Tissue-specific Expression of Two $\gamma$-Glutamyl Transpeptidase mRNAs with Alternative 5' Ends Encoded by a Single Copy Gene in the Rat*

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Two different cDNAs have been isolated and characterized from a rat kidney cDNA library. The two cDNA sequences are identical in the coding region and in the 144 bases upstream from the initiation codon but have alternate sequences (154 and 138 bases) at their 5' ends. Primer extension analysis on kidney mRNA reveals that both cDNAs are full-length and correspond to two mRNAs of nearly the same size (2142 and 2127 bases). Synthesis of two mRNAs with alternative 5' ends can be explained only by initiation at two separate promoters on the single rat $\gamma$-glutamyl transpeptidase (GGT) gene. The alternate 5' end nucleotide sequences were used as probes to detect the corresponding mRNAs in several rat tissues. In the kidney, the expression of both mRNAs was detected by in situ hybridization in the distal part of the proximal convolutions of the renal tubule. Northern blot analysis of kidney mRNAs reveals that the expression of both mRNAs increases from birth to the adult stage. Neither of these two transcripts is expressed in the liver or in seminal vesicles in which a larger mRNA (2.4 kilobase pair) is transcribed from the same gene. Thus, two GGT mRNAs, initiated on separate promoters on the single GGT gene, are expressed in the rat in a tissue-specific manner and coordinately regulated.

$\gamma$-Glutamyl transpeptidase (GGT)$^{1}$ (($\gamma$-glutamyl)-peptide: amino acid 5-glutamyltransferase, EC 2.3.2.2) is a component of the $\gamma$-glutamyl cycle. It catalyzes the degradation of glutathione into glutamic acid and cysteinyl glycine (1). The two GGT subunits, which are encoded by a common mRNA (2, 3), are located at the outer surface of the plasma membrane and play a key role in interorgan glutathione transport (4).

In the adult liver, GGT is expressed at a low level and is induced by alcohol (5, 6) and carcinogens (7). In the adult kidney, in which no regulation has been reported, the activity is 2000-fold higher than in the normal liver and is located in the proximal convolutions of the renal tubules at the basolateral and at the microvillar surfaces (8). The GGT catalyzes the degradation of the glutathione that reaches the kidney. The degradation products, which are taken up by the cells, permit the synthesis of GSH in that tissue. In order to study the mechanism of GGT expression as well as its cell specificity, we isolated previously a nearly full-length cDNA sequence from a rat kidney library (9, 10), and we demonstrated that, in the rat, GGT is encoded by a single copy gene (11). We now report the expression of the rat GGT gene in several tissues. We characterized two GGT mRNAs from the kidney, which differ only in their extreme 5' noncoding sequences. We show that their expression from two separate promoters on the single rat GGT gene is developmentally regulated in the kidney and under the control of tissue-specific factors.

EXPERIMENTAL PROCEDURES

MATERIALS

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNAse I, DNA polymerase I, S$\acute{e}$ and T7 RNA polymerases, and avian myeloblastosis virus reverse transcriptase were purchased from Promega Biotec, New England BioLabs, or Boehringer Mannheim. Radiolabeled nucleotides, nylon membrane (Hybond) used for RNA transfer, and film for autoradiography (Hyperfilm-MP) were obtained from Amersham Corp. The plasmid pGEM-3 was purchased from Promega Biotec.

METHODS

Isolation and Characterization of the GGT cDNA Clones—A rat kidney cDNA library made from poly(A)$^{+}$ RNA was screened for clones containing GGT sequences as described previously (9). Plasmid DNA, obtained by the alkaline lysis method, was purified on a cesium chloride gradient (12). Restriction enzyme digestions were carried out according to the recommendations of the supplier. The restriction fragments were electrophoresed on a 6% acrylamide gel under non-denaturing conditions, transferred to a nylon membrane, and hybridized to a nick-translated cDNA probe (10$^{6}$ cpm/µg) as described previously (11). The cDNA inserts from clones 12 and 17 (cDNA-12 and cDNA-17) were isolated from the plasmids by a PstI digestion, purified on a low melting agarose gel, and ligated into PstI-digested M13mp18 and M13mp19. The recombinants were introduced in the Escherichia coli TG$^S$ strain by the CaCl$_2$ procedure (13). Single-stranded DNA was sequenced by the dyeoxy chain termination method (14) from the universal oligodeoxynucleotide primer using the Sequenase kit from U. S. Biochemical.

The sequences from cDNA-12 and cDNA-17 (see Fig. 1) which extend upstream from the Real site (see Fig. 1), isolated by a PstI-Ral double digestion, were ligated into the PstI and Smal sites of pGEM-3. The recombinant plasmids (pGEM-3-12, pGEM-3-17) were introduced in the E. coli HB101 strain and sequenced as described for the recombinant phages.

* This work was supported by the Institut National de la Santé et de la Recherche Médicale and by the Université de Paris Val de Marne.

$\ddagger$ Recipient of a fellowship from the Fondation pour la Recherche Médicale and from the University of Paris Val de Marne.

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The abbreviations used are: GGT, $\gamma$-glutamyl transpeptidase; PIPES, piperezine-N,N'-bis(2-ethanesulfonic acid); MOPS, 3-(N-morpholino)propanesulfonic acid; kb, kilobasepair(s); ORF(s), open reading frame(s).
RNA Extraction—Rat tissues were frozen in liquid nitrogen, and RNA was extracted using the guanidium thiocyanate procedure (15). Polyadenylated RNA was isolated by oligo(dT)-trisacryl chromatography according to Aviv and Leder (16).

Synthesis of Oligodeoxynucleotides—Oligonucleotides complementary to the messenger RNA sequence were synthesized using a DNA synthesizer and purified on an Oligosil cartridge (Applied Biosystems) as recommended by the supplier. These oligonucleotides correspond to the sequence mapping at the position -145 to -190 from the initiation codon on cDNA-12 (oligonucleotide A) and on cDNA-17 (oligonucleotide B) and to the nucleotides -58 to -88 on the sequence common to the two cDNAs (oligonucleotide C) (see Figs. 1 and 3).

 Primer Extension Analysis—The synthetic oligonucleotides were 5'-end 32P-labeled using T4 polynucleotide kinase and [y-32P]ATP (300 Ci/mmol) at a specific activity of 3.10^10 cpm/µmol. An aliquot of 10^6 cpm was hybridized to 5 µg of kidney poly(A)+ RNA in 0.4 M NaCl, 10 mM PIPES, pH 6.5, and 1 mM EDTA. After ethanol precipitation, the oligonucleotide was extended 1 h at 4°C with 30 units of T7 polymerase and 200 µM dNTPs, 100 µM dithiothreitol, 1 mg/ml RNase A, 10 mM dithiothreitol, 0.5 mM ATP, CTP, and GTP; 1 unit/µl RNase. The reaction products were fractionated on a 6% polyacrylamide, 7 M urea gel and then analyzed by autoradiography. The size of the extended fragments was determined by comparison with an unrelated sequence ladder run on the same gel.

 RNA Analysis on Northern Blot—Poly(A)+ RNA (10-10 µg) in 30 µl of 20 mM MOPS, pH 7, 8 mM sodium acetate, 1 mM EDTA, 50% formamide, and 6% formaldehyde was heated at 65°C for 5 min; 2 µl of loading solution (50% glycerol, 0.5% bromophenol blue) was added to the samples which were then electrophoresed through a 1.5% agarose gel containing 2.2% formaldehyde and then transferred to a nynlon membrane.

The RNA sequences were cross-linked to the membrane by a 5-min exposure to a 302 nm UV lamp and baked at 80°C for 2 h. The blots were prehybridized overnight at 60°C in 50% formamide, 5% sodium dodecyl sulfate, 2 x SSC, 10 mM dithiothreitol, 1 mg/ml sheared salmon sperm DNA, 0.6 mg/ml yeast tRNA, and 1 x 10^6 cpm/ml 32P-labeled cRNA probe. In each experiment, a RNA sense probe of the same specific activity was used as a control. After hybridization, the slides were washed three times for 10 min at 52°C in 50% formamide, 2 x SSC and once in 2 x SSC. They were then incubated in 2 x SSC containing 10 µg/ml RNase A for 30 min at 37°C, washed using the conditions stated previously, and dehydrated in ethanol. The sections were then dried and kept at 4°C before autoradiography. Some sections were directly exposed to XAR-5 film before the addition of the autoradiographic emulsion. For autoradiography, the slides were dipped at 40-42°C in Kodak NTB-3 emulsion, diluted 1:1 with distilled water, exposed for 1-3 weeks at 4°C, developed in Kodak Dektol developer, and fixed in Kodak rapid fixer. The slides were subsequently stained with hematoxylin-eosin.

Genomic DNA Southern Blot Analysis—High molecular weight genomic DNA was extracted from Wistar rat kidney as described previously (11). DNA aliquots of 50 µg, digested by EcoRI, BamHI, or EcoRI-BamHI, were fractionated on a 0.8% agarose gel and transferred to a nynlon membrane. The blots were hybridized to a nick-translated cDNA probe (10^6 cpm/µg) as described previously (11).

RESULTS

Isolation and Characterization of the GGT cDNA Clones—The GGT cDNA sequence that we published earlier (cDNA-1) contains 2072 bases including a 227-base 5'-untranslated region (Fig. 1) (9). An oligonucleotide primer complementary to nucleotides -58 to -88 from the initiation codon (oligonucleotide C) (Fig. 1) was extended on kidney poly(A)+ RNA. A major extension product appears on the gel as a doublet with a size of 220 ± 2 bases (Fig. 2, I, lane C). A longer but minor extended cDNA (241 bases) is also synthesized but is hardly detectable on Fig. 2, I, lane C. This indicates that the 5'-end of the kidney mRNA maps at least at -277 + 9 bases upstream from the GGT initiation codon. In order to obtain the complete sequence, two alternative 5' end sequences from cDNA-12 and cDNA-17, which cover two alternative 5' end sequences in pGEM-3 vector for the synthesis of cRNA-12 probe and a 127-base cRNA-17 probe, respectively.
full cDNA sequence, we analyzed the other cDNA clones purified from our kidney cDNA library (9).

Restriction mapping and hybridization analysis reveal that among 40 GGT cDNA clones, 2 of them (cDNA-12 and cDNA-17) extend upstream the sequence of cDNA-1 (Fig. 1) referred to as clone 1 in (9). The restriction map of these clones reveals two different patterns (Fig. 1). cDNA-17 has a Sac1 site in its 5' part which is also present in cDNA-1. However, this restriction site is not present in cDNA-12 in which HindIII, PvuII, and HincII sites, absent from cDNA-1 and cDNA-17, are found.

The nucleotide sequences, determined from these two clones, are reported on Fig. 3. The cDNA-17 (331 bases) includes a 5'-untranslated region of 282 bases and extends up to the nucleotide +49 in the reading frame. This sequence is 100% homologous to the sequence reported previously for cDNA-1 but is longer by 55 bases at its 5' end. In cDNA-12 (317 bases), a sequence of 163 bases corresponding to its 5' end is also identical to the sequence found in cDNA-1, but its 5'-region (154 bases) exhibits no homology with the other clones. Therefore, there are two different GGT messenger RNAs in the kidney, the sequences of which are different only in their 5'-untranslated region. The divergent sequences are located upstream of the base -144 from the initiation codon (Fig. 3).

The 5'-untranslated sequence from cDNA-12 (288 bases) contains an ATG codon at -186 followed by a stop codon at -171, which delineate a short open reading frame (ORF) out of frame with the downstream GGT coding sequence, and which could code for 5 amino acids. In the 282-base-long 5'-untranslated region of the other cDNA (cDNA-17), one ATG was found at -270 and a second one at -262; they start two short ORFs of 48 and 34 amino acids, respectively, which are both out of frame with the GGT coding sequence. In that cDNA-17, a sequence of 18 bases (-191 to -174) that includes a g-base pair inverted repeat is 78% homologous to another cDNA-12 (oligonucleotide A) and cDNA-17 (oligonucleotide B) that has nearly the same inverted repeat. The oligonucleotide sequences (A, B, and C) are indicated. Vertical bars indicate identical bases in both cDNAs.

Two oligonucleotides complementary to sequences -145 to -190 of cDNA-12 (oligonucleotide A) and cDNA-17 (oligonucleotide-B) were hybridized to kidney poly(A)+ RNA. They were extended using reverse transcriptase to determine whether the two alternative sequences include the 5' end of the corresponding mRNAs. The longest product obtained from the two cRNA-12 (.oligonucleotide A) and cRNA-17 (oligonucleotide B) was found at -270 and a second one at -262; they start two short ORFs of 48 and 34 amino acids, respectively, which are both out of frame with the GGT coding sequence. In that cDNA-17, a sequence of 18 bases (-191 to -174) that includes a 6-base pair inverted repeat is 78% homologous to another sequence in the mRNA located near the start codon (-37 to -19) that has nearly the same inverted repeat.

Northern Blot Analysis of Rat Kidney mRNA—We examined the expression of the two mRNAs in the kidney during ontogeny. For this purpose, we subcloned the two alternative 5'-end sequences into an expression plasmid (pGEM-3) in order to synthesize an RNA probe complementary to the GGT mRNA with a high specific activity. Hybridization of these two probes to a Northern blot of kidney poly(A)+ RNA revealed that both sequences hybridize to a 2.2-kb mRNA (Fig. 4, A and B). From birth to adulthood, there is an 8-fold increase in the level of each mRNA species. At every developmental stage, the mRNA that hybridizes to cRNA-12 is predominant, and the ratio of both mRNAs (2.5) remains unchanged.

In Situ Hybridization of cRNA Probes to Rat Kidney Sections—Direct autoradiography of kidney sections from adult

**Fig. 2.** Analysis of the cDNA products synthesized on the 5'-terminal region of GGT mRNA by primer extension. 5' end-labeled oligonucleotide primers (100'000 cpm) were extended on 5 μg of kidney poly(A)+ RNA as described under "Methods." The extended cDNAs were analyzed on a 6% acrylamide denaturing gel. The autoradiography was performed for 48 h at -80 °C using an intensifying screen. In I, the oligonucleotide C (see Fig. 3) was used as a primer (lane C), and the size of the extended fragments was determined by comparison with 5' end-labeled fragments of cX74 DNA digested by HaeIII (lane M). A major extension product (220 ± 2 bases) (+) was obtained; the 5' end of this cDNA maps at -277 ± 2 bases from the GGT initiator codon. In II, oligonucleotide A or B (see Fig. 3) was used to prime the cDNA synthesis, and an unrelated sequence ladder was used to determine the size of the extended cDNA (lane M). From oligonucleotide A, a major cDNA (133 ± 2 bases) (−) and also a minor but longer one (153 ± 4 bases) (−) were detected (lane A). Thus, the 5' ends map at -277 and at -297 ± 4 bases from AUG (+1) From oligonucleotide B, the longest cDNA (132 ± 2 bases) (lane B) maps at -276 ± 2 bases from the AUG (+1).

**Fig. 3.** Comparison of the nucleotide sequences of GGT cDNA from pBR-12 and pBR-17. The sequence was determined on both strands using the dideoxy chain termination method as indicated under "Methods." The nucleotides were numbered from the 5'-end of the corresponding mRNA, located near the start codon (−37 to -19) that has nearly the same inverted repeat.
Kidney poly(A)+ RNA (1 fig) from rat at different developmental stages (lanes 1, newborn; lanes 2, 6 days old; lanes 3, 21 days old; lanes 4, adult) was electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized to the cRNA-17 probe (A) and cRNA-12 probe (B) as described under "Methods." Autoradiography was performed overnight using two intensifying screens at -80°C.

The signal is significantly lower after hybridization with the cRNA-17 GGT probe (Fig. 5B) than with the cRNA-12 GGT probe (Fig. 5A).

Optical microscopy of autoradiographs shows that both GGT mRNA transcripts are expressed in the proximal tubules localized in the inner cortex (i.e., pars recta), the outer medulla, and the cortical medullary rays of kidney from adult rat (Fig. 5, C and D). The localization of GGT mRNA transcripts is identical after hybridization with either cRNA-12 (Fig. 5C) or cRNA-17 (Fig. 5D) probes. However, the level of the signal in positive tubules, i.e., the number of GGT mRNA transcripts, is higher with the cRNA-12 probe than with the cRNA-17 probe. A similar distribution of the two GGT mRNA transcripts is observed in kidney sections from 18-day-old fetus (Fig. 5, E and F); the hybridization signal is lower than in the adult kidney, and the mRNA recognized by the cRNA-12 is expressed at a higher level (Fig. 5E) as in the adult kidney. In control experiments using the sense 35S-labeled probe, no signal was detected under the same conditions (data not shown). Proximal tubules have been identified as those exhibiting a brightly pink stained lumen after periodic acid-Schiff reaction.

**Northern Blot Analysis of GGT mRNA from Various Tissues**—The probes cRNA-17 and cRNA-12 were hybridized to Northern blots loaded with poly(A)+ RNA from several rat tissues (Fig. 6). GGT mRNA transcripts complementary to cRNA-17 and to cRNA-12 are detected only in the kidney sample (Fig. 6A and B, lanes 1). A second hybridization of these two blots to the cRNA-159 probe, which is complementary to 123 bases in the coding region, reveals after a 5-h autoradiography the 2.2-kb GGT mRNAs in the kidney (Fig. 6C, lane 1), which were already found after hybridization to cRNA-12 or to cRNA-17. This probe also revealed a 2.4-kb GGT transcript in the fetal liver (Fig. 6C, lane 3) and in the seminal vesicles (Fig. 6C, lane 4), which was not detected in A or B. No signal was detected in liver (Fig. 6C, lane 2) or in testis samples (Fig. 6C, lane 5). After 2 days autoradiography, the 2.4-kb GGT mRNA appears also in the liver but not in the testis (data not shown). These data reveal two different patterns for GGT mRNA expression. One is observed in the kidney and a second one in the liver or seminal vesicles. The relative amounts of total GGT mRNA transcripts determined by the densitometry of the autoradiogram (Fig. 6C) and expressed/μg of poly(A)+ RNA are as follows: kidney, 100; seminal vesicles, 4; and fetal liver, 2.

**Southern Blot Analysis of Rat Genomic DNA**—Two nick-translated cDNA probes, corresponding to the two alternate sequences of cDNA-12 and cDNA-17, were hybridized to genomic DNA digested by EcoRI and BamHI. Both sequences hybridized to the same DNA fragments of 15 and 18 kb.
obtained by EcoRI and BamHI digestions, respectively (Fig. 7).

**DISCUSSION**

Two GGT cDNAs are transcribed in the rat kidney; they have nearly the same size (2143 and 2127 bases) and cannot be separated on a Northern blot. The two mRNAs diverge only at the 5' end of their 5'-untranslated region and therefore may code for the same protein. According to the cDNA sequence of the cDNA-12, which is full-length, the initiation site of the corresponding mRNA maps at -298 bases upstream from the GGT initiation codon, and this corresponds to a minor cDNA obtained by primer extension analysis. However, the major products extended on that RNA are around 20 bases shorter. This could indicate that there are multiple initiation sites within the same transcript unit, yielding two mRNAs colinear in their 5' ends but of slightly different sizes. This has been described only for housekeeping genes that lack the conventional TATA and CAAT boxes (19-22); all the transcripts for these genes are initiated within 20 or 30 bases on the same transcription unit. Another possibility could be that the major cDNA fragment, mapping at -277 from the AUG, corresponds to a premature termination of the primer extension reaction caused by a secondary structure in the template, or it could be due to the fact that some nucleotides of the mRNA, engaged in a secondary structure, would have escaped the transcription reaction. The length of the other cDNA sequence (cDNA-17) and the size of the primer extension product, corresponding to the alternate messenger RNA, coincide within a few bases. Therefore, the two GGT cDNAs include the complete 5' end of the two mRNAs.

Among vertebrate mRNAs, only a few have 5'-untranslated regions of more than 200 bases (23), but there is no evidence that long 5'-untranslated regions alter the translation efficiency. The short ORFs found in the 5'-untranslated regions of the GGT mRNAs should not decrease significantly the efficiency of initiation at the downstream GGT initiation codon since they do not overlap with the GGT reading frame (24, 25); moreover the AUG codons in the 5'-untranslated regions of the GGT mRNAs are surrounded by sequences that fit poorly with the consensus sequence required for efficient initiation (23). According to the termination reinitiation model proposed for polycistrionic RNA translation, the initiation of GGT at an internal AUG can be very efficient since this codon is surrounded by a sequence that is almost identical to the consensus sequence (23, 26).

Several lines of evidence indicate that these two mRNAs with alternate 5' ends originate from two independent promoters on the same gene: (i) the sequence specific for each cDNA maps the 5' terminus of the corresponding mRNA; (ii) the two divergent sequences are colinear in a genomic DNA fragment of 15 kb; (iii) the GGT gene is represented only once per haploid genome (11). The difference in the level of these two GGT mRNAs in the kidney reflects either a different efficiency of these two promoters or an unequal stability of the two mRNAs. *In situ* hybridization, with specific cRNA probes, clearly demonstrates that the two GGT mRNA transcripts are expressed in the same segments of the distal part of the proximal tubules in the kidney from fetus and adult rat. Moreover, the difference in the level of these two GGT mRNAs is confirmed by these studies, *i.e.* a lower signal is observed with cRNA-17 than with the cRNA-12.

The two GGT mRNAs expressed in the kidney are not transcribed in the other tissues investigated, and a longer transcript (2.4 kb) is expressed in liver and in seminal vesicles. Therefore, three different GGT mRNAs are encoded from the single GGT gene. The two kidney transcripts are initiated on two separate promoters as discussed above. The 2.4-kb mRNA has been reported previously in liver cells treated with aflatoxin B1 or ethoxyquin (27); its sequence is identical to the two kidney mRNAs from the poly(A)" tail to the nucleotide 144 bases upstream from the initiation codon, except for two single-base modifications in the coding region and seven additional bases at the 3' end of the liver mRNA; thus, three GGT mRNAs have alternate 5' ends from that point (27). The complete sequence of the 5'-untranslated region of the 2.4-kb mRNA is not yet known, but it should account for the 200 extra bases found on this transcript by Northern blot analysis. The 2.4-kb GGT mRNA could be synthesized either by alternative splicing of the kidney primary transcripts or by a initiation on a third promoter on the rat GGT gene. Several eukaryotic genes are known to encode mRNAs differing only in their 5'-terminal ends by the use of two alternative promoters with or without alternate splicing (26, 28-31), but to our knowledge only the gene coding for insulin-like growth factor II is known to be transcribed from three promoters (32).

The GGT mRNA transcripts have alternative 5' ends associated with a common coding sequence, therefore they potentially produce the same protein. Synthesis of multiple transcripts from several promoters and/or by alternative splicing allows several possibilities for modulation of the gene expression at the RNA or at the gene level. At the RNA level, the stability and/or translational capacity of the mRNA can be differentially altered by sequences located upstream from the initiation codon. The sequence of 18 base pairs which is repeated with a high homology in the 5'-untranslated region of the GGT mRNA recognized by cRNA-17 is also repeated, and well conserved, in mRNA from HepG2, a human hepatoma cell line (33). These highly conserved sequences could come from stem-loop structures that could affect the translational capacity and/or stability of this mRNA. Recently, a cytosolic protein has been identified which recognizes a stem-loop structure in the 5'-untranslated region of human ferritin mRNA and acts as a repressor of ferritin translation (34).

Stem-loop structures are also involved in the mRNA stabilization (35). Differential mRNA expression can also be due to a particular 5'-untranslated sequence that is the target for
a hormonal stimulus (36) or that encodes a leader peptide from a short ORF upstream from the main reading frame (37).

Regulation of GGT expression can also occur at the gene level. On a multiple promoter gene, transcription can be regulated independently on one of the promoters in response to a particular stimulus (38), a tissue-specific factor (28), or a particular developmental stage (29). In the kidney, both GGT mRNAs are expressed at the several developmental stages that have been investigated, and the level of the two transcripts increases from the fetal to the adult state. This change in mRNA production and/or stability is parallel to the increase in GGT activity during kidney ontogeny (39). The synthesis of both 2.2-kb GGT mRNAs is also controlled by tissue-specific factors since they were not detected in liver, testis, or in seminal vesicles.

In conclusion, the expression of two GGT mRNAs with alternative 5' ends in the kidney reveals the existence of two independent promoters on the single-copy GGT gene. The expression of both transcripts is coregulated during kidney ontogeny and exhibits a strong tissue specificity. A third GGT mRNA species is transcribed in the liver and in seminal vesicles from a third promoter or by alternative splicing of a common primary transcript. Cloning of these promoters and of their flanking sequences will be a further step in the elucidation of the factors that control GGT gene expression in normal and neoplastic tissues and during ontogeny.

Acknowledgments—We wish to thank Drs. J. Hanoune, M. Aggerbeck, L. P. Aggerbeck, D. Stengel, and N. Ferry for their comments during the preparation of this manuscript and L. Rosario for her skillful secretarial assistance.

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