved upon mitochondrial import of the protein, thus reducing its size from the predicted value of 74,119 daltons to that of mature CPT II which is generally taken as ~70 kDa (21; but see footnote 4 in Ref. 1).

Available evidence indicates that unlike CPT I, which appears to be deeply anchored in the mitochondrial outer membrane, CPT II is less tightly associated with its inner membrane environment (2, 21). Although no strong predictions were available, transmembrane regions emerged from computer analysis, visual inspection of the hydrophobicity plot for CPT II (Fig. 5) and of the deduced amino acid sequence (Fig. 3) suggests the possible presence of a limited number of membrane spanning domains. One might be within the hydrophobic peak in the vicinity of amino acid 600. Studies are in progress to define the precise topographical relationship between membrane and enzyme.

Not surprisingly, when the nucleotide sequence for rat liver mitochondrial CPT II was compared with the GenBank and EMBL computer data banks, the two proteins with which it proved to be most similar were both of the acyltransferase class. These were rat liver carnitine octanoyltransferase (COT) and porcine choline acetyltransferase (ChAT), both of whose sequences have recently been reported (16, 17). CPT II was found to be identical over some 25-30% of its residues with each of these proteins and, allowing for conservative amino acid changes, the degree of similarity between CPT II and each of the other transferases appears to be ~50%. It seems reasonable to suppose that the binding sites for the substrates of CPT II, namely, acyl-CoA, CoA, carnitine and acylcarnitine, will be found to reside within these regions of similarity between CPT II and COT and/or ChAT.

In summary, the data presented provide the first insight into the primary structure of a mitochondrial isozyme of carnitine palmitoyltransferase. Although further studies are needed to determine the mechanism by which the nascent protein gains entry into the mitochondrion, where its targeting sequence is cleaved, and how the mature enzyme associates with the inner membrane, the present findings provide a first step toward these goals. They should also prove valuable in the analysis of CPT II isoforms and their genes in different tissues and species. Such knowledge will be indispensable in the quest to understand the genetic basis of the human CPT II deficiency syndromes.

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