Although use of multiple alternative first exons generates unique noncoding 5'-ends for γ-glutamyltransferase (GGT) cDNAs in several species, we show here that alternative splicing events also alter coding exons in mouse GGT to produce at least four protein isoforms. GGTα3 introduces CAG four bases upstream of the primary ATG codon and encodes an active GGT heterodimeric ectoenzyme identical to constitutive GGT cDNA but translational efficiency is reduced 2-fold. GGTα2–5 deletes the last eight nucleotides of exon 2 through most of exon 5 in-frame, selectively eliminating residues 96–231 from the amphipathic N-terminal subunit, including four N-glycan consensus sites, while leaving the C-terminal hydrophilic subunit intact. GGTα7 introduces 22 bases from intron 7 causing a frameshift and a premature stop codon so a truncated polypeptide is encoded terminating with 14 novel residues but retaining the first 339 residues of the native GGT protein. GGTα8–9 deletes the terminal four nucleotides of exon 8 plus all of exon 9 and inserts 24 bases from intron 9 in-frame so the C-terminal subunit of the encoded polypeptide loses residues 401–444 but gains eight internal hydrophobic residues. In contrast to the product of GGTα1, those derived from GGTα2–5, Δ7, Δ8–9 all lack transferase activity and persist as single-chain glycoproteins retained largely in the endoplasmic reticulum as determined by immunofluorescence microscopy and constitutive endoglycosidase H sensitivity in metabolically labeled cells. The development-stage plus tissue-specific regulation of the alternative splicing events at GGTα7 and GGTα8–9 implies unique roles for these GGT protein isoforms. The ability of the GGTα1 and GGTα7 to mediate the induction of C/EBP homologous protein-10, CHOP-10, and immunoglobulin heavy chain binding protein, BiP, implicates a specific role for these two GGT protein isoforms in the endoplasmic reticulum stress response.

The entire intron/exon structure of the mouse γ-glutamyltransferase gene (EC 2.3.2.2, GGT)† has been defined. This single copy gene is regulated by multiple alternative promoters that are coupled with alternative splicing mechanisms to generate several GGT cDNAs each with a unique 5'-noncoding region. But all encode the same protein, because coding exons appear to be spliced only in a constitutive fashion (1). The protein product is synthesized as a single-chain N-glycosylated propeptide, processed into an N-terminal amphipathic subunit and a smaller C-terminal subunit, and expressed on the cell surface where it functions as a key enzyme in glutathione metabolism (2).

Several human GGT cDNAs also exhibit unique 5'-ends and encode a protein that is processed in a similar fashion and shares 79% amino acid identity with that of the mouse (3). In addition, an alternatively processed human GGT cDNA has been described that contains an insertion of 22 bases within the coding domain. The extra nucleotide bases introduce a frameshift and a premature stop codon so that the predicted polypeptide would be a truncated GGT isoform. The protein product has never been characterized, but the elimination of the small subunit suggests that it would lack γ-glutamyltransferase activity. The identification of this alternative transcript implies that human GGT gene expression may be more complex than that of other species (4).

However, while characterizing the site of a point mutation in the GGTα8–9 mouse (5), we identified four previously unknown alternative splicing events involving coding exons in the normal mouse GGT gene (see Fig. 1). We studied these events to determine whether GGT protein isoforms are being generated through alternative processing of mouse GGT mRNA, to identify if these events are shared between mouse and human GGT, and finally to explore a potential role for these new mouse GGT protein isoforms in glutathione metabolism.

**EXPERIMENTAL PROCEDURES**

*Source of Probes and Tissues—* Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) was used according to the manufacturer's protocol to isolate total RNA from tissue or cells. Mouse genomic DNA was isolated from the lung. Human genomic DNA was provided by Dr. Qiang Yu from the Pulmonary Center of Boston University. Electrophoresis grade agarose was from International Biotechnologies, Inc. (New Haven, CT), and DNA standards were from Life Technologies, Inc. Materials for protein electrophoresis were from Bio-Rad (Richmond, CA), and protein standards were High-Range molecular weight markers from Amersham Pharmacia Biotech (Piscataway, NJ). X-OMAT and BioMax MR films were used for radiography and obtained from Eastman Kodak Co. (Rochester, NY). [α-32P]CTP, specific activity

*Dubbeco’s modified Eagle’s medium; Ham’s F-12, nutrient mixture Ham’s F-12; PBS, fetal bovine serum; CHOP, C/EBP homologous protein-10; BiP, immunoglobulin heavy chain binding protein; iCAR, human eosinophil and adenovirus cell surface receptor; hMUC, human mucin 1; RT, reverse transcription; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); PBS, phosphate-buffered saline.
the published sequences in the GenBank.

CHOP and BiP were generated by PCR after selecting primers based on

Unit of Boston University School of Medicine. The cDNA probes for

Primers were synthesized at the DNA/Protein Core Molecular Biology

CCTCCATCATCCTGAAGGTAGA. Primers M72 plus 10.1M were used

stitutive GGT cDNA amplification were M72, 5

CTGTCTGCGGTTTC as the upstream primer for both PCR reactions,

upstream hemi-nested primers were 22U, 5

9

TGCCAG and 24D, 5

CGGCAG and 24D, 5

GACAGGTGAGCGGTGCCTCC, for the secondary reaction.

was made on Kodak X-OMAT film, and the film was developed.

with 1

room temperature with 2

with QuickHyb (Stratagene) at 68 °C for 15 min. Radiolabeled probe

with a Stratagene UV cross-linker. The membrane was prehybridized

(5). RNA obtained from cell lines was quantitated by spectrophotometry

along with a common 3

primer an

product was predicted to have a molecular mass of 35.2 kDa with the

internal translation initiation site.

terminal 47 amino acid residues encoded from the vector. A secondary

was then added and incubated for 2 h. The filter was washed twice at

with 3% paraformaldehyde in PBS

for 12 min. After washing with 10

for 3.5 min at 90 °C in 50

b

m

l

m

Ci of [ 35S]Met/Cys for the times

with 30

l of goat anti-rat GGT antisera.

immunoprecipitates were resuspended in 80 l of 10 mM citrate buffer,

and radioactive protein bands imaged and quantitated from the dried gel using a PhosphorImager (Bio-Rad, Richmond, CA).

Mouse GGT Protein Isoforms

Enzyme Activity—Specific GGT enzyme activity was measured at

room temperature using the substrates γ-glutamyl-para-nitroanilide

Transient Expression of mGGT Isoforms in CHO Cells—Transient

expression of the mGGT isoforms was obtained in CHO cells using a

cowpox/bacteriophage T7 (t7TCP) expression system as described previously (6, 7). Confluent cultures of CHO cells were grown in 35-mm plastic dishes with a 1:1 mixture of DMEM and Ham's F-12 (1:1) supplemented with 3% FBS (normal culture media). Cells were washed for 3 times with PBS (normal culture media), then cultured in serum-free media with 30% Met/Cys (infection ~30) in 0.3 ml of the same media for 30 min. The media containing t7TCP was removed from the cells before transfection with a mixture of plasmid DNA (pCDNA3.1 with a T7 promoter) and Lipo-

fectAMINE (Life Technologies) at a 1:3 ratio in 1 ml of the same serum-free media for 3 h. The lipid and DNA mixture was then removed, and the cells were washed with 1 ml of DMEM media lacking Met and Cys before addition of fresh 0.14 M NaCl, 2.6 mM KCl, 15.2 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.5 mM MgCl₂ and 0.7 mM CaCl₂ then incubated with 0.5 Ci of [35S]Met/Cys for 30 min. After overnight rotation at 4 °C, the avidin-conjugated beads were removed, and the cells were washed with 1 ml of goat anti-rat GGT antisera.

immunoprecipitates of CHO cells expressing the mGGT isoforms were transfected with the mGGT isoforms (described above) were starved for Met and Cys for 15 min in 1 ml of DMEM media lacking Met and Cys before addition of fresh 0.14 M NaCl, 2.6 mM KCl, 15.2 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.5 mM MgCl₂ and 0.7 mM CaCl₂ then incubated with 0.5 Ci of [35S]Met/Cys for 30 min. After overnight rotation at 4 °C, the avidin-conjugated beads were removed, and the cells were washed with 1 ml of goat anti-rat GGT antisera.

immunoprecipitates were resuspended in 80 l of 10 mM citrate buffer,

b

m

l of goat anti-rat GGT antisera.

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immunoprecipitates were resuspended in 80 l of 10 mM citrate buffer,

b

m

l of goat anti-rat GGT antisera.

immunoprecipitates were resuspended in 80 l of 10 mM citrate buffer,

b

m

l of goat anti-rat GGT antisera.

immunoprecipitates were resuspended in 80 l of 10 mM citrate buffer,
the constitutive (Δ) splicing events of mouse GGT mRNA and the protein isoforms that result based on data in Table I and Figs. 2–8.

and glycyglycine as previously described (10). Protein was determined by the method of Lowry.

**RESULTS**

**Mouse GGTΔ1**—The majority of our PCR-derived GGT clones generated from lung and kidney RNA of wild type and GGTtrans−/− mice contained the trinucleotide insert CAG five bases upstream of the ATG initiation codon (details in Fig. 1). An examination of GGT cDNA sequences in the GenBank revealed an absence of this CAG insert in all mouse GGT cDNAs, as well as those from rat and pig, but revealed its presence in most, but not all, human GGT cDNAs. Certain human lung GGT cDNAs lacked this CAG insert (11). Because this CAG insertion is located at an intron/exon junction, we compared the intron sequences from the mouse, rat, and pig to determine whether alternative splicing could account for its presence (Fig. 2A). All three introns contain the highly conserved dinucleotides GT and AG at the 5′- and 3′-boundaries, respectively, and the AG is preceded by a cytosine residue. However, the mouse intron contains two CAGs in tandem at its 3′-intron boundary, whereas the rat and the pig intron each contain a single CAG. We then cloned and partially sequenced a corresponding human GGT intron sequence. We confirmed that this intron is identical in size to that of rat, mouse, and pig, ~0.5 kb, and contains two tandem CAG dinucleotides at the 3′-boundary like that of mouse.

To see if the proximity of this CAG insert to the ATG initiation site could affect the site of translation initiation, we used a transient VT7CP expression system to characterize the protein encoded by the GGTΔ1. Cow pox-infected CHO cells transfected with the GGTΔ1 plasmid were starved for Met and Cys and pulse-labeled in the same media with [35S]Met/Cys, before a chase period of 0 or 2 h. SDS-PAGE analysis of GGT-specific immunoprecipitates from the cell extracts revealed a single peptide of 83 kDa at t = 0, which was sensitive to treatment with endoglycosidase H and produced a product of 59 kDa (Fig. 3). The translation product from an internal initiation site is marked at 27 kDa. The primary translation product is marked at 35 kDa. The translation product from an internal initiation site is marked at 27 kDa.

gel pattern of the large subunit indicated that there is considerable microheterogeneity in the processing of these N-glycans, whereas the small subunit was more homogeneous despite its resistance to endo H treatment, indicating that the single N-glycan is minimally processed.

Similar data were obtained for the synthesis of GGTΔ1 in stable transfectants of CHO cells (Fig. 4). When these clonal CHO cells expressing GGTΔ1 were pulse labeled for 30 min and chased for 2 h prior to cell surface biotinylation with the membrane impermeant sulfo-NHS-SS-biotin, ~20% of the heterodimer was recovered from the immunoprecipitates with avidin-conjugated beads. This indicated that the GGTΔ1 protein did reach the plasma membrane (compare mock lanes in Fig. 4, A and B). This cell surface localization of the GGTΔ1 was confirmed by immunofluorescence analysis of COS cells 2 days after transfection with the GGTΔ1 cDNA (Fig. 5A). A similar pattern of immunofluorescence was observed in stably trans-
fected CHO cells expressing the GGT1 (data not shown). In both cases nearly all the GGT-specific immunofluorescence was found at the cell surface. An equally important result is that the clonal CHO cells expressing GGT1 exhibited a much higher γ-glutamyl transferase-specific enzymatic activity (280

**Table I**

| mGGT isoform | Peptide FW | N-Glycan consensus site | SDS gel Mr. (± end H) | Mr. difference | No. of N-Glycans estimated | Half-life† (CHO clone/VT7CP) |
|--------------|------------|-------------------------|-----------------------|---------------|--------------------------|-----------------------------|
| Δ1           | 61.2       | 7                       | 78/59                 | 24            | 7                        | 18.7/17.4                   |
| Δ2–5         | 46.7       | 3                       | 47/38                 | 9             | 3                        | 1.7/2.1                     |
| Δ7           | 38.2       | 5                       | 44/32                 | 12            | 4                        | 6.5/10.2                    |
| Δ8–9         | 57.6       | 7                       | 52/37                 | 15            | 5                        | ND/0.9                      |

a Formula weight was calculated from the predicted amino acid sequence.

b The consensus sequence for N-linked glycosylation is Asn-X-Ser/Thr.

c Apparent molecular weight of isoform on SDS gel as shown in Fig. 4.

d Difference in molecular weight of the isoform before and after treatment with endo H.

e Calculated number of N-linked glycans, assuming that removal of each glycan alters mobility by 3 kDa.

f Half-life was calculated from recovery of [35S]GGT isoforms after chase times of 3 and 18 h (Δ1 and Δ7) or chase times of 0 and 3 h (Δ2–5 and Δ8–9) as described under “Experimental Procedures.” A clonal CHO cell line expressing Δ8–9 was not available.

**Fig. 3.** Transient expression of mouse GGT isoforms in CHO cells. Confluent cultures of CHO cells in 35-mm wells were infected with cow pox for 30 min before transfection for 2 h with 18 μg of LipofectAMINE and either no plasmid (mock) or 6 μg of plasmid DNA encoding GGT1, GGTΔ2–5, GGTΔ7, or GGTΔ8–9. Cells were pulse-labeled for 15 min with [35S]Met/Cys prior to chase periods of 0 or 2 h, before extraction with octyl glucoside and immunoprecipitation of the GGT-related peptides with a goat polyclonal antibody and subsequent treatment overnight with (+) or without (−) endo H. Samples were subjected to SDS-PAGE and PhosphorImager analysis. Numbers to the right refer to the mobility of molecular mass standards in kDa.

**Fig. 4.** Only GGTΔ1 is expressed at the cell surface. Confluent cultures of clonal CHO cells stably expressing the GGTΔ1 in 21-mm wells were infected with cow pox for 30 min before transfection with 9 μg of plasmid DNA encoding GGTΔ2–5, GGTΔ7, or GGTΔ8–9 (mock received buffer and no DNA). The next day, cells were pulse-labeled for 30 min with [35S]Met/Cys prior to a 2-h chase period, before biotinylation of the cell surface on ice, extraction with octyl glucoside, and immunoprecipitation of the GGT. A portion of the immunoprecipitate (75%) was further incubated with avidin-conjugated beads to obtain the biotinylated cell surface GGT. Both a portion of the total immunoprecipitate (B, 25% of total) and the biotinylated surface GGT (A) were subjected to SDS-PAGE and PhosphorImager analysis. Numbers to the right refer to the mobility of molecular mass standards in kDa. The mobility of the large (L) and small (S) subunits of the GGTΔ1 heterodimer are shown on the left, and the asterisk indicates protein isoforms. The diffuse gel pattern of the GGTΔ1 large subunit indicates that there is considerable microheterogeneity in the processing of the N-glycans. This band is less evident in B, because these lanes represent only 25% of each immunoprecipitate.

**Fig. 5.** Immunofluorescence analysis. COS cells were transfected with plasmids encoding GGTΔ1 (A), Δ2–5 (B), Δ7 (C), and Δ8–9 (D). The steady-state localization of the expressed proteins were analyzed with rabbit anti-rat GGT antiserum followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG. COS cells (E) were stained for calnexin as a positive control for an ER protein, and primary antibody was omitted for a negative control (F). Surface staining is observed only for mGGTΔ1. Although Δ2–5, Δ7, and Δ8–9 predominantly show staining in the endoplasmic reticulum, a low level of juxtanuclear Golgi-like staining is also seen for all the proteins.
**Mouse GGT Protein Isoforms**

**Fig. 6. Characterization of GGT-Δ2–5.** A, an event resulting from usage of 5'- and 3'-splice sites within exon 2 and exon 5, respectively. The last eight nucleotides of exon 2, all of exons 3 and 4, and most of exon 5 are eliminated. B, the encoded protein loses amino acid residues 96 through 231, including four N-glycan consensus sites from the amphipathic N-terminal subunit, whereas the C-terminal hydrophilic subunit remains intact.

mU/mg) than nontransfected CHO cells (<1 mU/mg). And after overnight accumulation of GGTΔ1 in transfected cow pox-infected CHO cells, this enzyme activity was greatly increased (200–1000 mU/mg). Because this latter result indicated that the GGTΔ1 protein must be relatively stable, the half-life was determined for the radiolabeled GGTΔ1 during stable and transient expression by pulse-labeling cells for 30 min and immunoprecipitating GGTΔ1 after chase times of 3 and 18 h (Table 1). Calculation of the half-life from the percent loss of radiolabeled GGT during the two time points indicated the half-life for the GGTΔ1 is similar in stable (18.7 h) and transiently transfected (17.4 h) CHO cells. Thus GGTΔ1 encodes the normal mouse GGT protein. This protein exhibited normal synthesis, cell surface expression, stability, and enzymatic activity. Because the expression of GGTΔ1 in cultured cells was indistinguishable from that described previously for rat and human GGT, we next assessed whether the CAG insert in the mouse GGTΔ1 could affect translational efficiency using an in vitro transcription/translation assay. The synthesis of the primary translation product (35.2 kDa) from the transcript containing the CAG insert was reduced 2-fold when compared with synthesis from a transcript lacking the CAG sequence, whereas synthesis of an alternate protein from an internal ATG codon (27 kDa) was unchanged (Fig. 2B). Thus, the CAG appears to regulate translation of the GGT mRNA, not the protein product.

**Mouse GGTΔ2–5**—This alternative splicing event produces an in-frame deletion of the last eight nucleotides of exon 2, all of exons 3 and 4, and most of exon 5 (Fig. 6A). Nonconsensus 5'- and 3'-splice sites appear to have been utilized in exons 2 and 5, respectively. We could not identify a corresponding human intron sequence (Fig. 7A). The remaining 10 nucleotides at the 5'-end of the insertion were identified as intron 7 (Fig. 6B). The 22-base insertion results from an alternative splice site at intron 7—this alternative splicing event introduces a frameshift and a premature stop codon within the open reading frame (Fig. 7A). The encoded mouse protein is a truncated GGT-like protein that retains only the first 339 amino acids of the native GGT protein and gains 14 novel residues at the C terminus, the first seven of which are identical to that in the human (Fig. 7B). Because the intron/exon structure of mouse GGT is known and the last 10 nucleotides at the 3'-end of the insert agree exactly with the intron 7 sequences in the literature (1), it appears that the 22-base insertion results from an alternative splice site at intron 7. To confirm this, we cloned and partially sequenced the mouse GGTΔ2–5 (Fig. 7B). This 22-base mouse intron sequence is identical to the corresponding human intron sequence (Fig. 7C).}

When CHO cells transiently transfected with the GGTΔ2–5 were pulse-labeled with [35S]Met/Cys for 15 min, a single labeled protein of 47 kDa was immunoprecipitated after both 0- and 2-h chases (Fig. 3). At both time points the protein was sensitive to endo H treatment producing a protein of 38 kDa, consistent with the presence of three N-glycans, but which is notably smaller than the predicted sequence (46.7 kDa). Because the protein remains anchored to the membrane (data not shown), this indicates that the GGTΔ2–5 either is cleaved after the N-glycan consensus site in the small subunit or migrates anomalously on SDS gels.

The persistence of the endo H sensitivity of the GGTΔ2–5 protein after the 2-h chase also suggests that the protein is retained in the ER or early Golgi rather than moving to the cell surface. When this was tested by transient expression of GGTΔ2–5 in CHO-GGTΔ1 cells, only 1.5% of the GGTΔ2–5 was bionylated at the cell surface after a 2-h chase, whereas ~20% of the GGTΔ1 was bionylated in the same cells (Fig. 4). Localization in the ER was also evident by immunofluorescence microscopy of COS cells transiently expressing the GGTΔ2–5 (Fig. 5B). Determination of the half-life for the GGTΔ2–5 in both stable transfected (1.7 h) and transiently transfected (2.1 h) CHO cells indicates that this isoform of the mouse GGT is considerably less stable than the GGTΔ1 (Table 1). Enzyme assays of these cell extracts reveal no increase in γ-glutamyltransferase activity (<1 mU/mg) above the control levels observed in nontransfected CHO cells.

**Mouse GGTΔ7**—This alternative splicing event introduces a 22-base insertion within the coding domain. This insertion mimics that in humans and induces a frameshift and a premature stop codon within the open reading frame (Fig. 7A). The encoded mouse protein is a truncated GGT-like protein that retains only the first 339 amino acids of the native GGT protein and gains 14 novel residues at the C terminus, the first seven of which are identical to that in the human (Fig. 7B). Because the intron/exon structure of mouse GGT is known and the last 10 nucleotides at the 3'-terminus of the insert agree exactly with the intron 7 sequences in the literature (1), it appears that the 22-base insertion results from an alternative splice site at intron 7. To confirm this, we cloned and partially sequenced the ~3 kb of mouse intron 7 (Fig. 7A). The remaining 10 nucleotides at the 5'-end of the insertion were identified as intron 7 sequences, and they were preceded by a CAG as a 3'-splice site. This 22-base mouse intron sequence is identical to the corresponding human intron sequence (Fig. 7C). We used the 22-base insert as one of the primers in a PCR reaction to determine whether the GGTΔ7 splicing event is regulated in a developmental or tissue-specific fashion.
absent from that of the heart and the liver. It was also detectable in mRNA isolated from adult rat kidney and human peripheral blood mononuclear cells (data not shown).

To compare the relative abundance of GGTΔ7 to constitutive mGGT, we amplified the mRNAs from five different tissues using two sets of PCR primers that were common to both transcripts with a fully nested design (Fig. 7E). The 208-base pair signal amplified from constitutive mGGT was evident in all samples. The 230-base pair signal amplified from GGTΔ7 was only evident in lung, kidney, and brain and was less abundant than that of the constitutive GGT signal.

When CHO cells transiently expressing the GGTΔ7 were pulse-labeled for 15 min with [35S]Met/Cys a single product of 44 kDa was obtained after chase times of both 0 and 2 h (Fig. 3). Endo H treatment produced a peptide of 32 kDa in both cases, which was slightly smaller than the peptide size calculated from the predicted sequence (38.2 kDa), but indicates that N-glycans are present at four of the five consensus sites in the GGTΔ7. When the cell surface of CHO-GGTΔ1 cells transiently expressing the GGTΔ7 were in vitro translated after a 30-min pulse with [35S]Met/Cys and a 2-h chase, only 0.5% of the GGTΔ7 was recovered with avidin-conjugated beads while 37% of the GGTΔ1 was recovered from the same cells (Fig. 4). Immunofluorescence microscopy of COS cells transiently expressing the GGTΔ7 (Fig. 5C) revealed staining of the ER, which is consistent with both the persistence of endo H sensitivity and the minimal cell surface expression of this isoform. Enzyme assays for both CHO cells stably and transiently expressing GGTΔ7 revealed no increase in γ-glutamyltransferase activity (<1 mU/mg) above the control levels observed in non-transfected CHO cells. The half-life for the GGTΔ7 in these same cells (6.5 and 10.2 h, respectively) was approximately half of that observed for the GGTΔ1 (Table I).

Mouse GGTΔ8–9—The Δ8–9 alternative splicing event eliminates at least the last four nucleotides of exon 8 and all of exon 9 but introduces 24 novel bases (Fig. 8A). The last 10 of these 24 nucleotides are identical to the published sequence at the 3’-boundary of intron 9 (1). This intron was cloned and sequenced a corresponding human intron. The 67-nucleotide sequence at the 3’-intron boundary, then the 5’-splice site lies within exon 8 but it is a nonconsensus splice site. However, other nonconsensus dinucleotide sequences could also border these boundaries as denoted in Fig. 8A, so the exact location of this splice site is unclear. This GGT cDNA has not been described in humans and we did not detect any PCR product when this 24-base insert was used as a PCR primer with human cDNA (data not shown). To confirm this, we cloned and sequenced a corresponding human intron. The 67 nucleotides at the 3’-boundary of our clone matched perfectly with sequences in human GGT genes 3, 6, and 11 (data not shown). However, comparison with mouse intron 9 revealed neither conserved sequences in this region nor any conserved 3’-splice site sequences (Fig. 8B). Usage of the 24 corresponding human nucleotides also failed to produce a PCR product. Hence, this particular GGT splicing event may be specific to mouse.
producing a protein of 37 kDa, consistent with the presence of both time points the band was sensitive to endo H treatment immunoprecipitated after both 0- and 2-h chases (Fig. 3). At enzyme site of mGGT (are intron 9 with those from the corresponding human (Hs) intron reveals many differences. C, PCR analysis for developmental and tissue-specific expression of Δ8–9 was performed as in Fig. 7, but the upstream primer in the secondary PCR reaction was 24D. D, the amino acid residues that are eliminated from the small C-terminal subunit of mGGT are boxed, and the eight novel amino acids of Δ8–9 are boldfaced. These hydrophobic residues are inserted proximal to the active enzyme site of mGGT (underlined).

To determine if Δ8–9 was expressed in a developmental or tissue-specific fashion, we repeated the analysis outlined for GGTΔ7 but used the 24-base insert as one PCR primer. GGTΔ8–9 was not detected in RNA from the heart, the liver, or the lung during the neonatal period, even though it was present in the adult lung-positive control. In adult tissues, it was evident in RNA from the lung and the kidney but not that from the heart, the liver, or the thymus (Fig. 8C). Hence, GGTΔ8–9 exhibits developmental-stage and tissue-specific regulation, but the patterns differ from that of GGTΔ7.

The elimination of the terminal four bases of exon 8 plus all of exon 9 together with the insertion of 24 bases from intron 9 removes 44 amino acids residues from the C-terminal small subunit but adds 8 novel amino acids proximal to active enzyme site residues (Fig. 8D). When CHO cells transiently transfected with the GGTΔ8–9 were pulse-labeled with [35S]Met/Cys for 15 min, a single labeled protein of 52 kDa was immunoprecipitated after both 0- and 2-h chases (Fig. 3). At both points the band was sensitive to endo H treatment producing a protein of 37 kDa, consistent with the presence of five N-glycans on the protein. However, this is two less N-glycans than would be predicted from the GGTΔ8–9 sequence. In addition, the protein size for the GGTΔ8–9 calculated from the predicted sequence is considerably larger (57.6 kDa). The cumulative data would be most consistent with cleavage of the GGTΔ8–9 into an unstable heterodimer, immediate degradation of the small subunit, and retention of the residual large subunit within the ER. When the cell surface of CHO-GGTΔ1 cells transiently expressing the GGTΔ8–9 were biotinylated after a 30-min pulse with [35S]Met/Cys and a 2-h chase, only 2.2% of the GGTΔ8–9 was recovered with avidin-conjugated beads while 35% of the GGTΔ1 was recovered from the same cells (Fig. 4). Immunofluorescence microscopy of COS cells transiently expressing the GGTΔ8–9 (Fig. 5D) revealed staining of the ER, which is consistent with both the persistence of endo H sensitivity and the poor cell surface expression of this isoform. The half-life of the GGTΔ8–9 in transiently transfected cells was only 0.9 h, and there was no measurable γ-glutamyltransferase enzymatic activity. Although stably transfected cells were not available for these studies, the similar data obtained for the GGTΔ1, GGTΔ2–5, and GGTΔ7 between transient and stably transfected cells (Table I) indicates that the half-life and the enzyme activity data for GGTΔ8–9 in transiently transfected cells are reliable.

**Stress Response Induction in the Endoplasmic Reticulum**—Because GGTΔ1 is normally found at the cell surface, the localization of the GGTΔ2–5, Δ7, and Δ8–9 in the ER could simply indicate that these GGT isoforms are abnormally folded products and they are being retained for subsequent degradation. Alternatively, this subcellular localization could be consistent with a new previously undefined role for these proteins within the ER. The active GGTΔ1 enzyme is essential for turnover of glutathione at the cell surface. Although the other GGT isoforms lack this enzyme activity, they could still bind substrate and act as sensors of the critical glutathione redox levels found in ER. In support of this hypothesis, we found that the phenotype of the stable CHO cells for GGTΔ1 and Δ7 changed dramatically when the cell media was shifted from a 1:1 mixture of DMEM (cystine) and Ham’s F-12 (cysteine) with 3% FBS to DMEM alone with 3% FBS. Although the GGTΔ2–5 and Δ8–9 cells remained unchanged, the GGTΔ1 and Δ7 cells rounded, lifted, and detached from the culture plates within 12 h. The altered phenotype was reversed upon return of the cells to the original media containing cysteine as well as cystine. Because stress in the ER can be associated with this dramatic change in phenotype, we probed the cellular RNA from each cell line for the induction of CHOP-10 and BiP mRNAs, two markers of the ER stress response. CHOP-10 mRNA was not detected by Northern blot analysis in any of the cell lines before the change in media (data not shown) but was dramatically induced in the GGTΔ1 and Δ7, but not the GGTΔ2–5 and Δ8–9 cells, during the recovery period (Fig. 9). Actin mRNA was examined as a control for RNA loading and integrity and was unchanged. To be sure this induction of CHOP-10 was not due simply to overexpression of a recombinant protein, two additional cell lines expressing high levels of
the recombinant glycoproteins hCAR or MUC1 (12) were similarly characterized and found to not induce CHOP in response to the change in media. Although hCAR is a 46-kDa glycoprotein cell surface receptor for coxsackie and adenoviruses (13), MUC1 is a very heavily O-glycosylated mucin-like transmembrane protein ($M_r > 220$ kDa (12)). Finally, analysis of BiP mRNA levels in all these cell lines indicated that its expression paralleled that of CHOP-10.

**DISCUSSION**

Herein we have characterized four new GGT protein isoforms that are derived from alternative splicing events in mouse GGT cDNA. The mouse and the human not only generate GGT protein isoforms by this mechanism, but they share some of these splicing events in common. The encoded GGT protein isoforms can be expressed as transference active heterodimeric glycoproteins on the cell surface or as transferase inactive monomeric glycoproteins in the endoplasmic reticulum. These latter findings suggest potentially novel functions for native GGT protein and its protein isoforms within the endoplasmic reticulum in addition to the known role of the native protein as a cell surface ectoenzyme.

Alternative pre-mRNA splicing of nascent eukaryotic mRNAs is a post-transcriptional process that is known to be highly regulated and widely utilized to generate multiple alternative products from a single gene (14). A prime example of the power of alternative promoters to generate such mRNA diversity can be found in the mouse GGT gene itself. Six GGT cDNAs, each with a unique 5'-untranslated region, are generated from this single copy gene via six alternative promoters (15). Alternative splicing events within the open reading frame can generate protein isoforms by excluding specific exon sequences or including novel intron sequences in the mature mRNA transcript. However, prior to our study, only a constitutive splicing pattern had been identified in the mouse $GGT$ gene, which involves the invariant ligation of 12 coding exons, numbered by *Arabic numerals* 1 through 12, and one common noncoding exon, numbered by *Roman numerals* I as depicted in Fig. 1 (1).

The central difference between constitutive and alternative splicing lies in the selection and ligation of specific pairs of donor/acceptor splice sites. This is a complex process that is only partially understood, but it is clear that certain cis RNA sequences provide recognition sites for trans-splicing factors that form the spliceosome apparatus. These cis RNA sequences include the dinucleotides GT and AG located at the 5'- and the 3'-boundaries of an intron, respectively (16). These conserved dinucleotide sequences are found at all of the intron boundaries in the constitutively spliced mouse $GGT$ gene (1). The factors involved in the selection of these specific GT and AG residues from a much large number of potential choices are not yet fully known for any gene. However, additional consensus sequences surrounding these dinucleotides as well as internal consensus sequences at the branch point participate in the selection process. Selection of the AG residue at the 3'-splice site appears to involve a scanning process initiated from the branch point and a competition among various intervening AG dinucleotides with the nucleotide preceding the AG having a profound influence on its selection in the order CAG→TAG→AAG→GAG (16). In $GGT\Delta1$ and $GGT\Delta7$ the alternative splicing event was limited to the 3'-intron boundary and utilized an alternative AG dinucleotide that was preceded by a cytosine residue. In $GGT\Delta2$–5 and $GGT\Delta8$–9 the alternative splicing events involved 5'- and 3'-splice sites as well as non-GT/AG dinucleotides.

In $GGT\Delta1$ two CAGs in tandem are present at the 3'-boundaries of the corresponding introns in mouse and human GGT but absent from those of the rat and the pig. Our results show that alternative utilization of these sites can explain the presence or absence of a CAG trinucleotide insert upstream of the ATG initiation codon specifically in mouse and human GGT cDNAs. The scanning model predicts that the first CAG should identify the 3'-boundary of the intron while the second should be present in the cDNA. Our data agrees with this model, because 80% of the clones contained the CAG insertion. Despite the proximity of these bases to the translation initiation site, our protein expression studies clearly show that the heterodimer derived from $GGT\Delta1$ is a stable protein and identical to that described previously in the rat and in humans (9, 17). Hence, the mouse and humans express two GGT cDNAs that encode a GGT propeptide, which is heavily glycosylated, especially on the large subunit, and processed into the heterodimer before or after delivery to the cell surface. Our study now represents the first detailed characterization of the GGT glycoprotein in the mouse. The activity associated with this enzyme initiates the hydrolysis of reduced or oxidized glutathione at the cell surface, a process that is essential for recovery and uptake of cysteine/cystine. However, this metabolism also appears to generate the pro-oxidant hydrogen peroxide in the presence of iron (18). Hence, it is likely that the level of GGT activity is regulated. Our *in vitro* transcription/translation data shows that mouse and human GGT can use the insertion of this CAG sequence to down-regulate GGT protein production at the level of translation. The utilization of this same alternative splicing strategy to alter mRNA translational efficiency has already been described in the gene for human surfactant-associated protein A2, so it is not unique to GGT (19, 20).

$GGT\Delta7$ also utilizes a nearby AG dinucleotide located only 24 bases pairs upstream in intron 7 as the alternative 3'-intron boundary, and this is preceded by a cytosine residue. Two factors could have selected against this CAG as the constitutive 3'-splice site according to the scanning hypothesis. The first is a distance of less than 12 nucleotides from the branch point; the second is a location within a region of secondary mRNA structure such as stem loop. Our partial sequence analysis of mouse intron 7 supports the first mechanism. We found that there is a potential branch point consensus sequence that would place this alternative CAG within this distance restriction. There is also a second potential branch point sequence even further upstream that would also place it as the first downstream CAG trinucleotide (16). The factors that determine how these potential branch points are utilized in the
Mouse GGT gene are not yet known. The presence of these intron sequences in a human GGT gene together with their regulation in a developmental-stage and tissue-specific fashion in the mouse GGT gene suggests that they serve a common role in GGT gene expression in these two species. Determination of the degree of conservation will require further examination of the GGT genes of additional species such as the rat, the dog, the pig, and the cow. But these sequences are not found in the GGT gene(s) of bacteria, yeast, flies, or worms, suggesting that they are a relatively late addition to the genomes of metazoans. Further studies will be required to fully understand the complete role of these intron sequences for GGT gene expression. However, we note that the identical intron sequences are also found in the human lung-specific group II GGT cDNAs where they reside not in the coding domain but in the 5′-untranslated region (11). Similar, but not identical, sequences are also present in a human intronic GGT promoter (21).

Our PCR data on the relative expression of GGTΔ7 indicate that this alternative splicing event is minor compared with the constitutive event. However, we also found that the GGTΔ7 splicing variant was detectable in tissues where constitutive GGT mRNA was highly expressed, like the kidney, but not in tissues where GGT mRNA abundance is very low, like the liver. Hence, the expression of constitutive GGT and GGTΔ7 appears to be linked. An alternative splicing event like GGTΔ7 has previously been described in a human GGT mRNA, but three potential protein products were predicted based on the presence of different open reading frames (4). Our study in the mouse is the first to characterize the encoded protein product. Our characterization of GGTΔ7 expression in stable and transiently transfected CHO cells reveals that a single truncated form of GGT protein is synthesized. It is a glycoprotein of 44 kDa and has 14 novel residues at the C terminus. It is a relatively stable protein with a half-life of ~8 h and is localized to the ER rather than the cell surface. Because it lacks the C-terminal small subunit residues of the active site, it predictably has no transferase activity. However, it does retain the critical arginine 107 for substrate binding (23). Because recent studies have established an essential role for the GGT substrate glutathione in regulating the redox state of the ER (24), it is possible that GGTΔ7 can bind glutathione and acts as a sensor for glutathione levels within this compartment. Alternatively, the GGTΔ7 isoform may act as a chaperone for the synthesis of the native enzymatically active GGT, blocking its activity within the ER where degradation of glutathione would be unwanted. This would be supported by finding that GGTΔ7 predominately exists in tissues where high levels of GGT are synthesized, and the low level of GGTΔ7 would reflect that amount needed to balance the low transient levels of newly synthesized native GGT in the ER.

The dinucleotides delimiting the possible alternative 5′- and 3′-splice sites in GGTΔ2–5 and GGTΔ8–9 differ from the consensus GT and AG sequences. Hence, it is unclear exactly how these alternative events were processed but there are certainly many examples in other genes of splice sites that differ from the GT-AG consensus (25), including the human GGT genes (22). The presence of such sites led to the recent search and identification of an alternative intron subclass that is bounded by AT-AC dinucleotides. Therefore, other intron subclasses may also occur (26). Until further information becomes available in this area, the mechanism for these alternative splicing events in mouse GGT will remain uncertain. Nonetheless, the similarities between the developmental-stage and tissue-specific expression of GGTΔ8–9 and GGTΔ7 suggests that these GGT mRNA splicing events are highly regulated, whereas the differences in tissue-specific expression suggest they can be regulated independently. We were not able to find a human correlate for GGTΔ8–9, so this event may be specific to the mouse. The protein isoforms derived from GGTΔ2–5 and GGTΔ8–9 lack γ-glutamyltransferase activity, like GGTΔ7. In each case, a residue required for GGT activity, glutamic acid 108 (23) and aspartic acid 423 (27), respectively, is eliminated along with several other amino acids. GGTΔ2–5 and Δ8–9 were also localized to the ER. Coexpression studies failed to show any effect of these isoforms, including GGTΔ7, on total endogenous GGT activity or delivery of active transferase to the cell surface (data not shown). Hence, their exact function remains obscure. Certainly the more rapid turnover rates for Δ2–5 and Δ8–9 could indicate that these glycoproteins are sensed as abnormal GGT products within the ER and targeted for degradation.

Nonetheless, the expression of GGTΔ1 and GGTΔ7 protein isoforms appears to be able to impact the environment of the ER as suggested by their ability to trigger an ER stress response with a change in cell culture conditions. This stress was demonstrated by the parallel induction of the mRNAs for CHOP, a nuclear protein that is regulated by ER stress, and BiP, a chaperone whose expression during ER stress is coordinately regulated with CHOP (28). The message for CHOP is not normally expressed in cells but is strongly induced by ER stress. CHOP protein then forms stable heterodimers with C/EBP family members and binds to novel DNA target sequences to alter the pattern of cellular gene expression in response to ER stress. When the ER stress is severe, CHOP can activate a programmed cell death pathway. The ability to trigger this response appears to be limited to the full-length mouse GGT protein and the truncated GGTΔ7 protein isoform under the conditions tested here. But the change in cellular phenotype as well as the level of induction of CHOP mRNA was dramatic in these two cell lines. CHOP can be strongly induced by deprivation of nutrients such as glucose as well as the amino acids arginine, leucine, lysine, methionine, phenylalanine, and threonine, but none of these molecules were limiting in our conditions (29). However, reduced Cys is found in Ham’s F-12 and not in DMEM, which has only oxidized Cys, and this could directly impact the redox state of the ER. The lack of CHOP induction in two other stably transfected CHO cells lines overexpressing human glycoproteins hCAR or hMUC1 suggests that this triggering effect may be specific for these GGT isoforms. Further study will be required to understand the basis for the ER stress and the outcome of CHOP induction in these cell lines, but this will provide new insight into a role for GGT and its isoforms in this intracellular compartment. In addition, the GGTΔ7 mouse, an animal model of oxidant stress due to GGT deficiency, allows one to study how the loss of GGT enzyme activity affects the pattern of GGT mRNA splicing. In preliminary experiments, we found that the GGTΔ7 splicing event is increased in the lung of these animals, further supporting a connection between GGT activity and the Δ7 splicing event. The common expression of this event in human GGT suggests that the results of further studies on mouse GGT will be directly relevant to our understanding of human GGT gene expression and ultimately the role of GGT in mammalian cellular physiology and glutathione metabolism.

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