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CLONING AND NUCLEOTIDE SEQUENCE OF A FULL-LENGTH cDNA FOR HUMAN LIVER γ-GLUTAMYLCYSTEINE SYNTHETASE[1]

Jerry J. Gipp, Chawnshang Chang, and R. Timothy Mulcahy[2]

Department of Human Oncology, University of Wisconsin-Madison
Madison, Wisconsin 53792

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SUMMARY: We have cloned and sequenced a full-length cDNA for human liver γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in glutathione biosynthesis. The cDNA consists of 2634 bp containing an open reading frame encoding a protein of 367 amino acids and having a calculated M_r = 72,773. The nucleotide sequence of the cDNA for human liver GCS shares an 84% overall similarity with the composite rat GCS sequence deduced from three overlapping partial cDNAs (Yan and Meister, JBC 265: 1588-1593, 1990). The deduced amino acid sequences are 94% similar. Comparison of Northern blots of total RNA isolated from rat kidney or liver with that from human kidney revealed the GCS mRNA to be larger in the human tissue (~4.0 kb vs. ~3.7 kb). (The sequence for the human liver GCS cDNA has been assigned accession number M90656 in GenBank/EMBL databases.)

Glutathione (L-γ-glutamyl-cysteinyl-glycine; GSH) is a ubiquitous non-protein thiol involved in cellular metabolism, maintenance of cellular redox state, protection against oxidative injury and the detoxification of xenobiotics (1). Elevation of intracellular GSH has also been implicated in the resistance of human tumor cells to certain chemotherapeutic agents (2-5). Although GSH elevations are commonly detected in drug-resistant tumor cells, the mechanism(s) responsible for the increase has not been determined. This is at least partially attributable to a lack of appropriate molecular reagents for examining the expression and regulation of key enzymes involved in GSH homeostasis; thus prompting the current investigation.

Glutathione is synthesized from its constituent amino acids in two sequential ATP-requiring reactions catalyzed by γ-glutamylcysteine synthetase (GCS) and glutathione synthase, respectively (1). Under normal conditions, the upper limit of intracellular GSH concentration is regulated by GSH inhibition of GCS, the rate-limiting step in de novo GSH biosynthesis (1,6).

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[2]To whom correspondence should be addressed at K4/338, 600 Highland Avenue, Madison, WI 53792.

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γ-Glutamylcysteine synthetase purified from rat kidney can be dissociated into two distinct subunits of M_r 73,000 and 27,700 (7). The larger subunit possesses all the catalytic activity of the isolated protein and can be inhibited by GSH (7). Recently, Yan and Meister (8) determined the nucleotide sequence of the M_r 73,000 subunit of rat kidney GCS following isolation and sequencing of three overlapping partial cDNA clones. The amino acid sequence derived from these cDNAs showed little overall (8%) similarity with the sequence for E. coli GCS deduced from sequencing of genomic DNA (9).

In this report, we describe the cloning and sequencing of a 2634 bp full-length cDNA for GCS isolated from an adult human liver cDNA library. The sequence of the human GCS cDNA and the deduced amino acid sequence of the corresponding enzyme are compared with those of the rat. Hybridization of total RNA isolated from rat kidney and liver and human liver with probes prepared from both the rat and human sequences was compared.

MATERIALS AND METHODS

Isolation of cDNA clones and DNA sequencing. A λgt11 cDNA library was constructed using poly(A)$^+$ RNA isolated from human kidney. A 1010 bp probe ($rkGCS_{PCR}$), corresponding to nucleotides 51-1061 of the rat GCS (8) sequence, was generated from rat kidney RNA by polymerase chain reaction using synthetic 20-mer oligonucleotide primers corresponding to nucleotides 51-70 and 1042-1061 of the rat sequence. The probe was radiolabeled with ^{32}P using the random primer technique and used to screen the human kidney library (10). cDNA was prepared from phage stock by a plate lysis method (10).

Following EcoR1 digestion of phage DNA, a partial (1679 bp) cDNA insert (hkGCS-1) was isolated from low melting agarose gels, purified by phenol/chloroform extraction and cloned into the EcoR1 site of the phagemid pSK-Bluescript. The nucleotide sequence was determined using Sequenase (U.S Biochemicals) according to the manufacturer's instructions, using T3, SK, T7 and KS primers as well as synthetic primers corresponding to internal GCS sequences. Nucleotide sequence was verified by bi-directional sequencing reactions. Sequence analysis was conducted using DNASTAR (Madison, WI) software.

An 0.8 kb PstI fragment isolated from hkGCS-1 (Figure 1) was labeled with ^{32}P and subsequently used to screen a human liver cDNA library (Stratgene).

Northern analysis. Total RNA was isolated from rat kidney and rat liver by the guanidinium method according to Chomczynski and Sacchi (11). Human kidney RNA was purchased from Clontech. Northern blots were prepared according to Sambrook, Fritsch and Maniatis (10).

RESULTS AND DISCUSSION

Initially, four clones were isolated from the human kidney cDNA library following screening with the radiolabeled $rkGCS_{PCR}$ probe (solid box in rkGCS, Figure 1). However, of the four only one, the 1679 bp hkGCS-1, shared significant sequence homology with the published rat GCS sequence. Comparison with the rat sequence revealed that hkGCS-1 consisted of coding sequences for 387 amino acids on the carboxyl end of the protein and 398 nucleotides

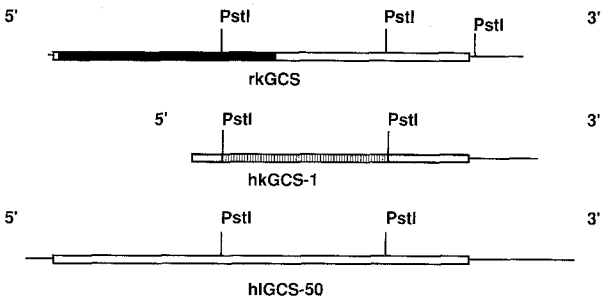

Figure 1. Restriction maps for rat (rkGCS) kidney cDNA, human kidney clone hkGCS-1 and the full-length GCS cDNA clone, hlGCS-50 isolated from human liver. Boxes represent open reading frame; the solid box in rGCS corresponds to the PCR fragment ($rkGCS_{PCR}$) used to screen the human kidney cDNA library. The striped box in hkGCS-1 corresponds to the 0.8 kb PstI fragment utilized to screen the human liver library.

of non-translated sequence containing a putative polyadenylation signal (AATAAC) (8). Repeated attempts to clone a full-length cDNA from the human kidney library were unsuccessful so the cloning strategy was modified to include screening of a human liver library. An 0.8 kb PstI fragment (stripped box in hkGCS-1, Figure 1) corresponding to sequences within the open reading frame of hkGCS-1 were used to identify positive clones. A total of 50 clones were isolated from the liver cDNA library and subjected to restriction analysis. Of these positive clones, only one, hlGCS-50, contained a full length insert. A schematic representation of the rat GCS cDNA, hkGCS-1, and hlGCS-50 are shown in Figure 1.

The nucleotide sequence of the sense strand of hlGCS-50 and the deduced amino acid sequence are compared with the corresponding rat sequences in Figure 2. The human cDNA consists of 92 bp of untranslated 5' sequence, a 1911 bp open reading frame starting at the ATG codon at position 93 and 628 bp of 3' sequence. The two cDNAs share an 89% nucleotide sequence similarity within the open reading frame, and 84% overall. The sequence AATAAC, suggested to be the putative polyadenylation signal in the rat GCS cDNA (8), was located at nucleotides 2221-2226 in hlGCS-50. However, the polyadenylation concensus sequence, AATAAA, was identified at nucleotides 2493-2498 in the distal 3' region of hlGCS-50; a sequence extending beyond that included in the rat cDNA as reported by Yan and Meister (8).

Both the rat and human cDNAs contain an open reading frame 1911 bp in length, encoding proteins of 637 amino acid residues. The calculated molecular weight of the protein encoded by hlGCS-50 is 72,773 Daltons, in good agreement with the estimated molecular weight of the large subunit of rat GCS (M_r=73,000). The deduced amino acid sequences share 94% similarity with the deduced sequence for the rat enzyme. These results indicate that, unlike the situation with E. coli GCS (8), a high degree of conservation exists between the human and rat proteins.

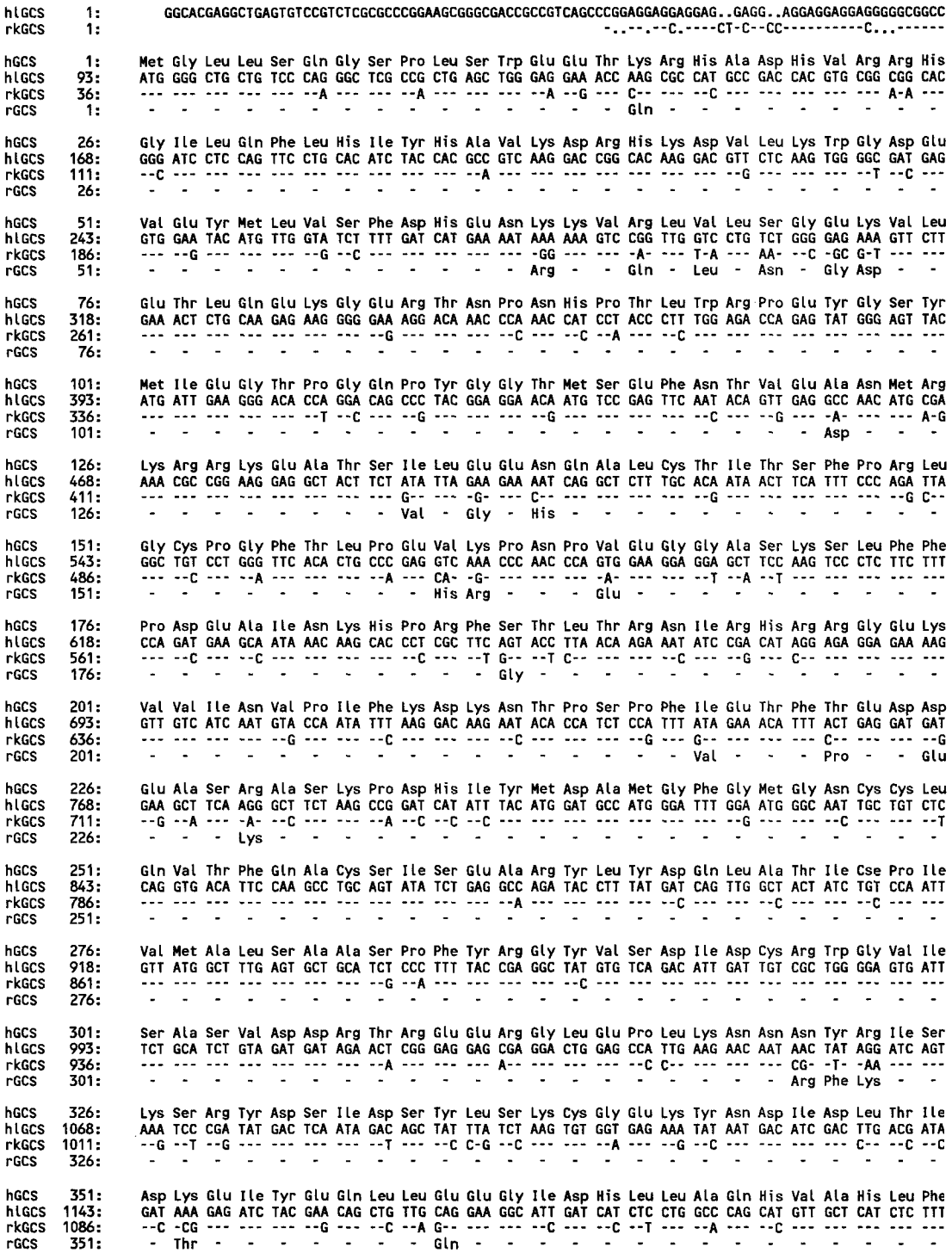

Figure 2. cDNA sequences and deduced amino acid sequences for rkGCS and hlGCS-50. The nucleotide and amino acid sequences are numbered on the left. The rkGCS residues that are identical to corresponding residues in hlGCS-50 are indicated by hyphens (-). Missing residues and gaps are shown as dots (.). * indicates the stop codon. The sequence for the human GCS cDNA has been assigned accession number M90656.

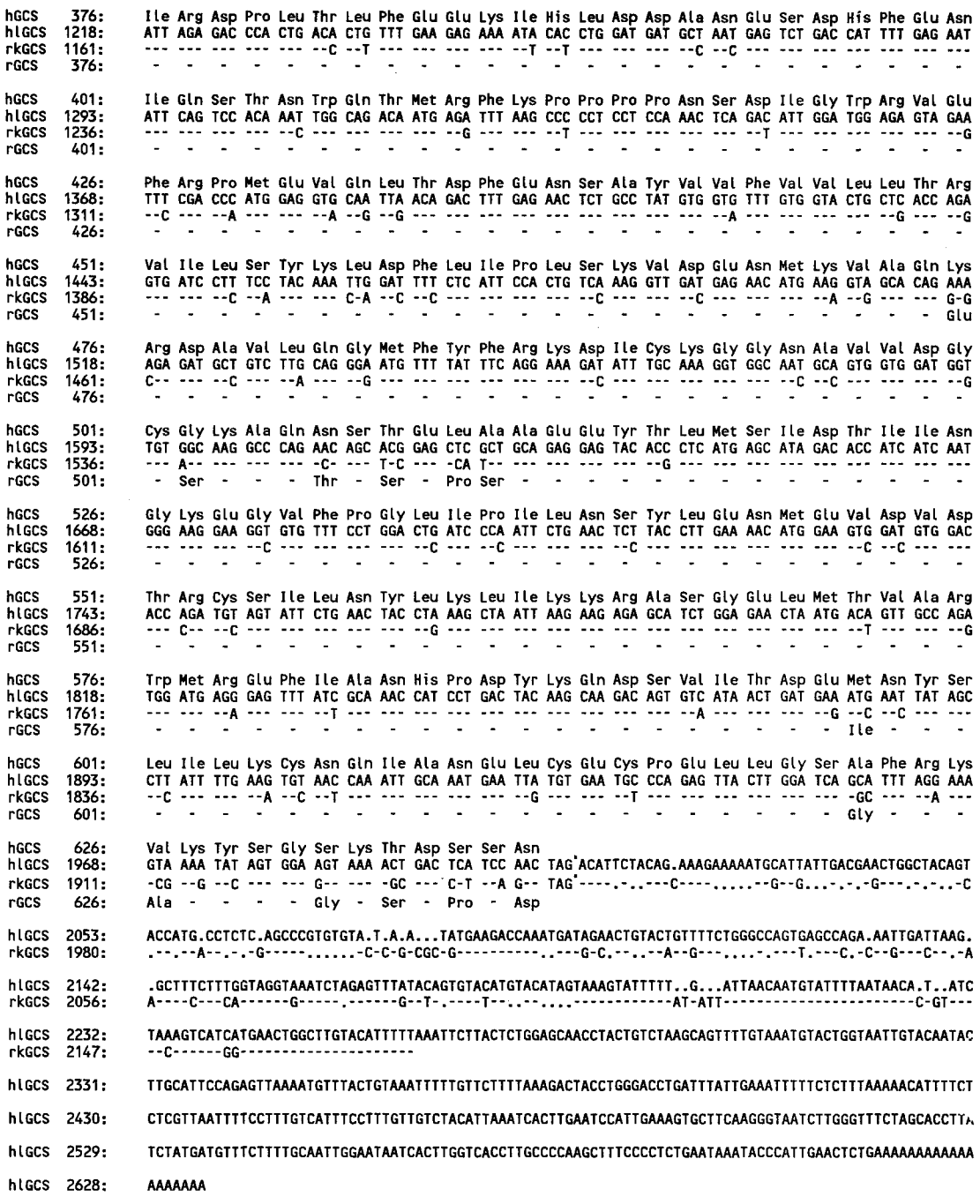

Figure 2-Continued

The 1.0 kb rkGCS$_{PCR}$ fragment and hkGCS-1 cDNA were used to probe Northern blots of total RNA isolated from rat kidney, rat liver and human kidney. Both probes identified similar RNA species (Figure 3). GCS mRNA was more abundant in rat kidney than in rat liver, consistent with previous observations (8). Both probes hybridized with a single 4.0 kb band in

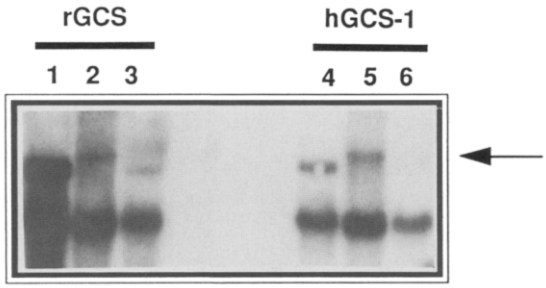

Figure 3. Northern blot analysis of total RNA isolated from rat kidney (lanes 1 and 4), rat liver (lanes 2 and 5) and human kidney (lanes 3 and 6). Ten μg of total RNA was loaded per lane. Lanes 1-3 were probed with the 1.0 kb $rkGCS_{PCR}$ fragment while lanes 4-6 were probed with hkGCS-1. The arrow indicates the position of the human kidney GCS message. The lower band in each of the lanes corresponds to β-actin message.

RNA isolated from human kidney. This RNA was approximately 300 base pairs larger than the species identified in the RNA isolated from the rat tissues. This species-specific size differential is similar to that reported for γ-glutamyl transpeptidase, another GSH metabolizing enzyme (12).

Glutathione plays a prominent role in cellular homoestasis and is particularly important in the response of cells to various types of injurious agents. The ability of cells to survive exposure to xenobiotics capable of inducing oxidative damage, ionizing radiation, heavy metals or alkylating agents has been correlated with intracellular levels of GSH. While the regulation of GSH levels in response to these insults has not been defined, there is ample evidence in the toxicological literature to support the role of GCS in many GSH-related adaptive responses (13-16). Defects in GSH synthesis and/or metabolism, particularly in red blood cells, have also been associated with various disease states (17). The availability of a full-length cDNA clone for human GCS will permit the investigation of GSH regulation at the molecular level; an approach which should provide insights to alternative means of manipulating GSH levels to experimental and therapeutic advantage.

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