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MULTIPLE mRNA FORMS OF HUMAN GTP CYCLOHYDROLASE I

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SUMMARY: To isolate full length cDNA clones encoding human GTP cyclohydrolase I, the first and rate-limiting enzyme in tetrahydrobiopterin biosynthesis, a cDNA library generated from human liver was screened by plaque hybridization. Analysis of the clones, hybridized with rat cDNA fragment, by restriction mapping and partial sequencing showed the existence of three kinds of cDNAs. All three cDNAs were identical in their central and 5' regions. They were, however, found to diverge at 3' ends. Furthermore, the three species of mRNAs corresponding to the three cDNAs were detected in human liver by reverse transcription-polymerase chain reaction (RT-PCR) analysis. These results indicate that, in humans, GTP cyclohydrolase I molecules are encoded by at least three distinct mRNAs.

(6R)-(L-erythro-1', 2'-dihydroxypropyl)-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (tetrahydrobiopterin, BH_4) is the natural cofactor for the three pterin-dependent monooxygenases which hydroxylate aromatic amino acids; phenylalanine (1), tyrosine (2), and tryptophan (3) hydroxylases. Tyrosine hydroxylase and tryptophan hydroxylase are the first and rate-limiting enzyme for the biosynthesis of the neurotransmitters, catecholamines and serotonin, respectively. Phenylalanine hydroxylase is essential for phenylalanine degradation. High concentrations of BH_4 are found in such tissues as brain, adrenal medulla, and liver where aromatic amino acid hydroxylases exist. Recent evidence has shown that BH_4 is also synthesized during multiplication and differentiation of cells that lack neurotransmitter biosynthesis and phenylalanine degradation (4-6). Furthermore, it has also been shown to be involved in the generation of the free radical nitric oxide in cardiovascular, immune, and nervous systems (7,8). Thus, recent studies indicate the multiple physiological roles of BH_4.

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Tetrahydrobiopterin is synthesized from GTP (9,10) by the following pathway: GTP $\xrightarrow{(a)}$ dihydroneopterin triphosphate $\xrightarrow{(b)}$ 6-pyruvoyl tetrahydropterin $\xrightarrow{(c)}$ BH_4 , and each step is catalyzed by (a) GTP cyclohydrolase I, (b) 6-pyruvoyl tetrahydropterin synthase, and (c) sepiapterin reductase. In the process of our study on gene expression and regulation of human tyrosine hydroxylase, we are involved in examining the regulation of the biosynthesis of BH_4 in man. We have determined the amino acid sequences of rat sepiapterin reductase (11) and human sepiapterin reductase (12). The conversion of GTP to D-erythro-7,8-dihydroneopterin triphosphate is the first and rate-limiting step in the BH_4 biosynthesis pathway and is catalyzed by GTP cyclohydrolase I. We have carried out molecular cloning and sequence analysis of cDNA for the human enzyme to investigate the molecular mechanism of enzyme regulation and to examine the genetic regulation of BH_4 biosynthesis under various physiological and pathological conditions. Hatakeyama *et al*. (13) isolated a cDNA clone encoding rat GTP cyclohydrolase I and reported the nucleotide sequence and predicted amino acid sequence. In the present study, we have cloned human GTP cyclohydrolase I, and demonstrate the existence of three different mRNA types in man.

MATERIALS AND METHODS

Screening of cDNA Library. A human liver cDNA library in λgt11 using random primer and EcoR1 linkers was a generous gift from Dr. Y. Ebina (The University of Tokushima, Japan). The rat GTP cyclohydrolase I cDNA probe was generated by reverse transcription-polymerase chain reaction (RT-PCR) with RNA from rat liver. The primer design was based on the rat cDNA sequence published by Hatakeyama et al. (13) with an additional BamHI site. The sequences of primers were 5'-GGATCCCATTTGTGGGAAGGGTCCA-3' and 5'-CGGATCCTCAGCTCCTGATGAGTGTG-3'. The PCR product contained 315 bp from position 412 to 726, and was subcloned into a plasmid vector. Sequencing of the clone revealed the identity with the reported one (13). The cDNA fragment of rat GTP cyclohydrolase I was labeled with [α-^{32}P]dCTP (110 TBq/mmol) by Megaprime DNA labeling system (Amersham) and used as probe. A total of 3 x 10^5 λ phage plaques on E.coli strain Y1088 lawn cells were screened. The phage plaques were lifted twice onto nylon membranes (Hybond-N, Amersham) and processed for hybridization. The membranes were prehybridized for at least 2 hrs at 65°C in 6 x SSC containing 5 x Denhardt's solution, 0.5% SDS and 0.1 mg/ml of salmon sperm DNA. Hybridization followed at 65°C for 17 hrs in the above specified buffer with a labeled probe. The membranes were washed with 2 x SSC containing 0.05% SDS at room temperature for 10 min twice, then soaked in the same solution at 42 °C for 20 min twice. The cDNA clones with positive signal were purified and subcloned into EcoR1 site of Bluescript KS (M13+).

DNA Sequence Analysis. The nucleotide sequences of the cDNA were determined by the dideoxynucleotide chain-termination method (14) using Sequenase (United States Biochemical Corp., Cleveland, OH) and synthetic primers.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA (2 μg) from human liver was reverse-transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories) using randam hexamer. The cDNA transcribed was used for PCR amplification (15) with the primer sets reflecting the distinct sequence in three cDNAs isolated in the present study. The following program was used for

PCR; initial template denaturation step, 3 min at 94 °C, and DNA amplification step, 15 sec at 94°C, 30 sec at 42°C, 30 sec at 72°C, for 30 cycles, using GeneAmp PCR System (Perkin Elmer/Cetus). The amplified DNA fragments were electrophoresed in 4% Nusieve GTG agarose (FMC Bioproduct) gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

The rat cDNA fragment labelled with [α-^{32}P]dCTP was used for screening through plaque hybridization of 3 x 10^5 recombinants from a human liver cDNA library. Eighteen substantially hybridized clones were isolated, subcloned into the EcoRI site of a Bluescript vector and analyzed. These clones were classified into three types, according to restriction mapping and partial sequencing. One clone of each type was selected and sequenced entirely. The cDNA inserts of their clones were found to diverge at their 3' ends and were designated human GTP cyclohydrolase I type 1, 2, and 3 (hGCH-1, 2, and 3) (Fig.1).

The complete nucleotide sequences of hGCH-1, hGCH-2, and hGCH-3 are shown in Fig.2. Both hGCH-2 and hGCH-3 were full length cDNA clones but hGCH-1 lacked 25 base pairs at its 5' end to be full length. The sequence of hGCH-2 was identical to that of hGCH-3, except for 3'-terminal sequence containing 12 nucleotides in coding region. hGCH-1 also contained a common or homologous 5' sequence and a nonhomologous 3' sequence. The junction of these shared and divergent sequences was observed at nucleotide 627 into the coding sequence. The result may indicate that the multiplicity of human cDNA from liver results from the alternative splicing. The 44th nucleotide in hGCH-3 was adenine instead of guanine in hGCH-1 and hGCH-2, causing an amino acid change between Gly and Asp at amino acid residue 15. This difference is thought to be due to allelic polymorphism. A poly(A)$^+$tail was not found in these sequences, because the cDNA library used was made using random hexamers for priming cDNA synthesis, not but oligo(dT).

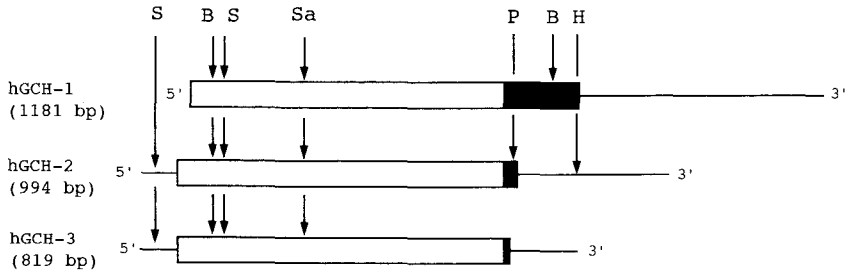

Fig.1. Structures and restriction enzyme maps of three clones of human GTP cyclohydrolase I cDNAs (hGCH-1, 2, and 3). Line, untranslated regions; box, coding region. White and black boxes represent the common and distinct regions of three cDNAs, respectively. Restriction enzyme abbreviations are: B, BamHI; H, HindIII; P, PstI; S, SmaI; Sa, SacI.

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                                                                5'-GGAGTTT   -61
          AGCCGCAGACCTCGAAGCGCCCCGGGGTCCTTCCCGAACGGCAGCGGCTGCGGCGGGTCC    -1
                                                                *
          ATGGAGAAGGGCCCTGTGCGGGCACCGGCGGAGAAGCCGCGGGGCGCCAGGTGCAGCAAT    60
          M  E  K  G  P  V  R  A  P  A  E  K  P  R  G  A  R  C  S  N
          GGGTTCCCCGAGCGGGATCCGCCGCGGCCCGGGCCCAGCAGGCCGGCGGAGAAGCCCCCG   120
          G  F  P  E  R  D  P  P  R  P  G  P  S  R  P  A  E  K  P  P
          CGGCCCGAGGCCAAGAGCGCGCAGCCCGCGGACGGCTGGAAGGGCGAGCGGCCCCGCAGC   180
          R  P  E  A  K  S  A  Q  P  A  D  G  W  K  G  E  R  P  R  S
          GAGGAGGATAACGAGCTGAACCTCCCTAACCTGGCAGCCGCCTACTCGTCCATCCTGAGC   240
          E  E  D  N  E  L  N  L  P  N  L  A  A  A  Y  S  S  I  L  S
          TCGCTGGGCGAGAACCCCCAGCGGCAAGGGCTGCTCAAGACGCCCTGGAGGGCGGCCTCG   300
          S  L  G  E  N  P  Q  R  Q  G  L  L  K  T  P  W  R  A  A  S
          GCCATGCAGTTCTTCACCAAGGGCTACCAGGAGACCATCTCAGATGTCCTAAACGATGCT   360
          A  M  Q  F  F  T  K  G  Y  Q  E  T  I  S  D  V  L  N  D  A
          ATATTTGATGAAGATCATGATGAGATGGTGATTGTGAAGGACATAGACATGTTTTCCATG   420
          I  F  D  E  D  H  D  E  M  V  I  V  K  D  I  D  M  F  S  M
          TGTGAGCATCACTTGGTTCCATTTGTTGGAAAGGTCCATATTGGTTATCTTCCTAACAAG   480
          C  E  H  H  L  V  P  F  V  G  K  V  H  I  G  Y  L  P  N  K
          CAAGTCCTTGGCCTCAGCAAACTTGCGAGGATTGTAGAAATCTATAGTAGAAGACTACAA   540
          Q  V  L  G  L  S  K  L  A  R  I  V  E  I  Y  S  R  R  L  Q
          GTTCAGGAGCGCCTTACAAAACAAATTGCTGTAGCAATCACGGAAGCCTTGCGGCCTGCT   600
          V  Q  E  R  L  T  K  Q  I  A  V  A  I  T  E  A  L  R  P  A
hGCH-1    GGAGTCGGGGTAGTGGTTGAAGCAACACACATGTGTATGGTAATGCGAGGTGTACAGAAA   660
          G  V  G  V  V  V  E  A  T  H  M  C  M  V  M  R  G  V  Q  K
2                              ACGTCTGCAGAACCATAGCTTCCACGCACCTGAACG
                               T  S  A  E  P
3                              ACGTAAGTCTGCATCTGCCTTTAGTAACGTCATAAT
                               T

hGCH-1    ATGAACAGCAAAACTGTGACCAGCACAATGTTGGGTGTGTTCCGGGAGGATCCAAAGACT   720
          M  N  S  K  T  V  T  S  T  M  L  G  V  F  R  E  D  P  K  T
2         AGCACAGAATGAACTGACGGTGGAAGACATTATGAGCTGTGTCCAACGTTTTAACCAAAG
3         GGTGCACTAGAAGTGATCTTGCTATTTAGTGCTTCTCATATTTTGTAGCACCAGGTGATG

hGCH-1    CGGGAAGAGTTCCTGACTCTCATTAGGAGCTGAGCTTCATTCAGTGTGTGTGCGTTGGTT   780
          R  E  E  F  L  T  L  I  R  S
2         CGTATCGTACCAACGATCTGTGAAAATGCACTGGAAGCTTCTGGTCCCGGTTTCCTTTGT
3         CCACACAACTGATATGATAACTGTAGATTTCG-3'

hGCH-1    GCCGATCGTACTGCCAGTAGCATTGTCTGTCTGTCCGGTCTTGTTTGTACATTCCATTTT   840
2         GGTCTATGTGGGTCTTGTCCTCATTGTAACTCCGTATATATGGTATAGGTATTTTAATCC

hGCH-1    CAATTGTTACAGATGTGAACTTTATTCCTTGTCACTAATTATATTTAAAATTATTTCTAG   900
2         TGGAAGCTGTTGCCTTATTAATGATTATCTTAAAATTTCCTCCATTGGGGCAGCGTGGGC

hGCH-1    GAAGTCAAAAAAATATAATAAAGGGTTGAGCCCTCTACTTTCTTCTTGCCACCTTTTTGT   960
2         CAAATTAAAACAAACAAAACCGCAACG-3'

hGCH-1    GGCAATATTAAAGTGAACTGCTAATAGTGTAAGTACGTGCACAAAACCACTGCCAGATAA  1020
hGCH-1    CCAGAGGGGCCTGGGAAGGGAGAAGAATTAGTGTATTTTTTTCAAATAGTACAGTAATTT  1080
hGCH-1    GCCTCATAAGCATAGGAGCATTGGGAATGAGAGGGAACTGTGCCCAGTATACTGTTTTTT  1140
hGCH-1    TTCTTCCTCCAATAAAAGTGGTGTGGTGCCGAAAGTGCTAAAATATTTAGTGCGGTATTG  1200
hGCH-1    CTCTGT-3'
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Fig.2. Nucleotide and deduced amino acid sequences of human GTP cyclohydrolase I cDNAs (hGCH-1, 2, and 3). The amino acid sequences are shown below the nucleotide sequences by one-letter code. The nucleotide sequences of hGCH-1, hGCH-2, and hGCH-3 were 1181 base pair (nucleotides 26 to 1206), 994 bp (-67 to 927), and 819 bp (-67 to 752), respectively. Termination codons are underlined. In the common sequence region (nucleotides -67 to 624), the 44th nucleotide in hGCH-3 was adenine and was different from that in hGCH-1 and hGCH-2, as shown with asterisk. The difference caused an amino acid change between Gly and Asp. The sequences in shaded boxes were used in RT-PCR analysis.

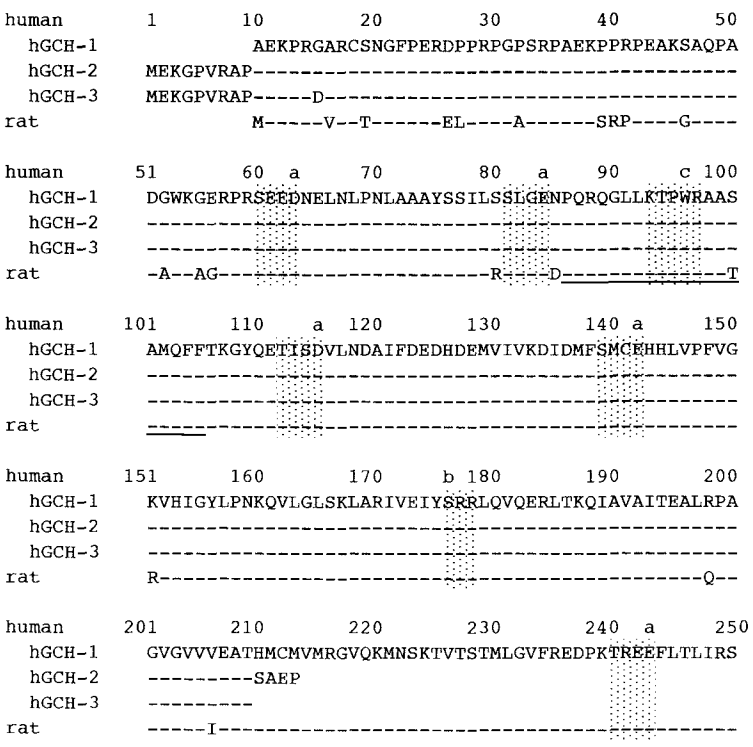

Fig.3. Comparison of the amino acid sequence of human and rat GTP cyclohydrolase I. The predicted amino acid sequence is shown by one-letter code. Identical residues are expressed by hyphens. A putative pterin binding site (13) is underlined. The possible phosphorylation sites for casein kinase II (a), protein kinase C (b) , and growth-associated histone HI kinase (c) are shown in shaded rectangles. Consensus sequences of casein kinase II, protein kinase C and growth-associated histone HI kinase are (S/T)XX(D/E)(14,15), (S/T)X(R/K)(16,17) and K(S/T)PXK (with the terminal K replaced by R in GTP cyclohydrolase)(11,18), respectively.

A comparison of the predicted amino acid sequences of human GTP cyclohydrolase I with that of the rat enzyme (13) is shown in Fig.3. The identity of hGCH-1, hGCH-2, and hGCH-3 with the rat sequence were 92.5%, 91.0% and 90.5%, respectively. hGCH-1 is comparable to the reported rat enzyme (13). In comparison with rat sequence, however, it was noted that an additional 9 amino acids at N-terminus existed in human sequence. Three sequences of hGCH-1, hGCH-2, and hGCH-3 conserved the possible phosphorylation sites for casein kinase II, protein kinase C, and growth-associated histone HI kinase. The corresponding sequence to a putative pterin binding site suggested previously (13) are also observed with high conservation. The C-terminal region including a hydrophobic peptide sequence in hGCH-1 and also in rat GTP cyclohydrolase I was deleted in hGCH-2 and hGCH-3. It may be necessary to examine the kinetic characteristics of the iso-enzymes, in order to explore the role of the hydrophorbic sequence region in regulation of the enzyme activity. Previously, Schoedon *et al.* (21) reported that the purified human GTP cyclohydrolase I appears to be composed of eight 50 kDa subunits as estimated from SDS/PAGE. In the present study, however, the molecular mass of hGCH-1,

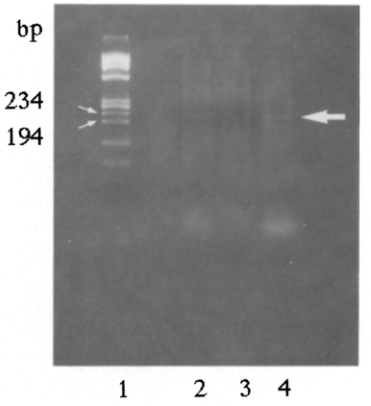

Fig.4. Agarose gel electrophoresis of human GTP cyclohydrolase I cDNAs amplified by PCR. RT-PCR analysis of RNA isolated from human liver was carried out with following primer set. As sense primer, 5'-TTGGTTATCTTCCTAACAAG-3'(primer 1) in common regions of hGCH-cDNAs (Fig.2) was used. 5'-GTGCTGGTCACAGTTTTGCT-3' (primer 2), 5'-TCTT CCACCGTCAGTTCATT-3'(primer 3) or 5'-AGCAAGATCACTTCTAGTGC-3' (primer 4), which is complementary to nucleotide indicated in Fig.2, was used as antisense primer of hGCH-1, hGCH-2, or hGCH-3 cDNA. Amplified cDNA fragments are indicated by an arrow. Lane 1, φX/HaeIII marker; lane 2, hGCH-3 detected with primer 1 and primer 4; lane 3, hGCH-2 detected with primer 1 and primer 3; lane 4, hGCH-1 detected with primer 1 and primer 2.

hGCH-2, and hGCH-3 were calculated to be 27,903, 23,515, and 23,189 daltons for the protein, respectively.

The occurence of multiple mRNAs in human liver was confirmed by RT-PCR analysis using a synthetic primer set. As shown in Fig.4, the corresponding mRNAs to hGCH-1, hGCH-2, and hGCH-3 were detected in human liver. Among these mRNAs, hGCH-1 was most abundant in liver from adult human. Both hGCH-2 and hGCH-3 also detected in liver slightly but substantially. It is well known that GTP cyclohydrolase I are widely distributed in the tissues and fluids of several mammalian species (22) and are involved in several distinct function (1-8). To understand the physiological significance of the multiplicity of human GTP cyclohydrolase I, it may be appropriate to examine the tissue and species specificity for the distribution of the multiple mRNAs.

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