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Characterization and sequence of a cDNA clone of gamma-glutamyltranspeptidase

Julio Coloma and Henry C.Pitot

McArdle Laboratory for Cancer Research, The Medical School, University of Wisconsin, Madison, WI 53706, USA

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ABSTRACT

We have isolated several cDNA's complementary to gamma-glutamyltranspeptidase (GGT) mRNA by screening a rat kidney library constructed in λgt11 with antibodies specifically reactive to the enzyme protein. The clone selected an mRNA that was translated into a 62 Kd peptide, corresponding to the GGT precursor. The longest clone isolated was 1842 bp long with an open reading frame coding for 565 amino acids. The length of the mRNA coding for GGT was estimated to be 2.2 kb long. The amino acid sequence derived from the nucleotide sequence matched the short sequences determined by us as well as by other authors.

INTRODUCTION

The characteristics, function, and possible role in carcinogenesis of the enzyme gamma-glutamyltranspeptidase have been the subject of a number of investigations during the past decade. The enzyme is found to be membrane-associated and widely distributed in adult, fetal, and neoplastic tissues (1). The enzyme is anchored to the membrane through the amino terminal portion of its heavy subunit (2). In both intestine (3) and kidney (4), the active site of the enzyme faces the luminal surface of the glandular cell in which it occurs. Multiple forms of the enzyme have been described both in humans (5) and in animals (6). Other evidence (8-10) has indicated that such multiple forms may be related to the carbohydrate moiety of the mature glycoprotein. The function of the enzyme in the cellular economy involves transpeptidation (11), and it has been suggested that this function serves to aid in the cellular synthesis of glutathione, especially noticeable in hepatocytes subjected to chemical toxicity (12).

The enzyme itself consists of two polypeptide chains, which are synthesized as a precursor form of a single polypeptide of 78,000 daltons, including the carbohydrate portion of the molecule (13-15). In vitro translation studies reveal that the precursor polypeptide itself has a molecular weight of 63,000 daltons (15). The presence of the enzyme has been used as a marker for the

appearance of preneoplastic foci in rat liver following the administration of various carcinogenic agents (12). In order to determine whether the appearance of the enzyme in early preneoplastic lesions represents the appearance of enzyme otherwise masked in normal hepatocytes or activation of genetic expression at the transcriptional and/or translational level, we have isolated a cDNA clone from a λgtll library of cDNAs of polyadenylated RNAs of rat kidney. Before this clone is used to study questions related to genetic expression, it has been characterized, this characterization being the subject of this paper.

MATERIALS AND METHODS

A rat kidney cDNA library constructed in phage λ gtll was kindly provided by Dr. M. Mueckler of the Whitehead Institute at M.I.T. GGT was purified from rat kidney after solubilization with Triton X-100 according to the procedure of Hughey and Curthoys (16). Antibodies against GGT were raised in rabbits and affinity-purified by absorption onto a GGT-Sepharose 4B column.

Screening of the library. Phages expressing GGT were identified by a modification of the method of Young and Davis (17,18). Approximately 30,000 recombinant phage particles were plated per 9-cm LB plate containing E. coli Y1090. Plaques were induced by incubation at 42°C for 4 hr. Plates were overlaid with nitrocellulose filters (BA85, Schleicher and Schuell) previously soaked in 10 mM isopropylthiogalactoside, and incubated a further 6 hr at 37°C. The filters were blocked and incubated overnight with the affinity-purified antibody (50 μg/ml). Positive plaques were located by the peroxidase-anti-peroxidase staining techniques (19). Positive plaques were picked up, and successive screening rounds were carried out with fewer and fewer phages at each step. After the third round, all the plaques were positive and well separated from one another.

Purification of inserts. Positive recombinants were grown in NZCYM medium, the phages isolated by CsCl gradient equilibrium centrifugation, and the DNA was purified by the SDS-EDTA protocol (20). cDNA inserts were purified by preparative agarose gel electrophoresis of the EcoRI digests of the recombinant phage DNA and subcloned in the EcoRI site of pBR322. Plasmids containing inserts were used to transform E. coli DH-1 by the $CaCl_2$/RbCl procedure (21). Cells carrying plasmids with inserts were grown on a large scale, the DNA was isolated by the alkaline lysis method (22), and the plasmid was purified by centrifugation in a CsCl/ethidium bromide equilibrium gradient.

Hybrid select translation. 10 μg of different clones were spotted on a small piece of nitrocellulose filter after being denatured in 0.5 M NaOH for

20 min at room temperature and neutralized in 0.2 NaCl/60 mM sodium citrate/ 0.1 M Tris pH 7.4. The filters were baked at 80°C under vacuum for 2 hr.

RNA was isolated from rat kidney by the guanidinium thiocyanate/CsCl centrifugation procedure (23,24). Poly(A)RNA was selected by two cycles of chromatography on oligo-dT cellulose (Collaborative Research, type III). Poly(A)RNA was hybridized with the cDNAs in 65% deionized formamide, containing 20 mM PIPES pH 6.4, 0.2% SDS, 0.4 M NaCl, and 100 µg/ml of tRNA, at 70°C for 10 min, followed by 3 hr at 50°C. Filters were washed ten times with 10 mM Tris pH 7.6, 0.15 M NaCl, 0.5% SDS, and 1 mM EDTA at 65°C, and three times with the same buffer without SDS. The RNA was eluted with a small volume of water containing 0.1 mg/ml of tRNA at 100°C for 1 min. After quick freezing in a dry ice/ethanol bath, the RNA was ethanol-precipitated.

The RNA was translated in a rabbit reticulocyte system (Promega Biotec, Madison, WI) in the presence of 1 µCi of ^{35}S-methionine. Translated products were immunoprecipitated as described (25), fractionated in a 10% SDS-polyacrylamide gel, and detected by fluorography.

Northern hybridization. 1 µg of rat kidney poly(A)RNA was denatured by heat at 55°C for 15 min in 2.2 M formaldehyde/50% formamide and fractionated by electrophoresis in a 1.4% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to a nitrocellulose filter and probed with a ^{32}P-labeled GGT clone (26). Mammalian and E. coli ribosomal RNAs were used as markers.

Restriction map. The locations of restriction enzyme cleavage sites were determined by digestion of the cDNAs with different restriction enzymes under conditions specified by the manufacturers (New England Biolabs, Promega Biotec). The size of the DNA fragments was determined by agarose gel electrophoresis.

Nucleotide sequence determination. Restriction fragments of different clones were subcloned into M13 mp18 and M13 mp19 vectors (27) and sequenced by the dideoxynucleotide chain terminator method (28) with a cloning and sequencing kit from New England Biolabs (Beverly, MA). Sequencing reactions were carried out with deoxyadenosine 5' (α-^{35}S triphosphate) (Amersham) and run on 8% polyacrylamide/7 M urea gels. The DNA sequence was analyzed with a computer program developed by the University of Wisconsin Genetics Computer Group.

Amino acid sequence determination. 0.5 mg of pure GGT was fractionated in a 10% preparative SDS-polyacrylamide gel. The band corresponding to the light subunit was cut and the protein recovered by electroelution. Amino acid analysis of the aminoterminal portion of the small subunit was performed with Applied Biosystems Protein Sequencer Model 478 (Foster City, CA), under the

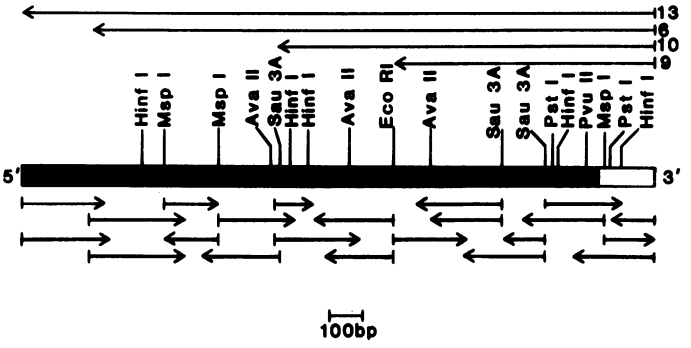

Figure 1. Partial restriction map and nucleotide sequencing strategy of GGT cDNA inserts. Horizontal arrows indicate the direction and extent of each sequence determination. EcoRI, Msp I, and Sau 3A restriction sites were used in different determinations. Long arrows in the upper part of the figure denote the length of the different clones. The solid black portion of the 5'-3' rectangle indicates the coding region.

supervision of Dr. R. Niece at the Biotechnology Center of the University of Wisconsin, Madison, WI.

RESULTS

In a primary screening of the cDNA library, 18 plaques out of 200,000 produced strong positive signals. All 18 positives were purified by three successive rounds of screening. The size of the inserts ranged from 0.7 to 1.8 kb. Most of the clones extended 740 bp from the 3' end to an internal EcoRI site. Only three clones--6, 10, and 13--contained a fragment beyond the EcoRI site, ranging 960, 340, and 1,100 bp respectively (Figure 1).

The cDNAs were further identified as GGT clones by analysis of the products obtained after the translation of the RNA selected by hybridization. Even though the incorporation of ^{35}S-methionine into the immunoprecipitable material was very low, a band was clearly visible after fractionation on an SDS-polyacrylamide gel (Figure 2). This protein had an estimated molecular weight of 62 kd, corresponding to the size of the single polypeptide precursor described for GGT (14,15). The low level of ^{35}S-methionine incorporation seems to be related to the high instability of the GGT mRNA (S. Thorgeirsson, personal communication).

Length of GGT mRNA. Poly(A)RNA was denatured and fractionated in a formaldehyde agarose gel as described in Materials and Methods. The hybridization was carried out with two different nick-translated probes, representing the

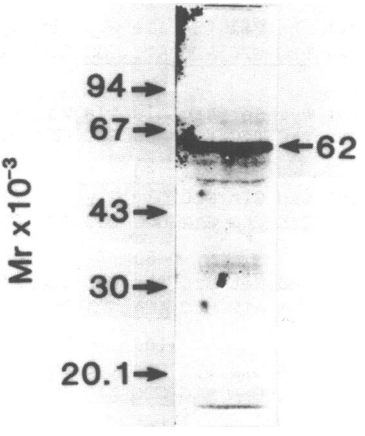

Figure 2. SDS-PAGE analysis of translation products from mRNA hybrid selected with cDNA. Clone 6 was bound to nitrocellulose and hybridized with kidney poly(A)RNA. Eluted RNA was translated in a rabbit reticulocyte lysate system. Products immunoprecipitated with affinity-purified GGT antibodies were fractionated in a 10% polyacrylamide gel. Molecular weight markers are indicated by the arrows.

two different EcoRI fragments of the GGT clone. As shown in Figure 3, both probes hybridized with the same single RNA species. No other bands were visible. The size of this RNA was estimated to be 2.2 kb in length.

Nucleotide and amino acid sequence of the GGT clones. Figure 1 shows the restriction map of the GGT cDNAs and the strategy used in the sequence procedure. The complete nucleotide- and amino-acid derived sequences are shown in Figure 4.

The longest clone isolated, 1,842 bp in length, included an open reading frame of 1,695 bp coding for 565 amino acid residues, a termination codon, and a 3' flanking region of 138 bp, containing 7 adenine residues of the poly(A) tail and the putative polyadenylation sequence AATAAA (29). Five possible N-

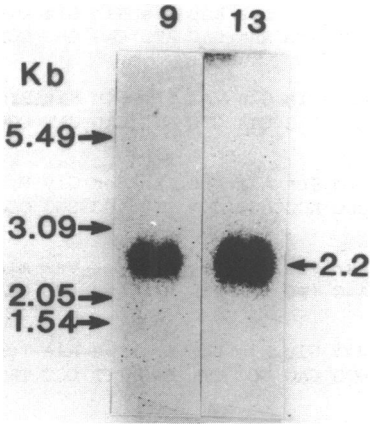

Figure 3. Estimation of the size of the mRNA coding for GGT. Kidney poly(A)RNA was blotted on nitrocellulose after being denatured and fractionated as described in Materials and Methods. Nick-translated clones 9 and 13 were used as probes. Molecular size markers are indicated by arrows.

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                          10                                  20
Arg Phe Leu Val Leu Gly Leu Val Ala Val Val Leu Val Phe Val Ile Ile Gly Leu Cys
CGG TTT CTG GTG CTG GGC CTG GTG GCG GTG GTT CTG GTG TTC GTC ATC ATC GGC CTC TGC

                          30                                  40
Ile Trp Leu Pro Thr Thr Ser Gly Lys Pro Asp His Val Tyr Ser Arg Ala Ala Val Ala
ATC TGG CTA CCC ACC ACC TCT GGG AAG CCT GAC CAT GTG TAC TCC AGG GCG GCC GTG GCC

                          50                                  60
Thr Asp Ala Lys Arg Cys Ser Glu Ile Gly Arg Asp Met Leu Gln Glu Gly Gly Ser Val
ACA GAT GCC AAG CGT TGC TCA GAG ATT GGG CGG GAT ATG CTA CAG GAA GGC GGC TCC GTA

                          70                                  80
Val Asp Gly Pro Ser Gln Ala Cys Cys Val Trp Gly Ser Leu Met Pro Thr Val Trp Ala
GTG GAC GGG CCA TCG CAA GCC TGC TGT GTA TGG GGC TCA TTA ATG CCC ACA GTA TGG GCA

                          90                                 100
Ser Gly Ala Ala Ser Ser Ser Pro Ser Thr Thr Ala Pro His Glu Lys Leu Lys Leu Ser
TCG GGG GCG GCC TCT TCT TCA CCA TCT ACA ACA GCA CCA CAC GAA AAG CTG AAG TTA TCA

                         110                                 120
Met Pro Val Lys Trp Leu Pro Gly Trp Pro Ile Pro Ala Cys Ser Ile Ile Leu Arg Thr
ATG CCC GTG AAA TGG CTC CCA GGT TGG CCA ATA CCA GCA TGT TCA ATA ATT CTA AGG ACT

                         130                                 140
Leu Lys Lys Glu Ala Phe Gln Trp Gln Phe Leu Gly Glu Ile Arg Gly Tyr Glu Leu Ala
CTG AAG AAG GAG GCC TTT CAG TGG CAG TTC CTT GGT GAA ATC CGT GGC TAT GAG CTG GCA

                         150                                 160
His Gln Arg His Gly Arg Leu Pro Trp Ala Arg Leu Phe Gln Pro Ser Ile Gln Leu Ala
CAC CAA CGG CAT GGC CGG CTA CCC TGG GCT CGC CTC TTC CAA CCC AGC ATC CAA CTG GCT

                         170                                 180
Arg His Gly Phe Pro Val Gly Lys Gly Leu Ala Arg Ala Leu Asp Lys Lys Arg Asp Ile
CGC CAT GGC TTC CCT GTG GGC AAG GGC TTG GCA AGA GCC TTG GAC AAA AAA CGG GAC ATC

                         190                                 200
Ile Glu Lys Thr Pro Ala Leu Cys Glu Val Phe Cys Arg Gln Gly Lys Val Leu Gln Glu
ATT GAG AAG ACA CCT GCT TTG TGC GAG GTG TTC TGC CGG CAA GGG AAG GTG CTT CAG GAA

                         210                                 220
Gly Glu Thr Val Thr Met Pro Lys Leu Ala Asp Thr Leu Gln Ile Leu Ala Gln Glu Gly
GGA GAG ACA GTA ACT ATG CCG AAG TTG GCC GAT ACG TTG CAA ATA CTG GCC CAG GAA GGG

                         230                                 240
Ala Arg Ala Phe Tyr Asn Gly Ser Leu Thr Ala Gln Ile Val Lys Asp Ile Gln Glu Ala
GCC AGG GCC TTC TAC AAT GGG AGC CTC ACA GCC CAG ATT GTG AAA GAC ATC CAG GAG GCT

                         250                                 260
Gly Gly Ile Met Thr Val Glu Asp Leu Asn Asn Tyr Arg Ala Glu Val Ile Glu His Pro
GGG GGC ATT ATG ACG GTT GAG GAC CTT AAC AAC TAT CGT GCG GAA GTG ATC GAG CAT CCG

                         270                                 280
Met Ser Ile Gly Leu Gly Asp Ser Thr Leu Tyr Val Pro Ser Ala Pro Leu Ser Gly Pro
ATG AGC ATC GGC CTC GGG GAC TCC ACC CTG TAC GTG CCC AGC GCC CCA CTC AGC GGG CCC

                         290                                 300
Val Leu Ile Leu Ile Leu Asn Ile Leu Lys Gly Tyr Asn Phe Ser Pro Lys Ser Val Ala
GTG CTG ATT CTC ATC TTG AAC ATC CTC AAA GGA TAC AAC TTC TCT CCA AAG AGC GTG GCA

                         310                                 320
Thr Pro Glu Gln Lys Ala Leu Thr Tyr His Arg Ile Val Glu Ala Phe Arg Phe Ala Tyr
ACC CCA GAA CAG AAG GCG CTG ACG TAT CAC CGT ATC GTG GAG GCC TTT CGG TTT GCC TAT
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                          330                                   340
Ala Lys Arg Thr Met Leu Gly Asp Pro Lys Phe Val Asp Val Ser Gln Val Ile Arg Asn
GCC AAG AGG ACC ATG CTC GGT GAC CCA AAG TTT GTC GAT GTG TCT CAG GTC ATC CGC AAC

                          350                                   360
Met Ser Ser Glu Phe Tyr Ala Thr Gln Leu Arg Ala Arg Ile Thr Asp Glu Thr Thr His
ATG AGT TCT GAG TTC TAC GCT ACT CAG CTT CGA GCC CGC ATC ACT GAT GAA ACC ACT CAC

                          370                                   380
Pro Thr Ala Tyr Tyr Glu Ala Glu Phe Tyr Leu Pro Asp Asp Gly Gly Thr Ala His Leu
CCA ACC GCC TAC TAT GAG GCT GAA TTC TAC CTT CCA GAC GAT GGG GGT ACC GCT CAC CTG

                          390                                   400
Ser Val Val Ser Glu Asp Gly Ser Ala Val Ala Ala Thr Met Thr Ile Asn Leu Tyr Phe
TCC GTG GTT TCC GAG GAT GGC AGT GCT GTG GCC GCC ACC ATG ACC ATC AAC CTC TAC TTT

                          410                                   420
Gly Ser Lys Val Leu Ser Arg Val Ser Gly Ile Leu Val Asn Asp Glu Met Asp Asp Phe
GGC TCC AAG GTC CTC TCT CGG GTC AGT GGC ATC CTT GTT AAT GAC GAG ATG GAT GAC TTC

                          430                                   440
Ser Ser Pro Asn Phe Thr Asn Gln Phe Gly Val Ala Pro Ser Pro Ala Asn Phe Ile Lys
AGC TCG CCC AAC TTC ACC AAC CAG TTT GGG GTA GCG CCC TCA CCA GCC AAC TTC ATC AAG

                          450                                   460
Pro Gly Lys Gln Pro Leu Ser Ser Met Cys Pro Ser Ile Ile Val Asp Lys Asp Gly Lys
CCA GGT AAG CAA CCG CTT TCA TCC ATG TGC CCC TCA ATC ATC GTG GAT AAG GAC GGC AAG

                          470                                   480
Val Arg Met Val Val Gly Ala Ser Gly Gly Thr Gln Ile Thr Thr Ser Val Ala Leu Ala
GTT CGG ATG GTG GTT GGA GCC TCG GGA GGT ACC CAG ATC ACC ACG TCT GTT GCA CTG GCC

                          490                                   500
Ile Ile Asn Ser Leu Trp Phe Gly Tyr Asp Val Lys Arg Ala Val Glu Glu Pro Arg Leu
ATC ATC AAC AGC CTG TGG TTC GGG TAT GAT GTG AAG AGA GCT GTG GAG GAG CCC CGT CTT

                          510                                   520
His Asn Gln Leu Leu Pro Asn Thr Thr Thr Val Glu Lys Asn Ile Asp Gln Val Val Thr
CAC AAC CAG CTT TTG CCC AAT ACC ACA ACA GTA GAG AAA AAT ATT GAT CAG GTG GTG ACT

                          530                                   540
Ala Gly Leu Lys Thr Arg His His His Thr Glu Val Thr Pro Asp Phe Ile Ala Val Val
GCA GGT CTG AAG ACT CGG CAC CAC CAT ACA GAG GTC ACA CCC GAC TTC ATC GCT GTG GTT

                          550                                   560
Gln Ala Val Val Arg Thr Ser Gly Gly Trp Ala Ala Ala Ser Asp Ser Arg Lys Gly Gly
CAG GCC GTC GTT CGA ACG TCA GGT GGT TGG GCA GCT GCC TCA GAT TCC AGA AAA GGC GGG

                    568
Glu Pro Ala Gly Tyr
GAG CCC GCT GGC TAC TGA GTGCCCGGAAGGGGCAAGACTGACCTCAGCCAAGAGACGAGAGTGGGACTCTGGA
GAACATGCTGCCCCTGGGTGGGAGAGAGCAGGATAATAAACAGAGGCCGCCGCCAAGTTGCGGGAAGCCTTTGCAGGCT
GGAAAAAAA
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Figure 4. Nucleotide and deduced amino acid sequence of GGT clones. The predicted amino acid sequence is shown above the corresponding codons. The underlined sequences correspond to the amino acid sequences determined by Matsuda et al. (23). Numbers correspond to the amino acid sequence starting at the third amino acid of the amino terminal end of the heavy subunit. The presumed polyadenylation signal is boxed.

Table 1. Amino acid sequence of the NH_2-terminal portion of the light subunit of GGT.

(1) (X)-(X)-His-Leu-Ser-Val-Val-(X)-Glu-Asp
(2) Thr-Ala-His-Leu-Ser-Met-Val
(3) ACC GCT CAC CTG TCC GTG GTT TCC GAG GAT

(1) Sequence determined at the University of Wisconsin, Madison. (2) Sequence determined by Matsuda et al. (34). (3) Nucleotide sequence reported in this study. (X) Not determined.

glycosylation sites were found, corresponding to the sequences Asn-X-Ser and Asn-X-Thr (30).

Another criterion we have used to identify this clone as that of the mRNA of GGT was the amino acid sequence of portions of the GGT protein. The sequence of the aminoterminal portion of the small subunit derived from the nucleotide sequence (amino acids 380-390, Figure 4) matched with the amino acid sequence determined by protein sequencing, performed at the University of Wisconsin (Table 1, 1) as well as the sequence reported early by Matsuda et al. (Table 1,2) (34).

DISCUSSION

We have isolated a cDNA clone apparently complementary to GGT mRNA by screening a rat kidney cDNA library constructed in a λgtll expression vector, using affinity-purified antibodies reactive with the enzyme. The identity of the clone was further confirmed by the facts that (1) the RNA selected by the cDNA was translated into a 62 kd protein, corresponding to the GGT precursor in molecular weight and immunoreactivity, and (2) the amino acid sequence predicted by the nucleotide sequence matched the known aminoterminal sequences of both subunits.

Although in the original screening technique of Young and Davis (17,18) both crude and purified antisera were used, we found that when crude antiserum was used, all the plaques gave a positive signal, even though the serum had been preabsorbed with *E. coli* and λgtll proteins. Only when affinity-purified antiserum was used did positive plaques give a strong signal clearly distinguishable from the background.

The 62 kd polypeptide immunoprecipitated from the translation mixture of the selected RNA was identical with that identified previously by Nash and Tate (14) and Finidori et al. (15) by immunoprecipitation with GGT antiserum

from translation reactions using kidney poly(A)RNA. This result, in addition to the fact that the two EcoRI fragments of the cDNA, representing basically the heavy and light subunits of the enzyme, hybridized with the same single RNA species (Figure 3), strongly supports the thesis that both subunits are synthesized as a single precursor, as was suggested by several investigators (14,15,32,33).

The largest clone we isolated was 1842 bp in length (Figure 4). Since the mRNA coding for GGT is 2.2 kb long (Figure 3) and assuming a poly(A) tail of 150-200 residues (31), the 5' flanking region can be estimated to be 200-250 bp in length.

This clone had a single open reading frame of 1,695 bp coding for 565 amino acids. The derived amino acid sequence contained the amino terminal sequences reported by Matsuda et al. (34) for the heavy and light subunits of the enzyme. This comparison showed that our clone lacked the first three amino acids (Met-Lys-Asp) of the aminoterminal portion of the heavy subunit. The sequence Gly-Pro-Pro-Leu described by these authors as the aminoterminal portion of the heavy subunit after papain treatment of the enzyme was not found in the nucleotide sequence reported herein. If the absence of the three N-terminal amino acids is considered, then the heavy subunit contains 379 amino acids having an estimated molecular weight of 42 kd, and the light subunit 189 amino acids with a molecular weight of 20 kd. These results are in agreement with the value of 62 kd for the protein precursor. Similar results are obtained for both subunits after deglycosylation of the native enzyme (35). These values are significantly smaller than those reported by Matsuda et al. (34), who found 436 and 210 amino acids for the heavy and light subunit respectively. The reason for these differences is not known.

The availability of this GGT cDNA clone will not only provide information on the structure and possible function of this enzyme, but it would also allow studies related to the regulation of the expression of the GGT gene in a variety of normal and neoplastic tissues. We are presently using the clone to study the expression of the GGT gene in preneoplastic hepatocytes and have found that these cells do express a dramatically higher level of GGT mRNA than do normal hepatocytes, which contain little or no GGT mRNA (Beer, D. and Pitot, H. C., unpublished observations).

Recently we have been made aware of the fact that Dr. J. Hanoune and his coworkers have isolated and characterized a full-length GGT cDNA clone from rat kidney. Their results will appear in the Proceedings of the National

Academy of Science, U.S.A. We appreciate Dr. Hanoune's willingness to share his findings with us and others.

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