An intronic promoter controls the expression of truncated human γ-glutamyltransferase mRNAs

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Abstract We have identified and characterized a genomic DNA fragment containing the coding sequences corresponding to the human γ-glutamyltransferase type 1 mRNA. The coding part of the gene spans over 16 kb and comprises 12 exons and 11 introns exhibiting a similar organization as for the mouse and rat GGT genes. The exons 1–7 encode the heavy subunit whereas exons 8–12 which encode the carboxy-terminal part of the heavy subunit (exon 8) and the light subunit are clustered in a 1.6-kb *Bgl*II fragment. Exons 7 and 8 are separated by a 3.9-kb intron containing in its 3′ part the sequences corresponding to the 5′-UTRs of the truncated GGT mRNAs described for human lung. Sequence analysis upstream this transcribed region exhibited putative promoter sequences and after transient transfection significant promoter activities were measured in V79 lung fibroblasts and KYN-2 hepatoma cells but not in A2780 ovarian cells. This specificity disappeared when only 550 bp upstream the transcription start site were used as promoter. These results argue for a promoter of truncated GGT mRNAs in intron 7, specifically regulated in human tissues.

Key words: γ-Glutamyltranspeptidase; Truncated mRNA; Intron; Promoter

1. Introduction

Catabolism of glutathione and glutathione conjugates begins with the cleavage of the γ-glutamyl moiety by γ-glutamyltransferase (GGT; EC 3.2.2.2), a heterodimeric glycoprotein which is located at the outer surface of the plasma membrane [1]. The highest GGT activity is found in the renal proximal tubules, small intestine, pancreas and other organs or tissues which have absorptive or secretory functions [2]. It is generally admitted that GGT participates in the active transport of amino acids [1] but also in other important biochemical pathways, such as detoxification or inflammatory responses, by processing GSH-conjugated electrophiles and leukotrienes [3,4]. Modulation of GGT activity is known to occur during development [5], but GGT activity is also elevated in numerous human tissue carcinomas [6,7], in sera from subjects with malignant diseases [8] and it is modulated during cell differentiation [9,10].

GGT is translated from a single mRNA as a single chain precursor polypeptide which is cleaved into two subunits (heavy subunit 55–60 kDa, light subunit 21–30 kDa) during the maturation process [11]. In rat and mouse several GGT mRNAs, having the same open reading frame (ORF) but differing in their 5′ untranslated regions (5′-UTR), have been described [12,13]. These mRNAs are transcribed from a single gene with several tissue specific promoters, each one controlling the expression of a specific mRNA [14–17].

Human GGT belongs to a multigenic family with at least 7 distinct members [18] mostly localized on chromosome 22 [19]. Type I GGT mRNA encodes for an enzyme with catalytic properties identical to the enzyme purified from human tissues [20]. Type I GGT mRNAs isolated from pancreas [21], placenta [22], fetal liver [23], and HepG2 hepatoma [24] have the same ORF but differ in their 5′-UTR, a situation similar to the one observed in mouse and rat. These different 5′-UTRs would play an important role in the regulation of the translation of the GGT mRNA [25].

Several truncated GGT mRNAs of ∼1.2 kb have been described in lung tissue [26]. One of them presents a coding sequence identical to the corresponding part of type I mRNA, has a unique 5′-UTR, and probably is transcribed from the same gene as the type I mRNA.

A determination of the structure of the human GGT gene is therefore necessary to elucidate the mechanisms involved in the generation of these multiple mRNAs. In this paper, we describe the cloning of the human GGT gene corresponding to the type I mRNAs and its structure as deduced from the nucleotide sequence. Furthermore we present evidence that the truncated mRNAs are transcribed in a tissue specific manner from a promoter contained within intron 7 of this gene.

2. Materials and methods

2.1. Genomic library screening

A human genomic library was generously provided by Dr. M. Goossens, Créteil, France and Dr. C.Y. Lau, San Francisco, USA. This library was prepared by insertion of *Mbo*I partially digested human genomic DNA at the *Bam*HI site of the cosmid pCV 105. 125 000 clones representing 1.25 copies of the human genome were screened with the HepG2 cDNA [24] as probe. Clones harboring the

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Abbreviations: GGT, γ-glutamyltransferase; kb, kilobase; UTR, untranslated region; Denhardt's, 1% Ficoll, 1% polyvinylpyrolidone, and 1% bovine serum albumin; PCR, polymerase chain reaction; 1×SSC, 0.15 M NaCl/0.015 M sodium citrate

The sequence reported here was submitted to EMBL databank under the accession number Y09833. Additional sequences of the human GGT gene (5′-UTR exons and coding exons) were submitted under the accession numbers AJ006806, AJ006854, AJ230125, AJ007378, AJ007379 and AJ007380.

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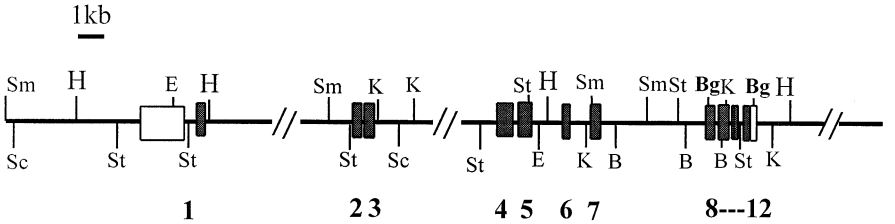

Fig. 1. Genomic structure of the coding region of human GGT gene. Exons are denoted by solid boxes, the 5′- and 3′-UTR are denoted in open boxes. Exons and introns are drawn to scale except for introns 1 and 3 where the length is approximative (see also Table 1). Enzymes used for the restriction maps are: B: *Bam*HI; Bg: *Bgl*II; E: *Eco*RI; E5: *Eco*RV; H: *Hin*dIII; K: *Kpn*I; P: *Pst*I; Sc: *Sac*I; Sm: *Sma*I; St: *Stu*I; X: *Xho*I.

sequences corresponding to the type I GGT mRNA were identified by the PCR/hybridization method developed by Courtay et al. [18].

2.2. Exon-intron structure determination

Purified cosmid DNA was subjected to restriction mapping and Southern blot analysis by standard techniques [27]. Three *Hin*dIII restriction fragments (9H1: ~4.5 kb, 9H2: ~6 kb and 9H3: ~7 kb), which hybridized to human GGT cDNA or oligonucleotide probes derived from the cDNA sequence, were gel purified and subcloned into pBluescribe or pUC19 vectors. The nucleotide sequence of the subclones was determined by manual dideoxy sequencing (T7 sequencing kit, Pharmacia), or in the case where some exons were localized into very large restriction fragments (exons 2–6) they were directly sequenced by ESGS (Montigny le Bretoneux, France) using internal primers derived from the cDNA sequence. The particular intron containing the 5′-UTRs of the truncated GGT mRNAs was amplified from the cosmid by PCR using Amp8 (5′-GCAGTCTCTA-GACCCGGG-3′) and Amp9 (5′-CAAGTTTGTGGATGTGACT-GA-3′) primers and the amplification product was subcloned in the pCRII vector (Invitrogen) for restriction mapping and sequencing.

2.3. RT-PCR analysis

Total RNA from human kidney (2 μg) and poly(A)$^+$ mRNA from human lung (0.5 μg) (Clontech) were reverse transcribed in 50 μl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each dNTP, 40 units RNasin and 200 units of MuMLV reverse transcriptase (Promega) for 2 h at 37°C.

Two μl of the above reaction were used for PCR amplification as described [18], either with Amp1-Amp2 primers or with Amp11 (5′-GGCAGCGAAGAACTCGGAGG-3′, position +4 to +24)-Amp12 (5′-AGGGAGTCAGACTGGTCA-3′, position −186 to −170) primers which were derived from the sequence of the truncated GGT cDNA [26]. PCR products were analyzed by agarose gel electrophoresis, blotted and hybridized with the ^{32}P-labelled oligonucleotide 5′-CTGCCGAGACCCCAGAGCTGGC-3′ which hybridizes in the 5′-UTR (position −13 to −34) of the truncated GGT mRNAs [26].

2.4. GGT-luciferase chimeric plasmids

A 3.6-kb fragment of the particular intron was amplified from cosmid DNA by PCR using 2 primers, Amp9S (5′-AGAGCTC-CAAGTTTGTGGATGTGACTGA-3′) which derives from the GGT intron sequence and comprises a *Sac*I restriction site in its 5′ end and Amp10 (5′-AAGATCTATACCAGTGTGACTCCCT-3′) which derives from the sequence of the 5′-UTRs of the truncated GGT mRNAs and comprises a *Bgl*II restriction site in its 5′ end and cloned in the corresponding sites of the pGL3 enhancer vector (Promega) to construct the pGL3-INT chimeric plasmid. The pGL3-INT was digested by *Bgl*II and *Bam*HI and the ~550-bp fragment, containing the most 3′ sequences of the putative promoter, was cloned in the *Bgl*II site of the pGL3 enhancer to construct the pGL3-INTBa chimeric plasmid.

2.5. Cell culture and transfections

The Chinese hamster lung fibroblasts V79, the human hepatoma cell line KYN-2 and the human ovarian cell line A2780 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. These cells were cotransfected with 2 μg of a luciferase construct and 0.5 μg of SV40-β-galactosidase per well using lipofectamine according to the manufacturer's protocol. The cells were cultured for 48–60 h and cell extracts were prepared by resuspending the cells in 1 ml of 1× Reporter lysis buffer (Promega). Luciferase activity was measured with the luciferase activity assay kit (Promega). β-Galactosidase activity was determined in every extract with the β-gal assay kit (Promega) to correct for differences in transfection efficiency.

3. Results

3.1. Structure of the human GGT gene

134 cosmids containing GGT related sequences were isolated after screening 1.25 copies of a human genomic DNA library using a HepG2 GGT cDNA [24] as probe. A clone, named c509, was selected for further studies as: (i) the amplification by PCR using Amp1-Amp2 primers generated 280 bp which hybridized to type I mRNA specific oligonucleotide

Table 1
Exon-intron organization of the coding region of the human GGT gene

Exon number	Position[a]	3′ splice acceptor[b]	Exon length (bp)	5′ splice donor[b]	Junction amino acids	Intron length (bp)[c]
1	−7/165	gtgcagCAGAGCCATG	168	GGG AGGgtgagcgg	R^{55}	≥3000
2	166/295	agGAT GCA	129	ACC ACA Cgtgagtgc	R^{99}	134
3	296/382	ccttctagGA AAA GCT	86	CAG AAG Ggtaagcca	G^{128}	≥3000
4	383/575	agGG GGG CTG	192	GTC TTG TGgtggttat	C^{192}	395
5	576/733	tgccccagT GAG GTG	157	GCG GCC Ggt	G^{245}	≥1500
6	734/884	cattgcagGG GGC ATT	150	CTC AAA GGgt	G^{295}	800
7	884/1020	atacggagG TAC AAC	136	ACT GAGgtaagggg	E^{340}	≈3800
8	1021/1210	cggcagGTG GTC	189	AAC CTC TAgt	Y^{403}	216
9	1209/1338	agC TTT GGC	129	CAG CCA Ggtaataga	G^{446}	88
10	1337/1449	tgcagcagGG AAG CAG	112	GCA CTGgtatgtgt	L^{483}	81
11	1450/1558	tgccccagGCC ATC	108	AAC ATT Ggt	D^{520}	286
12	1559-end	ctagACCAGG	>300			

[a]Exon position is indicated by nt numbers at its 5′ and 3′ limits according to the published cDNA sequence (accession no. X60069).
[b]Nucleotide sequences across the intron-exon junctions are shown, exon sequences in capital and intron sequences in lower-case letters.
[c]Sizes were determined either by complete sequencing or by estimating the distance between exons on the restriction map.

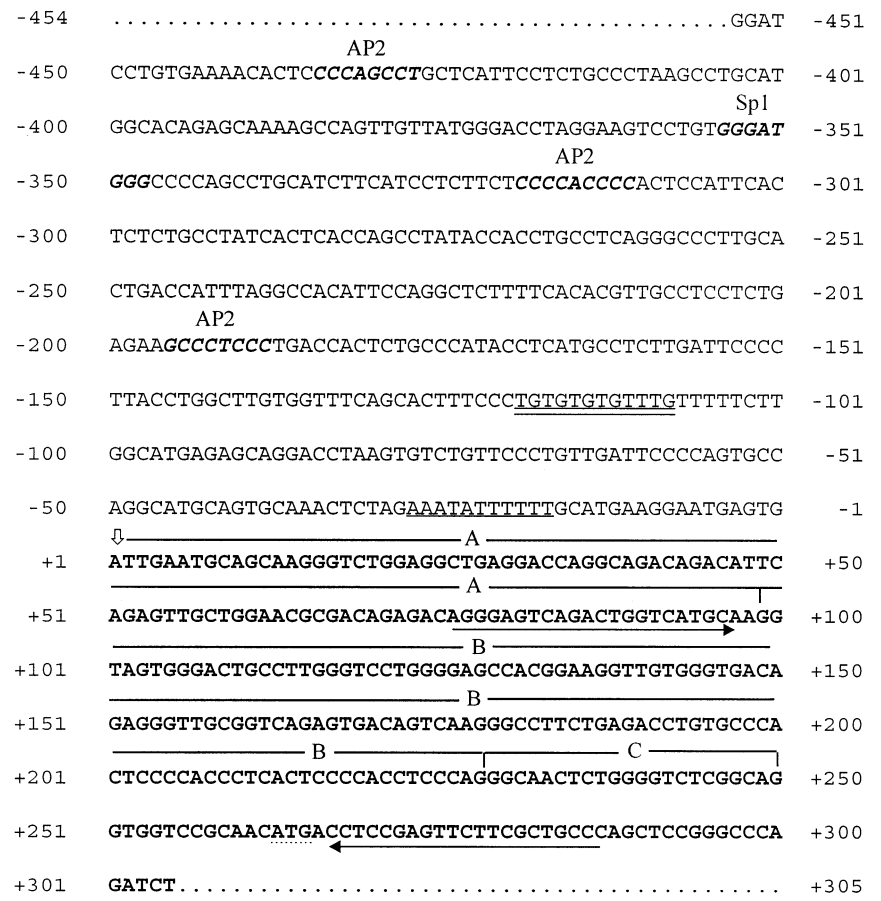

Fig. 2. Partial nucleotide sequence of the intronic promoter. Nucleotides are numbered from the first nucleotide (showed by an arrow) of the published mRNA sequence [26]. Nucleotides present in the mRNA are printed in bold face. Regions A, B, and C of the 5′-UTR are delimited by upper solid lines. The ATG initiator is dashed underlined. The A/T rich region is underlined and the GT repeat is double underlined. The consensus sequences for Sp1 and AP2 transcription factors are in bold italic. The location of Amp11 and Amp12 primers are underlined by arrows.

[18]; (ii) they hybridized with an oligonucleotide corresponding to nucleotides +1 to +20 of the type I GGT mRNA [21]; (iii) their partial restriction map fitted with the corresponding map of gene 6 [18] (results not shown), and the sequence of the exons was determined and was found to be 100% identical to the type I GGT mRNA.

As shown in Fig. 1 cosmid 509 contains all the coding exons, the 3′-UTR and a part of the 5′-UTR corresponding to bases 199–480 of the HepG2 GGT mRNA [24]. The coding sequences of the human GGT gene are divided in 12 exons, spanning over approximatively 16 kb, and they are arranged in a similar manner as for the mouse and rat GGT genes [12,13].

The intron-exon junctions were determined by comparing the gene and mRNA sequences [21] and are summarized in Table 1. Exon 1 contains the last 7 bp of the common 5′-UTR, the ATG start codon, the first 54 codons and part of codon 55. Exons 1–7 correspond to the mRNA sequences encoding for ~98% of the heavy subunit and are spread over 16 kb. Exon 8 encodes for the end of the heavy subunit and the beginning of the light subunit, where the proteolytic cleavage of the precursor polypeptide occurs. Exons 9–12 encode for the remaining of the light subunit and are comprised in a 1.6-kb *Bgl*II fragment in accordance to Courtay et al. [18]. Exon no. 12 consists of 151 bp of coding sequence, the TGA stop codon and the 3′-UTR including the polyadenylation signal. All the intron-exon junctions are in good agreement with the GT/AG rule.

3.2. *Transcription of truncated mRNAs is controled by an intronic promoter*

Exon 7 and exon 8 are separated by a ~3.9-kb intron (intron 7, Fig. 1). Partial sequencing of this intron revealed that it contained in its 3′ end the distinct 5′-UTR of the truncated GGT mRNAs described for human lung [26]. A 759-bp *Bam*HI-*Bgl*II fragment corresponding to the 3′ part of this intron was subcloned and sequenced (Fig. 2).

The value +1 was attributed to the base corresponding to the first nucleotide of the truncated mRNAs as described by Wetmore et al. [26] and was confirmed by in vitro transcription/primer extension experiments (not shown). Thus bp +1 to +262 correspond to the 5′-UTR of the truncated GGT mRNAs and are organized in three regions (Fig. 2): region A (+1 to +99), region B (+100 to +228) and region C (+229 to +250), and are separated by GT/AG intron-exon junctions, consistent with the proposed alternative splicing events at the origin of various types of truncated GGT mRNA [26]. Upstream of the 5′-UTR there is no TATA box but an A/T rich 'TATA-like' region (AAATATTTTT) centered at −23. No CAAT boxes are present but consensus sequences for

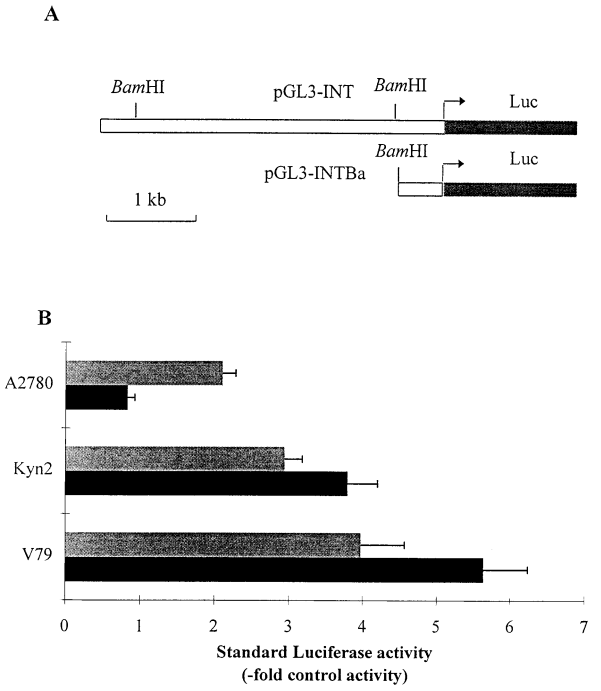

Fig. 3. Analysis of promoter activity by transient expression. A: Schematic representation of constructs driving the luciferase reporter gene. B: Analysis of the expression of GGT-luciferase constructs. Transfections were carried out with the pGL3-INT (■) (a ∼3.6-kb insert from the intron used as promoter) or the pGL3-INT B (▨) (∼550 bp of the most 3′ region of the above fragment) in V79, KYN-2, and A2780 cells. Each value was corrected for transformation efficiency as reported in Section 2 and expressed as -fold activity of the control (promoter-less) pGL3 vector and is the average of three values obtained from three separate experiments.

potential binding of the Sp1 factor [28] were found at positions −355 to −348 and for the AP2 factor [29] at positions −435 to −428, −320 to −312 and −196 to −189. A GT motif (TGTGTGTGTTTG) homologous to the core sequence of many enhancers [30] is found between nucleotides −120 and −109.

To ascertain whether the truncated mRNAs arose from tissue specific splicing of the complete GGT mRNA or from a distinct promoter we subcloned two DNA fragments in the reporter pGL3 enhancer vector (Fig. 3A): (i) the whole intron as amplified with primers Amp9S and Amp10 (corresponding to nucleotides +94 to ∼−3600, pGL3-INT); (ii) the ∼550-bp fragment corresponding to nucleotides −454 to +94 (pGL3-INTBa).

V79 lung fibroblasts transfected with the whole intron (pGL3-INT) as promoter showed the highest activity (5.5-fold over the control promoterless pGL3 plasmid) representing approximately 50% of the luciferase activity driven by the late SV40 promoter (Fig. 3B). In the same cells the pGL3-INTBa construct showed a 4-fold enhancement of luciferase activity. Promoter activity in the KYN-2 hepatoma cells was also important but less than in the V79 cells, while the pGL3-INT was ineffective in the A2780 ovarian cancer cells. These results show that this intron exhibits a promoter activity and that its expression is tissue specific.

3.3. Expression of the truncated mRNAs in lung and kidney tissues

The tissue expression of the truncated mRNAs was examined by specific RT/PCR in RNA from human kidney and lung. As expected Amp1-Amp2 amplifications gave one major band of 217 bp (Fig. 4A, lanes 2 and 4) for both tissues, while Amp11-Amp12 amplification gave a less intense 211 band, which corresponds well to the theoretical size, and many faint smaller bands (Fig. 4A, lanes 1 and 3). Upon hybridization with the probe specific for 5′-UTR of the truncated mRNAs several bands were revealed. The 211-bp band and a 140-bp band were present in both tissues, while in lung two additional bands of 174 bp and 95 bp hybridized with the probe (Fig. 4B, lanes 1′ and 3′). As expected the products from the Amp1-Amp2 amplifications gave no signal (Fig. 4B, lanes 2′ and 4′). The 211-bp band should correspond to a truncated mRNA whose 5′-UTR is composed of the regions A, B and C (Fig. 2) and the 95-bp product could correspond to the 5′-UTR composed of the regions A and C (Fig. 2). These results are in good agreement with the splicing events proposed by Wetmore et al. [26]. The additional intermediate fragments of 174 bp and 140 bp indicate that region B cannot be considered as unique and most probably contains internal splicing acceptor and donor sites.

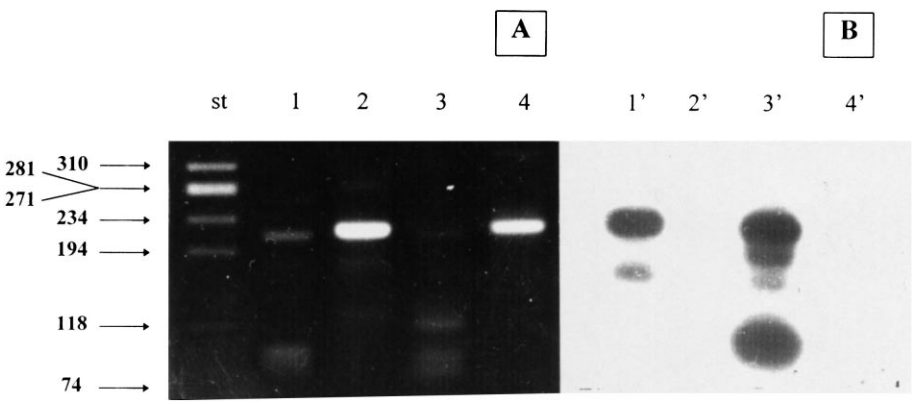

Fig. 4. RT-PCR analysis of the expression of the truncated mRNAs in human tissues. A: Ethidium bromide stained gel; st ϕX174/*Hae*III fragments (the bp length is shown on the right); lanes 1 and 2: RT/PCR of human lung poly(A)$^+$ mRNA; lanes 3 and 4: RT/PCR of human kidney RNA; in lanes 1 and 3 PCR was performed with the Amp11-Amp12 primers specific for the truncated mRNAs; in lanes 2 and 4 PCR was performed with the Amp1-Amp2 primers. B: Southern blot of the above gel using a ^{32}P-labelled oligonucleotide specific for the truncated GGT mRNAs; samples in lanes 1′, 2′, 3′ and 4′ correspond to lanes 1, 2, 3, and 4 in A.

4. Discussion

We report here the cloning and characterization of the GGT gene which is ubiquitously expressed in human tissues. The coding sequence perfectly corresponds to the sequences of GGT mRNAs isolated from pancreas, placenta, fetal liver and HepG2 hepatoma [21–24], confirming that these mRNAs are transcripts from the same gene. The coding sequence is divided in 12 exons which are organized in 5 regions containing exon 1, exons 2 and 3, exons 4 and 5, exons 6 and 7 and exons 8–12, respectively. These regions are separated by large introns (>1500 bp) whereas within each region the introns are smaller (<800 bp). A similar pattern is observed for rat and mouse GGT genes but the corresponding introns are smaller than in human [12,13].

In mouse and rat, the different GGT mRNAs are transcribed from a single gene under the control of multiple tissue specific promoters [14–17]. This organization leads to the expression of multiple mRNAs with an identical open reading frame differing in their 5′-UTR. Furthermore, no truncated mRNAs have ever been detected in rodent tissues. In human, the organization of the GGT gene and the presence of mRNAs differing in their 5′-UTR supposes a similar regulation and expression pattern. The major difference is that altered and/or truncated human GGT mRNAs have been reported.

Such altered GGT mRNAs have first been described for fetal and adult liver and were demonstrated to be present in many other tissues [31,32]. They exhibit a 22-bp insertion which leads to a premature stop codon and shortens the open reading frame to 1098 bp in comparison to 1710 bp for type I mRNAs. These extra 22 bp are localized at the 3′ end of intron 7, confirming that these mRNAs are transcribed from the same gene as the type I mRNAs. Inside these truncated transcripts a second putative open reading frame is observed with its initiation codon in a favorable environment for the initiation of translation [32]. This second open reading frame matches perfectly with the one reported for the truncated mRNAs found in lung tissue [26]. In this intron and upstream of these 22 bp we observed the sequences of the 5′-UTR of the truncated mRNAs. These sequences contain the three adjacent regions A, B, and C observed in the cDNA sequence [26] and which are not interrupted by introns. Complex splicing events seem to take place in a tissue specific manner resulting in a marked heterogeneity of this 5′-UTR. By specific PCR we have shown that these 5′-UTRs can be composed of regions A and C, or of regions A, B and C (found in kidney and lung tissues) or of regions A, C and a part of region B (found in lung tissue only).

Another interesting feature of the human GGT gene is that the truncated mRNAs are transcribed from a promoter present in the intron containing the specific 5′-UTRs. Transfection studies show that the $\sim$3.6-kb sequence immediately upstream of the supposed first mRNA base has a high promoter activity in lung fibroblasts while this activity is undetected in ovarian cells. Promoter activity is also supported by a 545-nucleotide sequence 3′ fragment which contains AP2 and Sp1 binding sites and a GT repeat. In contrast to the $\sim$3.6-kb fragment, the 550-bp 3′ fragment exhibits a promoter activity in ovarian cells, indicating that the upstream sequences could contain a putative *cis* acting element conferring tissue specificity to the promoter.

In conclusion, our data indicate that three mRNA with different ORFs can arise from a single GGT gene: one type contains the 'normal' ORF which is translated to an active enzyme; the second contains a 22-bp insertion due to an additional unspliced intronic sequence; and finally the third type comprises the truncated mRNAs transcribed from the intronic promoter.

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