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Research Article

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Carbamyl Phosphate Synthetase I Deficiency

One Base Substitution in an Exon of the CPS I Gene Causes a 9-Basepair Deletion due to Aberrant Splicing

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Abstract

Carbamyl phosphate synthetase I (CPS I; EC6,3,4,16) is an autosomal recessive disorder characterized by hyperammonemia. We studied the molecular bases of CPS I deficiency in a newborn Japanese girl with consanguineous parents. Northern and Western blots revealed a marked decrease in CPS I mRNA and enzyme protein but with a size similar to that of the control, respectively. Sequencing of the patient's cDNA revealed a nine-nucleotide deletion at position 832–840. Sequencing analysis of the genomic DNA revealed a G to C transversion at position 840, the last nucleotide of an exon in the splice donor site. This substitution altered the consensus sequence of the splice donor site and the newly cryptical donor site in the exon caused the 9-bp in-frame deletion. This report seems to be the first complete definition of CPS I deficiency, at the molecular level. (*J. Clin. Invest.* 1993. 91:1884–1887.) Key words: splice donor site • urea cycle • consanguinity • mutation • hyperammonemia

Introduction

Carbamyl phosphate synthetase I (CPS I[1]; EC6,3,4,16), the first enzyme in ureagenesis, catalyzes the synthesis of carbamyl phosphate from HCO_3^-, 2ATP, and NH_4^+. CPS I is a mitochondrial enzyme located in the matrix compartment and is expressed in the liver and in epithelial cells of intestinal mucosa (1).

Deficiency in the enzyme results in an autosomal recessive disorder associated with hyperammonemia, protein intolerance, as well as impaired mental and physical development. The incidence of CPS I deficiency in Japan is 1:800,000 (2). Two clinical patterns of CPS I deficiency have been described, the neonatal form (3) and the delayed onset form (4, 5). The neonatal form is characterized by severe hyperammonemia from the neonatal period and sometimes results in neonatal death and the other form is milder in terms of protein intolerance. Enzyme defects are severe in the neonatal form and a partial deficiency can occur in the late onset form. Molecular events in the CPS I gene, as related to the enzyme defects have not been elucidated.

The enzyme, purified from a variety of mammalian sources (6–8), proved to be a single polypeptide with a molecular weight of 165,000. Human CPS I cDNA cloned and characterized in our laboratory showed that CPS I is composed of 1,500 amino acid residues, the calculated molecular weight of the precursor form is 164,828, and that of the processed mature form is 160,438 or 160,324 (9).

Elucidation of the primary structure of the human CPS I prompted us to analyze CPS I deficiency, at the molecular level. We describe here an exon mutation that resulted in aberrant splicing and partial deletion in mRNA.

Methods

Patient. The patient was the progeny of first cousin parents and two siblings had died in the neonatal period. A severe hyperammonemia (956 μg/dl) associated with lethargy and grunting developed on the second postnatal day. Intensive treatment including exchange blood transfusion was ineffective and she died 4 d later. Liver CPS I activity decreased to 11% of the control.

Material. Liver samples taken from the patient within 1 h of death were homogenized in the cold using 0.1% cetyltrimethyl-ammonium bromide at a 10:1 ratio of buffer volume/tissue wet weight. The homogenate was used for enzyme assay and Western blot analysis. The enzyme activity was measured by the method of Brown and Cohen (10).

Western blot analysis. SDS-PAGE was performed according to standard procedures (11). Transfer onto nitrocellulose was done at 4°C for 14 h at 20 V in 20 mM Tris and 200 mM glycine (pH 8.3). Immunodetection was performed using an antiporcine CPS I polyclonal antibody prepared in our laboratory (12). Protein concentrations were determined using BCA protein assay reagent kits supplied by Pierce Chemical Co. (Rockford, IL). Laser densitometry, using a computing densitometer (model 300-A; Molecular Dynamics, Sunnyvale, CA), was used to quantitate the relative signal intensity of the bands obtained.

Northern blot analysis. Total RNAs were isolated from the frozen autopsied liver and a human control liver, using the guanidium thiocyanate/cesium chloride centrifugation method (11). 10 μg of total RNA was denatured with 3% formaldehyde separated by electrophoresis in a 1.2% agarose gel, and transferred on to a nitrocellulose membrane. The filter was hybridized with ^{32}P-labeled, full-length human CPS I cDNAs probe and a human arginase cDNA probe (9). Prehybridization, hybridization, and wash were performed using standard procedures (11). Autoradiography was done using an intensifying screen for 24 h at −70°C. The density of the obtained bands were measured by laser densitometer.

cDNA analysis. First strand cDNA was generated from 10 μg of total RNA, using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) with specific antisense oligonucleotides primers (13). PCR amplification of cDNA performed for 30 cycles consisted of denaturation 94°C for 1 min, annealing at 44°C for 1 min, and extension at 72°C for 3 min (14). The amplified cDNAs were subcloned and nucleotide sequences of

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1. *Abbreviation used in this paper:* CPS I, carbamyl phosphate synthetase I.

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insert cDNA were determined (T7 Sequenase Kit; United States Biochemical Corp., Cleveland, OH).

Genomic DNA analysis. Gene fragment covering mutation was obtained from a genomic library using the plaque hybridization technique (11). The library was constructed from BamHI partial digests of human placenta genomic DNA. Approximately 2.5×10^6 EMBL-3 phages of total DNA library were screened, using human CPS I cDNA as a probe. Phage DNAs of positive clones were characterized by restriction mapping and Southern hybridization analysis. The DNA of surrounding deletion was subcloned and sequenced. The genomic DNA for the family analysis was purified from white blood cells, according to standard procedures (11).

Results

Western blot analysis. The liver sample obtained from the patient at autopsy was analyzed for CPS I protein and for enzyme activity. The SDS-PAGE and Coomassie staining of proteins revealed that a protein band corresponding to CPS I that is usually seen in normal liver samples was absent in the patient (data not shown). A Western blot using a specific antibody against CPS I showed that immunoreactive protein related to CPS I of a normal size was present in the liver of the patient, however the amount of the protein was markedly decreased. For quantitative analysis of CPS I protein, different amounts of samples were subjected to SDS-PAGE, Western blots, and densitometric analyses. The patient's CPS I protein was decreased to 1.7% of the control (Fig. 1 *A*).

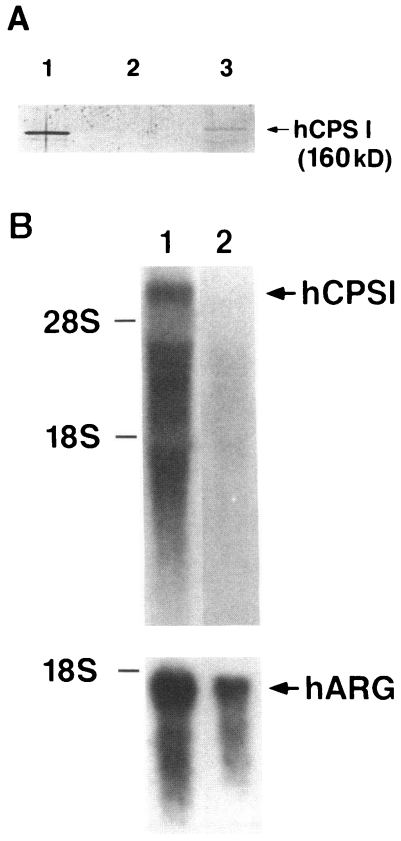


Figure 1. Western blot analysis (*A*) and Northern blot analysis (*B*) of CPS I deficiency patient. *A*, Western blot analysis of protein obtained from a human liver, the sample was homogenized and analyzed as described in Methods. (Lane *1*) 0.2 μg of protein from control liver. Lane *2*, 0.2 μg of protein from the patient's liver. Lane *3*, 2.0 μg of protein from the patient's liver. An arrow indicates the 160-kD protein of CPS I. *B*, Northern blot analysis of RNA obtained from a human liver. RNA was prepared and analyzed as described in Methods. Lane *1*, 10 μg of total RNA from a control liver. Lane *2*, 10 μg of total RNA from the patient's liver. The blot was probed with a labeled human CPS I cDNA and arginase cDNA. An arrow indicates size of the human CPS I mRNA and arginase mRNA.

Northern blot analysis. Northern blot analysis of the total RNA from the liver of the patient revealed that the mRNA for CPS I was present and that the amount of the mRNA was markedly decreased to 3% of the control. The level of human arginase mRNA was 80% of the control (Fig. 1 *B*).

cDNA analysis. We synthesized cDNA for CPS I from the mRNA and amplified the entire coding region of the cDNA, using six pairs of primers (Fig. 2), then the amplified fragments were subcloned and sequenced. Sequence analysis indicated a 9-bp deletion at positions 832–840 (Fig. 3 *A*). No other mutation was detected in the coding region of CPS I cDNA.

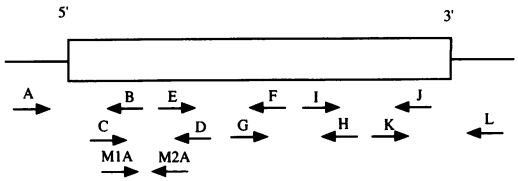
Genomic DNA analysis. To search for cause of the 9-bp deletion, we analyzed the CPS I gene. As the structure of human CPS I gene has not been elucidated, we obtained the gene fragment that covered the region of the mutation by screening of a genomic library. A phage clone named M-8 was found to cover the region of the mutation. Subsequently the nucleotide sequence of the phage clone was determined. The 9-bp sequence deleted from the mRNA of the patient was located in the 3′ end of an exon composed of the coding sequence of nucleotide residues 832–840. We prepared a specific primer set which allows for amplification of the region related to the mutation (Fig. 2, *M1A* and *M2B*). The nucleotide sequence of the amplified DNA from the controls and the patient revealed that DNA from the patient had a G to C transversion at position 840, the last nucleotide of the exon which confers a conserved sequence of the splice donor site (Fig. 4). The G to C transversion at position 840 generated a new enzyme site of MaeII (5′-A/CGT-3′). Thus, the PCR products from the patient and the controls, which included the splice donor site of the exon, were digested with MaeII. As expected, the DNA fragment from the patient but not from the control was cleaved into two fragments (Fig. 5). Thus, the patient was homozygous for the G to C transversion. A family survey of the mutation revealed that the parents, the sister, and the brother were heterozygous for the G to C transversion (Fig. 5). These studies confirmed that the mutation had been transmitted from the consanguineous parents. Analysis of mRNA and genomic DNA from the patient suggested that the 9-bp deletion in the mRNA was the result of aberrant splicing due to point mutation in the consensus sequence of the splice donor site of the exon.

We found that the human CPS I cDNA nucleotide sequence reported by Haraguchi et al. (9) had an error of one base addition of T at base number 834 (counted from ATG) and one base omission of A at base number 837.

The 9-bp deletion in the cDNA sequence provided a new restriction site for the enzyme SspI (5′-AAT/ATT-3′). As shown in Fig. 3 *B*, the 72-bp fragment obtained by PCR amplification (Fig. 2, *M1A* and *M2A*) of the patient's cDNA was cleaved into 40- and 32-bp fragments. To investigate the presence of minor mRNA of a normal or different length, the cDNA fragment from the patient was reamplified after digestion with SspI, but no fragment was detected. A PCR fragment with primers A and B (Fig. 2) showed one band of an almost normal length, thus exon skipping had not occurred. These results suggest that almost all of the mature mRNA in the patient carried the 9-bp deletion.

Discussion

We obtained evidence for a point mutation in which the last nucleotide of the exon had G replaced with C in the CPS I gene



Primer sequences were as follows

A -64 : 5'-GTGTTATCACACAATCTCAT-3' : -55
B 1079 : 5'-GTTTGATCGTTGACATTCAC-3' : 1060
C 607 : 5'-GGAGTTTCAACCAAGGATGT-3' : 626
D 1869 : 5'-CTCCACCAGAATTTGGTTGG-3' : 1850
E 1423 : 5'-GGCTTAAAGCAAGCGGATAC-3' : 1442
F 2855 : 5'-GGGTATTCTGCAGCCAGTGT-3' : 2836
G 2574 : 5'-TGATGACAACATGTCCCTTG-3' : 2593
H 3645 : 5'-GATGGTTTGTGTGGGCAGCA-3' : 3626
I 3388 : 5'-CCTTCCTATGTTTTGAGTGG-3' : 3407
J 4484 : 5'-CTGTACTGCCTGTAGTGGAA-3' : 4465
K 3905 : 5'-CTGTTGACTATGTTGCAATT-3' : 3924
L 5079 : 5'-CTCTGTCACATTTCTCAACTTCAAT-3' : 5055
M1A 797 : 5'-GGAACCCAGCTCTTGCAGAACCAC-3' : 820
M2A 877 : 5'-TTCCAAACAATGGCTCCTTGCGATCAC-3' : 851
M2B : 5'-CCTGATAAATAAAATGTATCACA-3' :

Figure 2. Reverse transcription PCR and sequencing strategy for human liver CPS I cDNA. Arrows indicate the orientation and the region of primers used for synthesis of the first strand cDNA and PCR amplification. Sequences of the primers are shown in the lower panel.

in a CPS I deficient patient. As a result of the exon mutation a cryptical donor site appeared within the exon and there was a 9-bp deletion in the coding region in the mRNA (Fig. 6).

While there are mutations that affect mRNA splicing at a variety of locations within the gene, there are few examples of splice donor site mutation within the last nucleotide of the exon and which affect mRNA splicing. The precedents are β^+ thalassemia, the spf^{ash} mutation at the ornithine transcarbamylase locus of mouse, and ry^{5208} mutation at the rosy (xanthine dehydrogenase) locus of *Drosophila melanogaster* (15–17). β^+ Thalassemia had the same G to C transversion at the last position of the exon. In the case of β^+ thalassemia, the relative efficiency of the correct splicing of mRNA was decreased to 1.7% of the control.

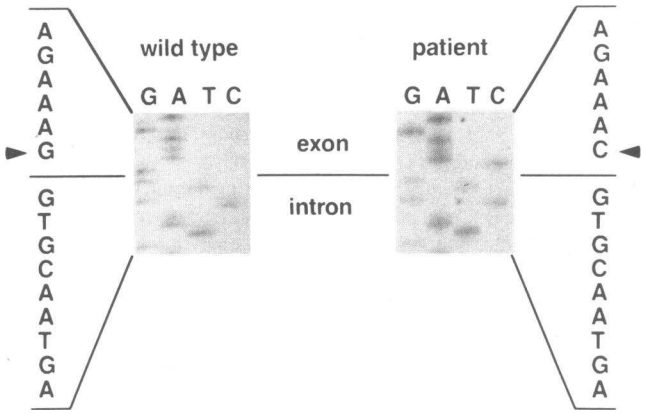


Figure 4. Partial nucleotide sequences of wild type and mutant genomic DNA. Each autoradiogram represents the genomic sequence encompassing the mutation of the exon/intron junction in CPS I gene. Arrows indicate the position of mutation.

A scoring system described by Shapilo and Senapathy (18) gives a numerical value for any proposed 5′ or 3′ consensus sequence. The score of proper splice site of the exon of the CPS I gene was decreased from 65.1 to 51.6% by the G to C transversion and the score of the newly appearing splicing site in the exon was 71.5%. A CPS I with normal length mRNA was not detected in the autopsied liver of the patient, as analyzed by PCR amplification. Almost the entire mRNA of CPS I appeared to be spliced, using the cryptical site, and the product seemed to be unstable, as deduced from Northern blots. The decreased production of mRNA might result in a deficiency in the mature form of CPS I protein.

The same mutation was absent in two other patients with CPS I deficiency (data not shown), thus the mutation may be heterogeneous in CPS I deficiency.

This report seems to be the first molecular analysis of CPS I deficiency. A prenatal diagnosis and carrier detection of CPS I deficiency is difficult because the enzyme is expressed only in the liver and intestine. Fetal liver biopsy for a prenatal diagnosis was done by other workers (19, 20) and a restriction frag-

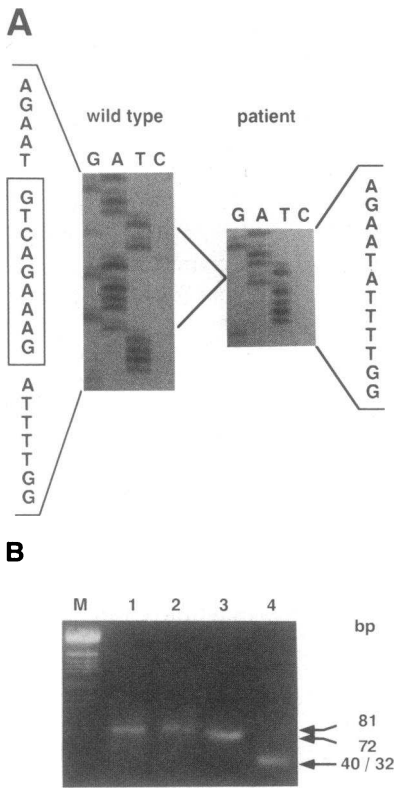


Figure 3. Partial nucleotide sequences of wild type and mutant cDNA (*A*), and agalose gel electrophoresis of PCR amplified DNA after digestion with SspI (*B*). *A*, Each autoradiogram represents the cDNA sequence encompassing the deletion. The nucleotide sequence of cDNA from the patient revealed an in-frame 9-bp deletion. *B*, PCR amplification with primers M1A and M2A. Lane *1*, normal control fragment. (Lane *2*) normal control fragment after digestion with SspI. Lane *3*, fragment from the patient. Lane *4*, fragment from the patient after digestion with SspI. Lane *M*, marker.

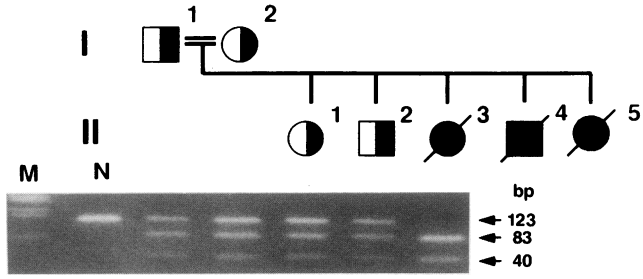


Figure 5. Pedigree of the CPS I deficient family; the CPS I gene fragment spanning the mutant region was digested with MaeII. The 123-bp fragment cleaved to 83 bp and 40 bp in the mutant allele. II-4,5 are siblings of the patient. *N*, normal control; *M*, marker.

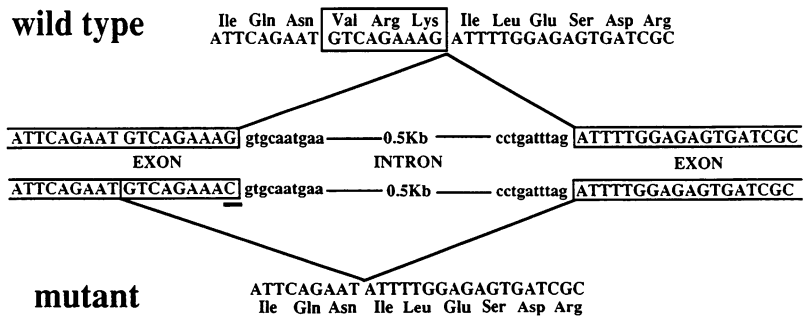


Figure 6. Organization of genomic, cDNA and amino acid sequence, as well as schematic representation of CPS I gene, together with the wild type and the patient's cDNA and amino acid sequence. The cDNA alteration seen in the mutant CPS I predicts the in-frame deletion of the three amino acids. The mutation is underlined.

ment length polymorphism using restriction enzyme BglI was reported (21). A DNA analysis of CPS I deficiency makes for safety and accuracy.

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