Cloning of the Human Carnitine-Acylcarnitine Carrier cDNA and Identification of the Molecular Defect in a Patient

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Summary

The carnitine-acylcarnitine carrier (CAC) catalyzes the translocation of long-chain fatty acids across the inner mitochondrial membrane. We cloned and sequenced the human CAC cDNA, which has an open reading frame of 903 nucleotides. Northern blot studies revealed different expression levels of CAC in various human tissues. Furthermore, mutation analysis was performed for a CAC-deficient infant. Direct sequencing of the patient's cDNA revealed a homozygous cytosine nucleotide insertion. This insertion provokes a frameshift and an extension of the open reading frame with 23 novel codons. This is the first report documenting a mutation, in the CAC cDNA, responsible for mitochondrial β-oxidation impairment.

Introduction

The carnitine-acylcarnitine carrier (CAC) shuttles acylcarnitine esters, in exchange for free carnitine, across the inner mitochondrial membrane (Pande 1975; Ramsay and Tubbs 1975). This transport is an essential step in the process of long-chain fatty-acid oxidation (Stanley 1987; Coates and Tanaka 1992; Stanley et al. 1992). The oxidation of fatty acids in mitochondria plays an important role in energy production. During fasting, fatty acids are used for hepatic ketone-body synthesis. Furthermore, fatty acids are an important source of energy for heart muscle and also for skeletal muscle, during exercise, whereas ketone bodies are excellent substrates for the brain (Stanley 1987; Coates and Tanaka 1992). The overall fatty-acid oxidation in mitochondria requires the concerted action of at least 17 different proteins, including 16 enzymes and the transporter CAC. Genetic defects have been identified in most of these proteins (Stanley 1987; Stanley et al. 1992). These defects generally present in early infancy, with acute, potentially life-threatening episodes of hypoketotic hypoglycemic coma induced by fasting. The clinical phenotypes are very similar and can be attributed to one of three major types of presentation, with predominantly hepatic, cardiac, or skeletal muscle involvement (Kelly and Strauss 1994; Pande and Murthy 1994; Pollit 1995). So far, six cases of CAC deficiency have been reported (Stanley et al. 1992; Pande et al. 1993; Brivet et al. 1994, 1996; Niezen-Koning et al. 1995; Ogier de Baulney et al. 1995). The main features in these severely affected patients with onset in the neonatal period are hypoketotic hypoglycemia, mild hyperammonemia, variable dicarboxylic aciduria, hepatomegaly with abnormal liver functions, various cardiac symptoms, and skeletal muscle weakness. In all cases, CAC activity in cultured skin fibroblasts is below detectable levels. However, so far, the CAC deficiency has not been characterized at the molecular level, in any patient.

Fundamental properties of eukaryotic CAC have been investigated extensively in intact mitochondria (Pande 1975; Ramsay and Tubbs 1975; Pande and Parvin 1980; Idell-Wenger 1981; Murthy and Pande 1984) and after purification and reconstitution into liposomes (Indiveri et al. 1990, 1991*a*, 1991*b*, 1992, 1994, 1995). The carrier is embedded in the inner mitochondrial membrane and has an apparent molecular mass of 32.5 kD in rat liver (Indiveri et al. 1990). It governs a one-to-one exchange, between long-chain acylcarnitine esters and nonesterified carnitine, across the inner mitochondrial membrane and also the unidirectional transport of carnitine across this membrane, although less efficiently (Pande and Parvin 1980; Indiveri et al. 1991*a*). Incorporated into liposomes, the purified carrier protein has substrate specificity and inhibitor sensitivities similar to

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those in intact mitochondria (Indiveri et al. 1990, 1991*a*, 1991*b*). CAC operates according to a ping-pong mechanism (Indiveri et al. 1994).

Very recently we described the cDNA and amino acid sequence of the rat CAC (Indiveri et al. 1997). These studies have shown that CAC belongs to a protein family that, so far, has been found to comprise 10 biochemically well-characterized mitochondrial carriers and also several other members of unknown function that are beginning to emerge with the advance of genomic DNA sequencing (Walker and Runswick 1993; Palmieri 1994; Crabeel et al. 1996; Palmieri et al. 1996). These proteins have evolved from a common ancestor, by two-tandem gene duplication, and have related structures and mechanisms (Walker and Runswick 1993; Palmieri 1994).

We report on the nucleotide sequence of the human CAC cDNA and the corresponding amino acid sequence, as well as the distribution of CAC mRNA in human tissues. For the first time, a mutation has been found in the CAC cDNA of a unique, now 9-year-old, CAC-deficient patient.

Patient and Methods

Case Report

This 9-year-old girl with a normal family history survived a severe neonatal condition consisting of hypoglycemia, cardiac arrest, hepatomegaly, and hepatic dysfunction. These features are often observed in patients with a fatty-acid oxidation disorder. Thereafter episodes of lethargy and hepatomegaly occurred only during mild viral infections. Acylglycines were nondiagnostic, and acylcarnitines showed increased medium- to long-chain hydroxy and unsaturated derivatives. A liver biopsy showed fatty infiltration. During the last few years, she has had a rather normal physical and neurophysiological development: she has done well with prolonged exercise, and in between the attacks she is an alert, cooperative, and pleasant young girl. Her symptoms are much milder than those in the other CAC-deficient patients who have been described. Cultured skin fibroblasts were sent to one of us (R.W.) to investigate a possible defect in mitochondrial fatty-acid oxidation.

Fibroblast Study

Overall β-oxidation in fibroblasts was measured as described previously by Olpin et al. (1992). The CAC activity in fibroblasts was measured essentially according to the study by Pande et al. (1993).

Cloning and Sequencing of the Human CAC

Oligonucleotides were designed on the basis of the cDNA sequence of the rat liver CAC (Indiveri et al. 1997), at the following nucleotide positions: 125–150 (sense 1F, 5′-CCTGGTGTTTGTGGGGCACCCCTTG-3′); 455–480 (antisense 1R, 5′-CTGAATCTGCAGTAAGCATTTGATC-3′); 405–430 (sense 2F, 5′-GGGATGTTATCTGGTGTGTTCACCA-3′); and 937–962 (antisense 2R, 5′-ACAAGTTGGGGGCAATCCAATTGAG-3′). These primers (Pharmacia Biotech) were used in PCRs, to amplify cDNA fragments encoding the human liver CAC. The template was first-strand cDNA reverse transcribed, with oligo(dT) by AMV reverse transcriptase (Boehringer Mannheim) for 1 h at 55°C, from human liver mRNA (Clontech). PCR reactions were carried out for 30 cycles (94°C for 30 s, 60°C for 1 min, and 72°C for 2 min). At the end, a single incubation at 72°C for 7 min was added. The extension to the 3′ end was performed by priming from poly(A), with two nested forward primers (AGAAGAAGGAGTCACCTCCTTGTA and CAAAGGGTTCAATGCAGTCATGA, corresponding to nucleotides 831–854 and 855–877, respectively, of the rat cDNA sequence). To extend the sequence to the 5′ end, a touchdown PCR (Don et al. 1991) was performed on an adaptor-ligated

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1    GGGCTCGAGC GGCCGCCCGG GCAGGTCGAG AACTGACAGA CGGAGTGACA GACGGACTGA CC ATG GCC GAC CAG
1                                                                        M   A   D   Q

75   CCA AAA CCC ATC AGC CCG CTC AAG AAC CTG CTG GCC GGC GGC TTT GGC GGC GTG TGC CTG GTG TTC GTC GGT
5    P   K   P   I   S   P   L   K   N   L   L   A   G   G   F   G   G   V   C   L   V   F   V   G

147  CAC CCT CTG GAC ACG GTC AAG GTC CGA CTG CAG ACA CAG CCA CCG AGT TTG CCT GGA CAA CCT CCC ATG TAC
29   H   P   L   D   T   V   K   V   R   L   Q   T   Q   P   P   S   L   P   G   Q   P   P   M   Y

219  TCT GGG ACC TTT GAC TGT TTC CGG AAG ACT CTT TTT AGA GAG GGC ATC ACG GGG CTA TAT CGG GGA ATG GCT
53   S   G   T   F   D   C   F   R   K   T   L   F   R   E   G   I   T   G   L   Y   R   G   M   A

291  GCC CCT ATC ATC GGG GTC ACT CCC ATG TTT GCC GTG TGC TTC TTT GGG TTT GGT TTG GGG AAG AAA CTA CAA
77   A   P   I   I   G   V   T   P   M   F   A   V   C   F   F   G   F   G   L   G   K   K   L   Q

363  CAG AAA CAC CCA GAA GAT GTG CTC AGC TAT CCC CAG CTT TTT GCA GCT GGG ATG TTA TCT GGC GTA TTC ACC
101  Q   K   H   P   E   D   V   L   S   Y   P   Q   L   F   A   A   G   M   L   S   G   V   F   T

435  ACA GGA ATC ATG ACT CCT GGA GAA CGG ATC AAG TGC TTA TTA CAG ATT CAG GCT TCT TCA GGA GAA AGC AAG
125  T   G   I   M   T   P   G   E   R   I   K   C   L   L   Q   I   Q   A   S   S   G   E   S   K

507  TAC ACT GGT ACC TTG GAC TGT GCA AAG AAG CTG TAC CAG GAG TTT GGG ATC CGA GGC ATC TAC AAA GGG ACT
149  Y   T   G   T   L   D   C   A   K   K   L   Y   Q   E   F   G   I   R   G   I   Y   K   G   T

579  GTG CTT ACC CTT ATG CGA GAT GTC CCA GCT AGT GGA ATG TAT TTC ATG ACA TAT GAA TGG CTG AAA AAT ATC
173  V   L   T   L   M   R   D   V   P   A   S   G   M   Y   F   M   T   Y   E   W   L   K   N   I

651  TTC ACT CCG GAG GGA AAG AGG GTC AGT GAG CTC AGT GCC CCT CGG ATC TTG GTG GCT GGG GGC ATT GCA GGG
197  F   T   P   E   G   K   R   V   S   E   L   S   A   P   R   I   L   V   A   G   G   I   A   G

723  ATC TTC AAC TGG GCT GTG GCA ATC CCC CCA GAT GTG CTC AAG TCT CGA TTC CAG ACT GCA CCT CCT GGG AAA
221  I   F   N   W   A   V   A   I   P   P   D   V   L   K   S   R   F   Q   T   A   P   P   G   K

795  TAT CCT AAT GGT TTC AGA GAT GTG CTG AGG GAG CTG ATC CGG GAT GAA GGA GTC ACA TCC TTG TAC AAA GGG
245  Y   P   N   G   F   R   D   V   L   R   E   L   I   R   D   E   G   V   T   S   L   Y   K   G

867  TTC AAT GCA GTG ATG ATC CGA GCC TTC CCA GCC AAT GCG GCC TGT TTC CTT GGC TTT GAA GTT GCC ATG AAG
269  F   N   A   V   M   I   R   A   F   P   A   N   A   A   C   F   L   G   F   E   V   A   M   K

939  TTC CTT AAT TGG GCC ACC CCC AAC TTG TGA GGCTGAAGGC TGCTCAAGTT CACTTCTGGA TGCTGGAAGC TGTCG
293  F   L   N   W   A   T   P   N   L

1014 TTGAGGAGAA GGAGTAGTAA GCAGAACTAA GCAGTCTTGG AGGGCAAGGG GAGGGGAATG GTGAGATCCG AGCCC

1089 TGTGCATGGA CTTGGTGAGA CTGTTGCCTT AATGACATCC TGCACCGTGT ATAACTTAGT GTGTCATTTT GAAACTT

1166 GAATTCAT TCTTATCAAT TTAAGGGATC TTAAAAGGAT TTGAATGGAA CAAGTAGCTT CCAGACCAGA TAC TACCTGT
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Figure 1 Nucleotide sequence of human CAC cDNA. The deduced amino acid sequence of the open reading frame of 903 bp (301 codons) is shown below the nucleotide sequence. Human CAC amino acids that differ from those of rat CAC (Indiveri et al. 1997) are shown in underlined, italicized print. The cDNA and deduced protein sequences are in GenBank (accession no. Y10319; http://www.ncbi.nlm.nih.gov/Web/Genbank/index.htm).

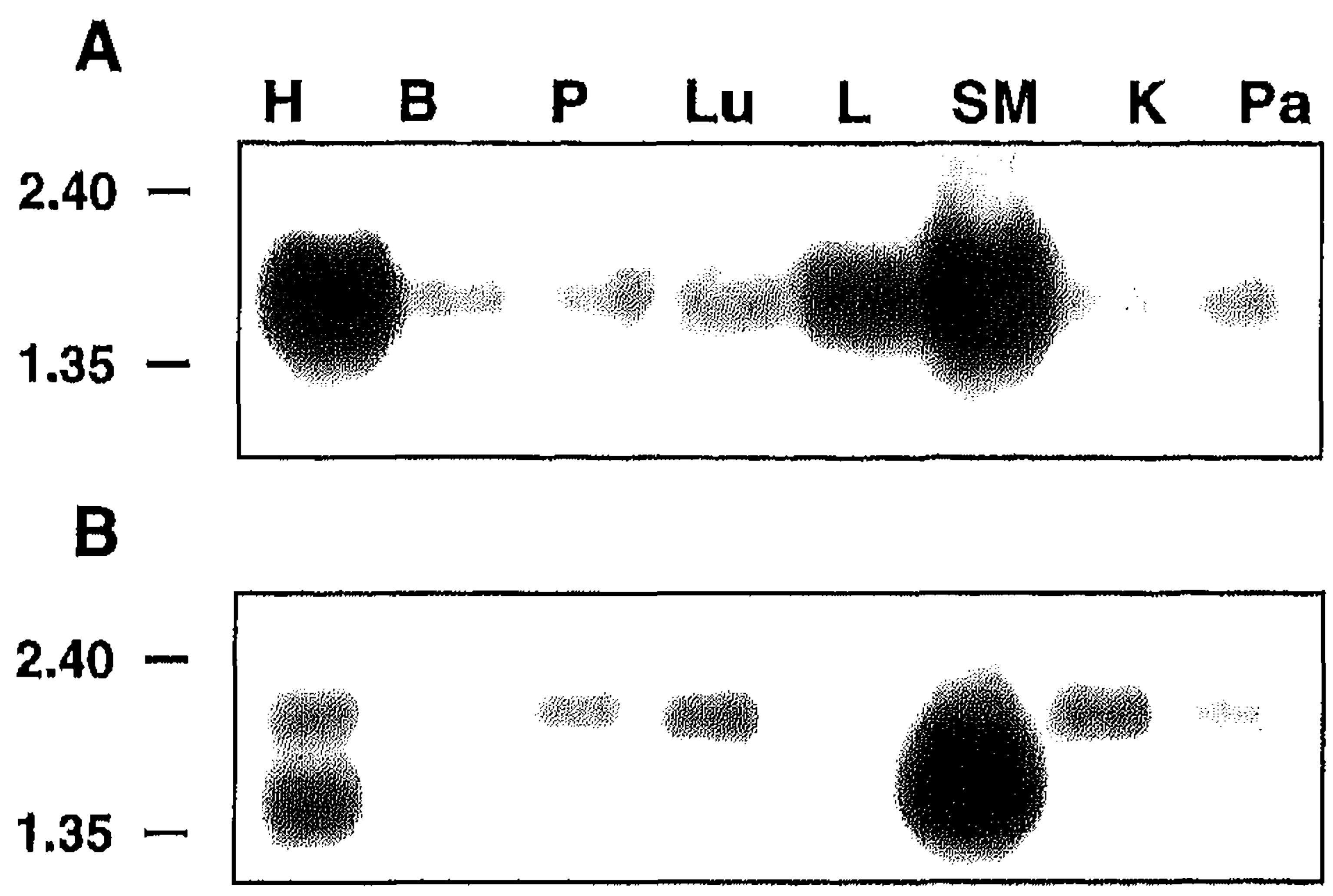

Figure 2 Expression of CAC mRNA in human tissues. Northern blots (2 μg poly[A] RNA/tissue) hybridized with a human CAC probe (*A*) and an actin probe (*B*) are shown. The molecular weight is indicated in the figure: human CAC mRNA is ~1.8 kb. Results for the following tissues are shown: heart (H), brain (B), placental (P), lung (Lu), liver (L), skeletal muscle (SM), kidney (K), and pancreatic (Pa).

double-stranded human liver cDNA (Clontech). In this case we employed two nested forward adaptor primers and two reverse-specific primers (TTGTGTCTGCAGTCGGACCTTGAC and CCAGAGGGTGACCGACGAACACCAGGC, corresponding to nucleotides 257–278 and 121–146, respectively, of the rat cDNA sequence). DNA-sequence analysis was performed according to established procedures (Indiveri et al. 1997).

Northern Blot Analysis

The sequence corresponding to nucleotides 421–967 of the human CAC cDNA was used as a probe in northern blots performed on mRNA derived from various human tissues (Clontech). Northern blot analysis was performed according to standard protocols, with high stringency. The filters were autoradiographed at −80°C. For normalization of the hybridization signals a probe encoding part of human actin (Clontech) was employed.

Mutation Detection

Total RNA was extracted (Chomczynski and Sacchi 1987) from cultured skin fibroblasts and was stored as an ethanol precipitate at −80°C. A 5-μg sample of RNA was reverse transcribed to cDNA, in 1 h at 42°C, with 200 U of Superscript II reverse transcriptase (Life Technologies), by use of oligo(dT) and random hexamer primers. Five microliters of this first-strand cDNA was subjected to PCR amplification. By PCR, we generated three overlapping fragments that covered the entire coding region, using the following synthetic oligonucleotide primers (Perkin Elmer): for fragment 1, 5′-GCAGGTCGAGAACTGACAGAC-3′ (sense F1) and 5′-TTTCTCCTGAAGAAGCCTGAA-3′ (antisense R1), at positions 21–41 and 481–501, respectively, of the human CAC cDNA sequence (fig. 1); for fragment 2, 5′-CCTGGAGAACGGATCAAGTG-3′ (sense F2) and 5′-CAATTAAGGAACTTCATGGCAA-3′ (antisense R2), at positions 450–469 and 928–949, respectively; and, for fragment 3, 5′-GCAGTGATGATCCGAGCCTTC-3′ (sense F3) and 5′-ACAGGTAGTATCTGGTCTGGAA-3′ (antisense R3), at positions 873–893 and 1224–1245, respectively. For each fragment, 35 cycles of PCR were performed (92°C for 60 s, 60°C for 60 s, and 72°C for 90 s). The cycles were preceded by an initial denaturation step at 95°C for 3 min and were followed by a final extension at 72°C for 10 min. Ten microliters of each PCR reaction was analyzed on 1.0% agarose gels with 0.5 μg ethidium bromide/ml in 1 × Tris borate–EDTA. The nucleotide sequences of the PCR products were analyzed by direct sequencing using the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's recommendations.

Results

Cloning and Sequencing of Human CAC cDNA

The recently reported rat cDNA of CAC (Indiveri et al. 1997) was used for cloning and sequencing of the human CAC homologue. Four overlapping sequences were amplified, by PCR using human liver cDNA as a template, with synthetic oligonucleotide primers based on the rat cDNA sequence and, to extend the sequence to the 5′ end, with two nested forward primers complementary to adaptors that were added to the 5′ extremities of the human liver cDNA. The obtained cDNA sequence was 1,243 bp in length and had an open reading frame of 903 bp (fig. 1). Assignment of position 63 as the first nucleotide of the initiation codon was deduced from comparison with the CAC cDNA of rat liver (Indiveri et al. 1997), since no stop codon was found in the 5′ UTR. The sequence, which extended into the 3′ noncoding region, did not contain the polyadenylation signal. The protein encoded by the human CAC cDNA contains 301 amino acids, and its calculated molecular weight is 32.9 kD.

Expression of CAC mRNA in Human Tissues

A hybridization probe consisting of nucleotides 421–967 of the human liver CAC cDNA was employed in northern blot experiments. Figure 2 shows the presence of one band for CAC mRNA, ~1.8 kb in length, in various human tissue types. The CAC mRNA was highly expressed in heart, skeletal muscle, and liver tissues. A much lower level of expression was found in brain, placental, pancreatic, and kidney tissues and especially in lung tissue. These differences in the level of expression of the CAC transcript in the various tissues

Table 1

Overall β-Oxidation Rates and CAC Activities in Cultured Skin Fibroblasts of the Patient and of Controls

	Patient	Controls (n)[a]
Overall β-oxidation (nmol/h/mg):		
[9,10-^{3}H]Myristic acid	4.1	6.0 ± 2.3 (61)
[9,10-^{3}H]Palmitic acid	2.4	8.8 ± 4.1 (33)
CAC activity (pmol/min/mg):		
Produced $^{14}CO_2$	ND[b]	47 ± 16 (5)

[a] Data are the mean ± SD. n = no. of controls.
[b] ND = not detectable.

tested were not due to variations in the amounts of RNA loaded on the gel, since this was checked in a control experiment with an actin probe.

Biochemical Studies in the Index Patient

Biochemical measurements of intact cultured skin fibroblasts showed a diminished oxidation rate of ^{3}H-labeled myristic and palmitic acid (66% and 27%, respectively, of the control mean), as shown in table 1. The oxidation rate of ^{14}C-labeled octanoate and butyrate was found to be normal (not shown). The activities of the various mitochondrial fatty acyl-CoA dehydrogenases, the enoyl-CoA hydratases, the 3-hydroxyacyl-CoA dehydrogenases, and the 3-ketoacyl-CoA thiolases, as well as of carnitine palmitoyltransferases I and II, were all normal (not shown). As shown in table 1, the activity of CAC was not detectable.

Mutation Analysis

The CAC mRNA from cultured skin fibroblasts from the patient was reverse transcribed, and the cDNA was PCR amplified in three overlapping fragments. Sequencing of these fragments revealed an insertion of a cytosine in the cytosine-rich region of bp 955–959, as shown in figure 3. This insertion changes the sequence of the CAC protein from amino acid 300 (asparagine to glutamine) to the carboxy terminus and expands the length of the protein by 21 amino acids, to 322 amino acids (fig. 4*A*).

Hydrophilicity calculations were performed according to the Kyte-Doolittle method, for the peptide sequence of the wild-type and the patient's CAC. The values obtained were used for Chou-Fasman predictions of the secondary structure of the protein. The Chou-Fasman two-dimensional plot (fig. 4*B*) showed a dramatic conformational change, at the C-terminal region, between the wild-type and the patient's CAC protein.

Discussion

Primary Structure of Human CAC

The protein encoded by the human CAC gene is 301 amino acids long, and its calculated molecular weight is 32.9 kD. The amino acid sequences of human and rat CAC are highly conserved: there is ~90% identity between these species (fig. 1) (Indiveri et al. 1997). Human CAC differs from its rat counterpart in 29 amino acids, 15 of which are nonconserved. The identity between the human and rat CAC is less than that found for some other mitochondrial carriers. In fact, 97% identity was found between the human and rat 2-oxoglutarate carriers (Iacobazzi et al. 1992; Dolce et al. 1994*b*), and 95% identity was found between the human and rat citrate carriers (Kaplan et al. 1993; Iacobazzi et al. 1997) and phosphate carriers (Ferreira et al. 1989; Dolce et al. 1994*a*). Three repeated homologous domains, each ~100 amino acids in length, can be distinguished in the human CAC, a characteristic previously recognized in other mitochondrial transport proteins (Walker and Runswick 1993; Palmieri 1994). These domains are related to those found in the mitochondrial carrier-protein family (Walker and Runswick 1993; Palmieri 1994).

The cDNA and deduced protein sequences are in GenBank (accession no. Y10319; http://www.ncbi.nlm.nih.gov/Web/Genbank/index.htm). A Blast search for

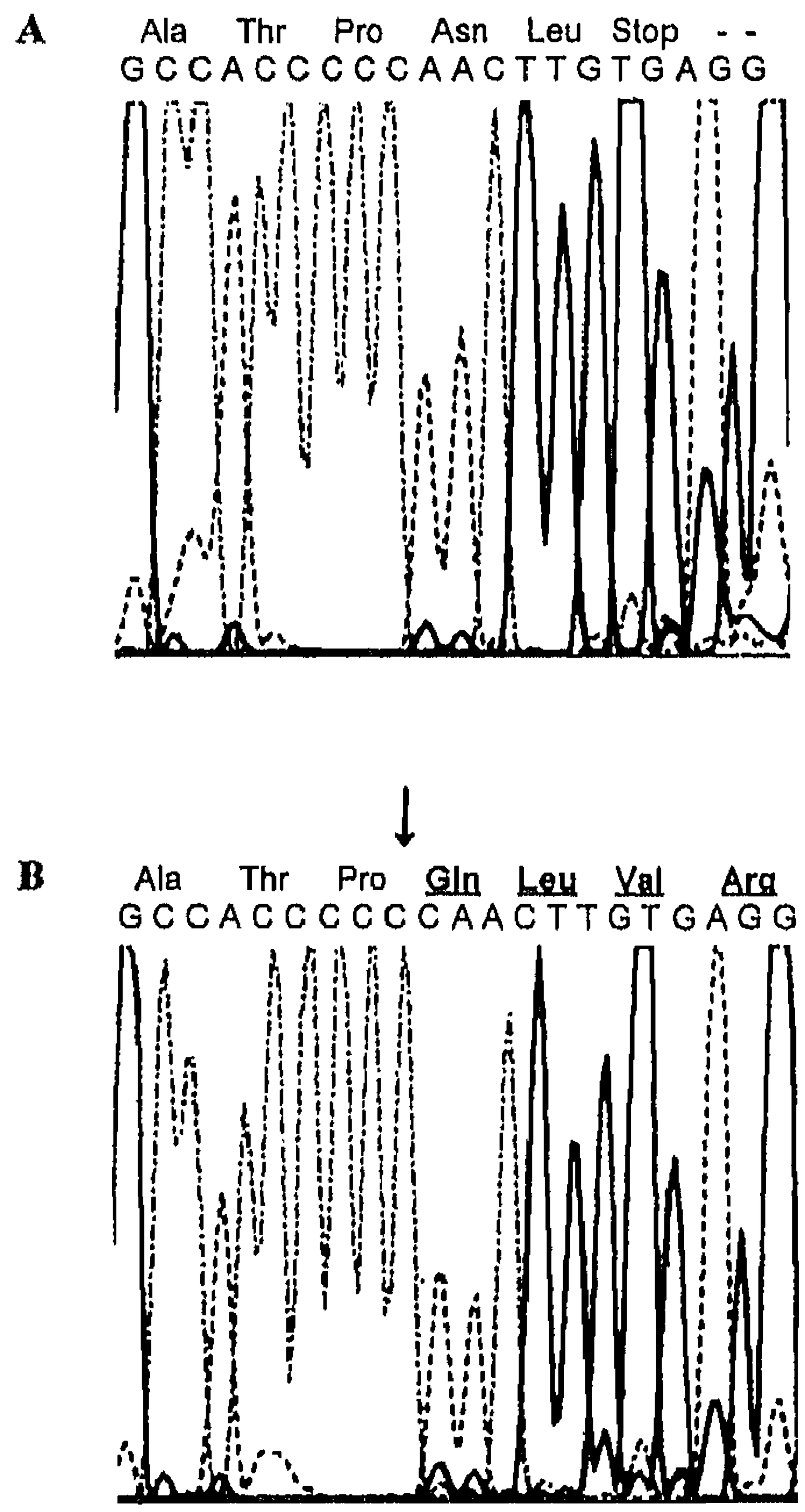

Figure 3 Sequences of CAC cDNA segments, with their accompanying amino acid codes, for a control (wild type) (*A*) and the patient (*B*). For the patient, a C insertion, resulting in a frameshift and an extension of the C terminus of the CAC protein (*underlined*), is shown (*arrow*).

A

C-terminus wild type: ... W A T P N L (296 … 301)

C-terminus patient: .....W A T P **Q L V R L K A A Q V H F W M L E A V V E E K E** (322)

B

wild type:

patient:

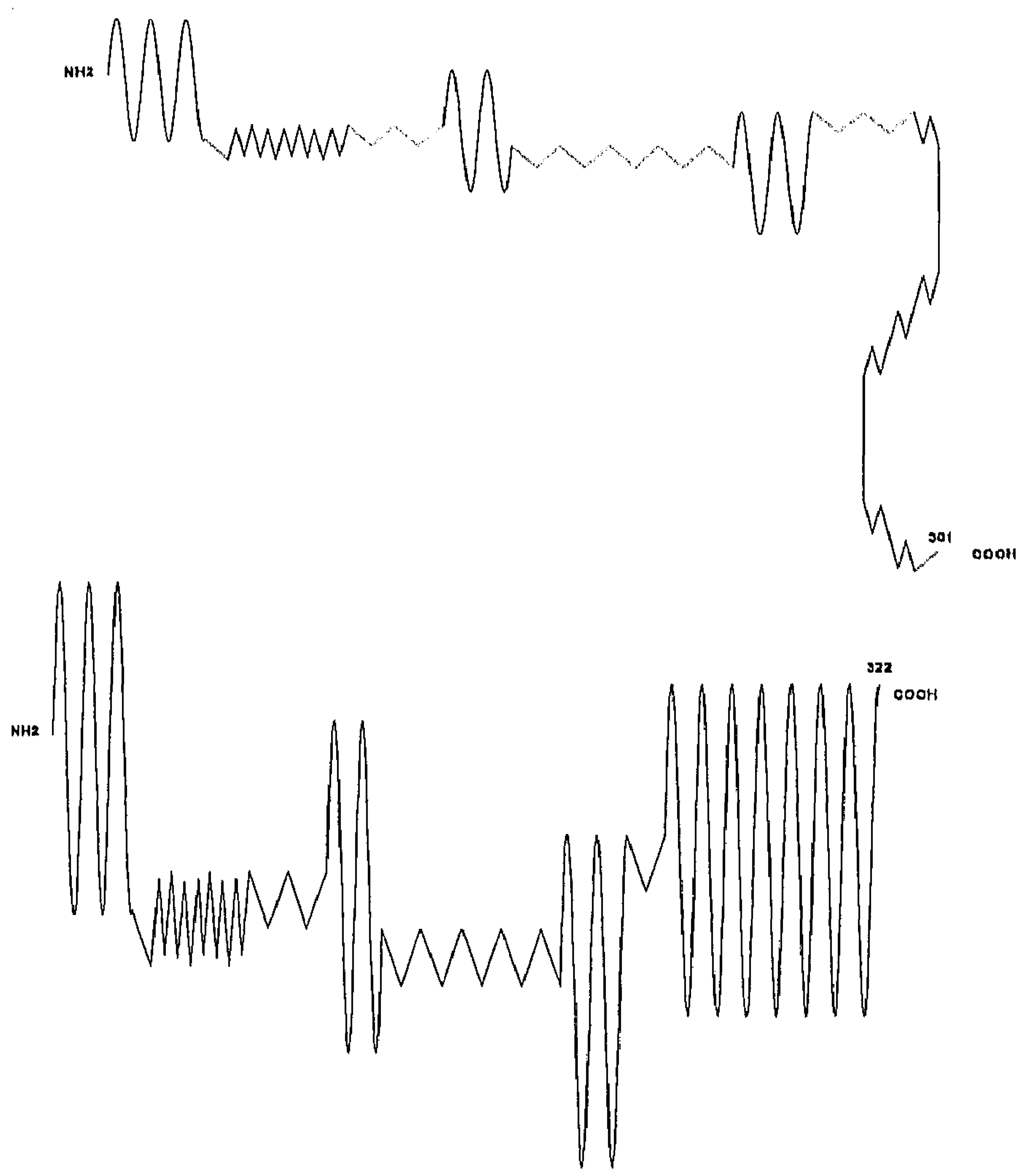

Figure 4 *A*, Primary C-terminal amino acid sequence of wild-type (*top*) and patient's (*bottom*) CAC protein. *B*, Predicted secondary structure, according to the Chou-Fasman algorithm, of wild-type (*top*) and patient's elongated (*bottom*) CAC.

homologous sequences revealed seven different expressed-sequence-tag (EST) clones (AA305590, R11780, F08483, Z28872, AA378439, N77642, and N87428) that originated from different human tissues. These EST clones have sequences that are homologous with different parts of the CAC protein and are presumed to encode a carrier protein of unknown function.

Tissue Distribution of CAC mRNA

The CAC defect was detected in the patient's fibroblasts. It is likely that CAC deficiency occurs in all tissues, since no evidence for the existence of various tissue-specific isoforms has been found. As shown in figure 2, high levels of CAC mRNA transcripts were found in heart, skeletal muscle, and liver tissues, which is in fair agreement with the clinical involvement of these tissues in the patient. Much lower levels of expression were found in brain, placental, kidney, and pancreatic tissues and especially in lung tissue.

CAC-Deficient Patient

We have identified CAC deficiency in cultured skin fibroblasts from a child who survived a stormy neonatal period due to her fatty-acid oxidation disorder, which invariably has been fatal in other patients (Stanley et al. 1992; Pande et al. 1993; Brivet et al. 1994, 1996; Niezen-Koning et al. 1995; Ogier de Baulney et al. 1995). Direct sequencing of the entire cDNA of the patient revealed the presence of a homozygous insertion of a cytosine nucleotide in the only cytosine-containing region, bp 955–959, resulting in a frameshift. We repeated sequencing of the cDNA of this patient and of three controls, in three independent experiments, all of which revealed the homozygous insertion in the patient's cDNA. This excludes the possibility of a mistake made by the *Taq* DNA polymerase in the PCR reaction. The region containing the insertion consists of five cytosine nucleotides, which makes confirmation of the insertion by restriction-enzyme analysis impossible. Unfortunately, no material from the other family members was available for further investigations.

The CAC protein of the patient has an obviously changed C terminus: amino acids 300 and 301 are changed from asparagine and leucine, respectively, to glutamine and leucine, respectively, and the protein has been elongated by 21 amino acids. A Chou-Fasman prediction shows a dramatically changed secondary structure. The C terminus is changed from a turn (in wild-type CAC) to a helix structure, in the patient. The molecular basis of the transport process mediated by the CAC is still unrevealed. A proper folding and orientation of the CAC protein in the mitochondrial membrane is crucial for adequate functioning. The novel extension in the patient contains a hydrophobic domain, which may be embedded in the mitochondrial inner membrane instead of protruding into the intermembrane space. A diminished entrapping or binding capacity of the positive charge of the quaternary nitrogen of carnitine to the negative carboxylate of the CAC C-terminus can be hypothesized for the patient. The alterations of the patient's CAC also may lead to instability of the protein. An impairment of the patient's CAC in substrate binding or translocation also can be considered. The functional consequences of the molecular defect apparently are restricted, in view of the mild clinical phenotype of the patient. Surprisingly, no CAC activity was detectable in fibroblasts.

In the present study, we determined the sequence of the human cDNA of the mitochondrial CAC, showed differences in human tissue distribution, and defined the first molecular defect in a CAC-deficient patient. This study provides the techniques necessary to resolve the molecular basis of CAC deficiency. This may be of great importance for the reliable diagnosis of patients at risk and also may guide the way toward prenatal diagnosis of this severe type of inborn error in metabolism.

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