Identification of a Second Promoter Which Drives the Expression of γ-Glutamyl Transpeptidase in Rat Kidney and Epididymis†

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ABSTRACT: In rat, γ-glutamyl transpeptidase (GGT) is encoded by multiple mRNAs ($mRNA_I$, $mRNA_{II}$, $mRNA_{III}$, and $mRNA_{IV}$) that differ only in their 5′ untranslated regions and are transcribed from a single-copy gene. Using oligonucleotides designed from the 5′ untranslated sequences of the GGT $mRNA_{II}$ and $mRNA_{III}$, we amplified a 3.4-kb genomic sequence which contains the promoter region for $mRNA_{II}$. The sequence flanking the two initiation start sites for $mRNA_{II}$ contains consensus motifs for several potential regulatory proteins and a TATA-like element at the expected position 26 bp upstream from the predominant start site. The sequence from positions −528 to +72 associated with the chloramphenicol acetyltransferase (CAT) reporter gene drives a promoter activity in LLC-PK1, a pig kidney cell line. Deletion analysis revealed that the region from nucleotides −528 to −322 mediates an activation of the promoter activity, whereas the sequence from −322 to −114 has a negative effect. Furthermore, the structural organization of the 5′ end of the GGT gene reveals that the GGT $mRNA_{III}$ is transcribed from a third promoter located upstream from the promoter II on the GGT gene. By Nothern blot analysis, the promoter II was found to be expressed only in the kidney and in the epididymis. We also identified two new mRNA species which are expressed in the H5 hepatoma cells. Therefore, the GGT gene expression reveals a strong tissue- or cell-specific pattern which is based on the transcription of several mRNA species from multiple promoters.

γ-Glutamyl transpeptidase (GGT)[1] [(5-glutamyl)-peptide: amino-acid 5-glutamyltransferase, EC 2.3.2.2.] is a heterodimeric glycoprotein located at the outer surface of the plasma membrane (Tate & Meister, 1981). This enzyme is involved in the metabolism of glutathione and other γ-glutamyl compounds (Tate & Meister, 1981). In rat, as well as in humans, the highest GGT activities are found in the epithelial cells of the kidney, epididymis, pancreas, and small intestine (Tate & Meister, 1981), tissues which are very active in cell detoxication or in protein synthesis. Unlike hepatocytes, all these tissues rely largely on an extracellular supply of cysteine to support the active synthesis of proteins and glutathione occurring in these organs (Meister, 1984). This process involves the extracellular degradation of glutathione by GGT, thus allowing the recovery of extracellular cysteine by the cell (Meister, 1984).

In the kidney and in the epididymis, there is a strong increase in GGT activity during the ontogeny (Wapnir et al., 1982; Agrawal et al., 1988) in contrast to the liver where a sharp decrease occurs from the fetal to the adult stage (Wapnir et al., 1982). In the adult liver, however, there is an induction of that GGT activity in hepatobiliary disorders (Chobert et al., 1989) and during carcinogenesis (Cameron et al., 1978).

GGT is synthesized as a propeptide which is cleaved into its two mature subunits (Barouki et al., 1984). In rat, a single-copy GGT gene (Pawlak et al., 1988) codes for several messenger RNAs which differ only in their 5′ untranslated regions (Chobert et al., 1990; Griffiths et al., 1989; Darbouy et al., 1991). The $mRNA_I$ (2.2 kb) and $mRNA_{II}$ (2.2 kb) have alternate 5′ termini (Chobert et al., 1990) whereas the sequence reported for the GGT $mRNA_{III}$ (2.4 kb) is not full length (Griffiths et al., 1989) (Figure 1). The sequence for $mRNA_{IV}$ (2.5 kb) has not been cloned but experiments have revealed that it differs also in its 5′ untranslated region from the other GGT mRNA species (Darbouy et al., 1991). The organization of these sequences on the GGT gene is partially known (Kurauchi et al., 1991; Rajapopalan et al., 1990). In fact, the most 5′ GGT genomic sequence cloned so far extends 2.7 kb upstream from the initiation codon into the leader exon for $mRNA_{II}$ and includes only the transcription start site for the GGT $mRNA_I$ (Kurauchi et al., 1991).

In the present work, we isolated a 3.4-kb genomic sequence which includes the leader exon for $mRNA_{II}$ and extends to the 59-bp sequence which is specific for the $mRNA_{III}$ cloned from an ethoxyquin-induced liver cDNA library (Griffiths et al., 1989). We mapped the different sequences which are specific for these three mRNAs on the GGT gene; their organization reveals that they are transcribed from three separate promoters. We characterized a second GGT promoter coding for the $mRNA_{II}$ which was found to be expressed in the kidney and in the epididymis.

EXPERIMENTAL PROCEDURES

Genomic DNA was prepared from the liver of Wistar rats (Pawlak et al., 1988). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, T7 RNA polymerase and the plasmid pGEM-3Z were obtained from Promega Biotec; the plasmid pCH110 (β-galactosidase plasmid), which contains the LacZ gene in front of the SV40 early promoter, was obtained from Pharmacia; the deoxyribonucleotides and S1 nuclease were from Boehringer Mannheim, and the Taq DNA polymerase was from Stratagene. Radiolabeled nucleotides, [^{14}C]chloramphenicol, nylon membrane, and hyperfilms MP

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[1] Abbreviations: GGT, γ-glutamyl transpeptidase; CAT, chloramphenicol acetyltransferase.

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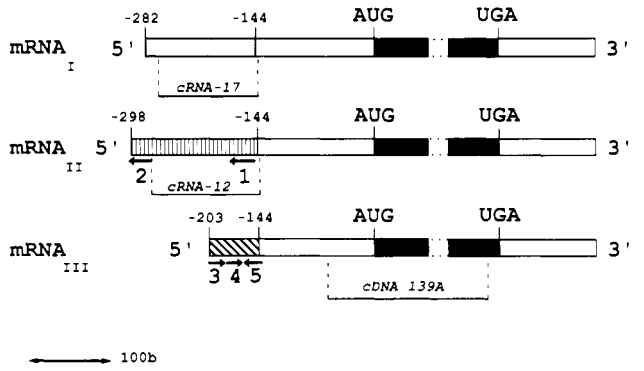

FIGURE 1: Schematic diagram of the GGT mRNA sequences. Oligonucleotides used in the amplification experiments and probes hybridized to the Northern blots are mapped on the GGT mRNA sequences. Probe cRNA-17 is specific for $mRNA_I$; oligomers 1 and 2 and the probe cRNA-12 are specific for $mRNA_{II}$. Oligomers 3, 4, and 5 are specific for $mRNA_{III}$. The cDNA probe 139A is common to the three GGT mRNA species. The coding region (shaded area), the noncoding region common to the three GGT mRNAs (open area), and the sequences specific for $mRNA_I$ (stippled area), $mRNA_{II}$ (hatched area), and $mRNA_{III}$ (striped area) are shown. The numbering is relative to the first base of the initiation codon which is designated +1.

for autoradiography were purchased from Amersham Radiochemical Centre.

The oligodeoxyribonucleotides, designed from the rat kidney cDNA sequences (Chobert et al., 1990) and from the rat liver cDNA sequence (Griffiths et al., 1989) (Figure 1), were synthesized on an Applied Biosystems Model 380B DNA synthesizer and purified on an OPC cartridge as recommended by the supplier. Five oligonucleotides were used as primers in polymerase chain reaction (PCR) experiments: oligomer 1 (5'AGAGGGGCAAGAGGTCAG3'), oligomer 2 (5'GGCAGCAGCAAGGAGGC3') and oligomer 5 (5'AGTGTGAACCCAGGGGAA3') are complementary to the RNA strand; oligomers 3 (5'CCGCAGCACCAGAATAAC3') and 4 (5'CAGCAAGCTCCTGTGTCC3') correspond to the sequence on the RNA strand.

DNA Amplification. The amplification of genomic DNA fragments was carried out in a DNA thermal cycler from Perkin Elmer-Cetus Instruments. Genomic DNA samples (1 μg, digested by *Bam*HI) were subjected to 30 cycles of amplification using 100 ng of each set of two primers and 2.5 units of Taq DNA polymerase in a 100-μL volume. Samples were denatured at 94 °C for 1 min, cooled to 55 °C over 1 min for primer annealing, and heated to 72 °C for a 3-min elongation. After the last cycle, the samples were incubated for an additional 5 min at 72 °C to ensure completion of the final extension step.

Analysis of the Amplified DNA. An aliquot (20 μL) of the amplified DNA was analyzed on a Southern blot by two successive hybridizations to the oligonucleotides 2 and 4, labeled at their 5' end by the T4 polynucleotide kinase with [γ-^{32}P]ATP (Kurauchi et al., 1991). The blot was washed twice for 1 h at 50 °C in 6× SSC, 0.1% SDS.

Cloning and Sequencing of the Amplified DNA. The DNA fragment of 3.4 kb which was amplified from the oligonucleotides 1 and 3 (Figure 2) and which is represented in Figure 3 (fragment A) was digested partially by *Pvu*II and fully by *Hin*dIII. A *Pvu*II–*Hin*dIII fragment of 600 bp was subcloned in the *Sma*I–*Hin*dIII sites of pGEM-3Z, using standard recombinant DNA procedures. The resulting plasmid pGEM-3-1 (Figure 3) was used as a template and hybridized to synthetic oligonucleotides for DNA sequencing. Sequencing reactions were performed using the Sequenase kit from United States Biochemical Corp. in the presence of [α-^{35}S]dATP.

S1 Nuclease Mapping Analysis. Mapping of the 5' end of the kidney GGT mRNA onto the gene was performed according to the Berk and Sharp procedure (Berk & Sharp, 1977). The plasmid pGEM-3-1 was digested by *Hin*dIII, 5'-end-labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP, and further digested by *Bgl*II. The resulting 358-bp *Bgl*II–*Hin*dIII fragment labeled only at the 5' end of the RNA complementary strand was purified on a 1% low-melting agarose gel. The probe (5 × 10^6 cpm) was hybridized to kidney poly(A)$^+$ RNA and digested with 200 units of S1 nuclease for 1 h at 37 °C (Kurauchi et al., 1991). The S1 protected fragments were resolved by electrophoresis on a 6% acrylamide, 7 M urea gel along with a sequence ladder.

GGT-CAT Plasmid Constructions. Several GGT-CAT plasmids were constructed by subcloning GGT genomic sequences, from the nucleotide −528 upstream from the major transcription initiation site to the nucleotide +72 downstream, into the pMW1-CAT, a vector which carries the CAT reporter gene (Gorman et al., 1982). *Sac*I–*Hin*dIII and *Pvu*II–*Hin*dIII fragments from pGEM-3-1 (Figure 3) were cloned at the corresponding sites in the pMW1-CAT vector. The resulting clones contain GGT sequences from nucleotides −528 to +72 (pII −528 +72) and from nucleotides −322 to +72 (pII −322 +72). Four other inserts were prepared from DNA amplified sequences delineated by oligomers designed from nucleotides −322 to −305 (a), −114 to −98 (b), and −30 to −13 (c) on the RNA strand and the oligomer d extending from nucleotides +54 to +72 on the complementary strand (Figure 3). An *Xba*I restriction site was added at the 5' end of oligomer a and a *Sac*I site was added at the 5' end of oligomers b, c, and d. This allowed the insertion of the following sequences into the pMW1-CAT vector: (a) the sequence −322 to +72 in the reverse orientation into the *Sac*I and *Xba*I sites (pII +72 −322); (b) the sequence −114 to +72 into the *Sac*I site in both orientations (pII −114 +72; pII +72 −114); (c) the sequence −30 to +72 into the *Sac*I site (pII −30 +72). The pRSV-CAT plasmid has been previously described (Gorman et al., 1982).

Cell Culture and Transfection Experiments. The rat hepatoma cell line HTC (Richards et al., 1982) and the pig kidney cell line LLC-PK1, derived from the proximal convolution of the renal tubule (Stevens et al., 1986), were cultured in Dulbecco's-modified Eagle's medium (Gibco) supplemented with 5% fetal calf serum, 2 mM glutamine, 1 mM pyruvate, penicillin (200 units/mL), streptomycin (50 μg/mL), and fungizone (0.5 μg/mL). The rat hepatoma cell line H5 (Deschatrette et al., 1974) was cultured in a modified Ham F12 medium (Gibco) (Coon & Weiss, 1969) supplemented with 5% fetal calf serum, penicillin (200 units/mL), streptomycin (50 μg/mL), and fungizone (0.5 μg/mL). Twenty-four hours prior to the transfection, LLC-PK1 subconfluent cells dishes were trypsinized, and 5 × 10^5 cells were plated per 10-cm-diameter tissue culture dish. These cells were cotransfected with 10 μg of a CAT construct and 10 μg of the β-galactosidase plasmid per dish using the calcium phosphate technique followed by a glycerol shock (Wigler et al., 1979). The cells were cultured for 40 h, and cell extracts were prepared by three cycles of freezing and thawing. CAT assays were performed at 37 °C for 2 h in the presence of 4 mM acetyl-CoA and 62.5 nCi of [^{14}C]chloramphenicol, as described (Gorman et al., 1982). The β-galactosidase activity was determined in every extract to correct for differences in transfection efficiency, as descirbed (An et al., 1982). Proteins were

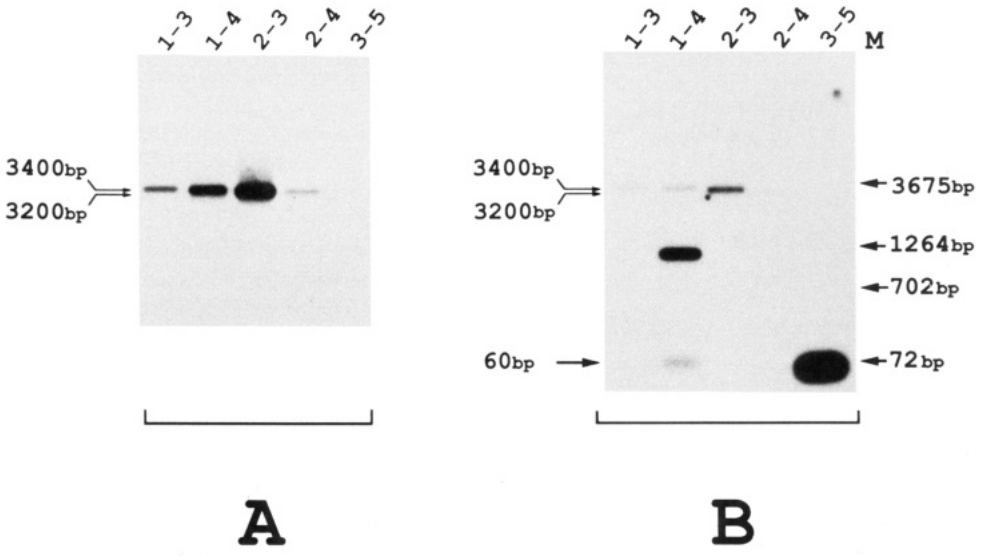

FIGURE 2: Southern blot analysis of the amplified GGT genomic sequences. Five sets of two GGT specific primers (1–3; 1–4; 2–3; 2–4; 3–5) were used for the amplification of GGT genomic DNA sequences. The amplified genomic sequences were run on a 1% agarose gel along with DNA molecular weight markers (M) and transferred to a Hybond membrane. The GGT products were revealed by hybridization. (A) Hybridization to oligonucleotide 2. (B) Hybridization to oligonucleotide 4.

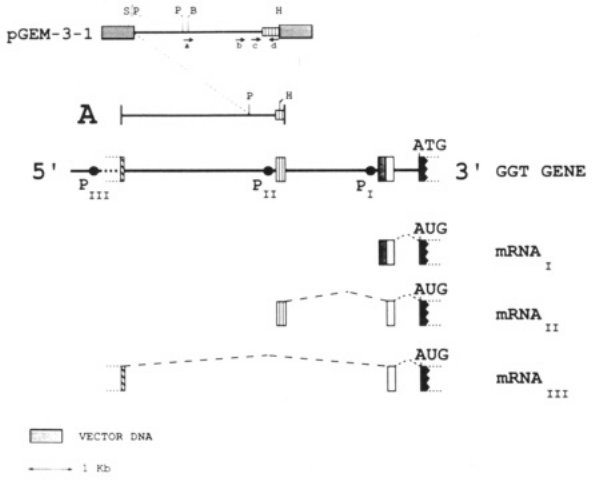

FIGURE 3: Organization of the 5′ end of the GGT gene and synthesis of alternative transcripts. Exons are represented by boxes, and introns are shown by solid lines connecting the exons. The black box with ATG (first coding exon) and the open box (5′ noncoding sequence) are common to the three mRNAs transcribed from this gene. The shaded box corresponds to the 5′ part of the leader exon for $mRNA_I$, the striped box corresponds to the leader exon for $mRNA_{II}$, and the hatched box, 6.1 kb upstream from the ATG, corresponds to the sequence specific for $mRNA_{III}$. Fragment A represents the genomic sequence of 3.4 kb amplified between oligomers 1 and 3 (see Figure 2, lane 1–3). Magnification of the *Pvu*II–*Hin*dIII 600-bp fragment cloned in pGEM-3-1 is shown at the top of the figure; a, b, c, and d represent the oligonucleotides used in the preparation of the GGT-CAT constructs. Restriction sites: H, *Hin*dIII; B, *Bgl*II; S, *Sac*I; P, *Pvu*II; and [P], *Pvu*II not regenerated in the construct.

assayed according to the method of Bradford (Bradford, 1976).

Northern Blot Analysis. Total RNA was extracted from the tissues and from HTC and H5 hepatoma cells using the guanidinium thiocyanate procedure (Chirgwin et al., 1979). Poly(A)$^+$ RNA was purified by two successive purifications on an oligo-dT trisacryl column, resolved on a 1.5% agarose gel, transferred onto a nylon membrane, and hybridized to RNA or cDNA probes complementary to the GGT mRNA sequences. Briefly, labeled cRNA probes were obtained by in vitro transcription of plasmids pGEM-3-12 (cRNA-12) and pGEM-3-17 (cRNA-17) (Chobert et al., 1990), in the presence of 60 μCi of [α-^{32}P]uridine 5′-triphosphate (UTP). These probes are mapped to their complementary sequences on the GGT mRNAs (Figure 1); cRNA-12 (132 bases) and cRNA-17 (128 bases) hybridize specifically to $mRNA_{II}$ and $mRNA_I$, respectively. Conditions of hybridization and of washing have been described previously (Chobert et al., 1990). The cDNA 139A was obtained by a *Pvu*II–*Pst*I digestion of pGEM-4-139 (Chobert et al., 1990) and labeled using the random priming kit developed by Amersham. The Northern blots were prehybridized overnight at 42 °C in 50% formamide, 1% sodium dodecyl sulfate, 5× SSC, 1× Denhardt, 50 mM NaH_2PO_4, and 250 μg/mL denatured salmon sperm DNA. The hybridization was carried out in the same medium at 42 °C for 20 h. The blots were washed three times in 0.1× SSPE (15 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM EDTA), 0.5% sodium dodecyl sulfate for 1 h at 65 °C.

RESULTS

Amplification and Cloning of GGT Genomic Sequences. The oligonucleotides used for in vitro DNA amplification were designed from the 3′ end (oligomer 1) and the 5′ end (oligomer 2) of the leader sequence which is specific for $mRNA_{II}$ and from the 59-bp sequence (oligomers 3, 4, and 5) specific for $mRNA_{III}$, as reported in Figure 1. Genomic DNA amplifications from the combination of the oligonucleotides 1 and 3 (1–3) or 2 and 3 (2–3), which are mapped on cDNA sequences in Figure 1, yield fragments of 3.4 kb and 3.2 kb which both hybridize to oligomers 2 and 4 (Figure 2A,B). It reveals that the $mRNA_{III}$-specific sequence from which oligomer 3 was designed is located 3.2 kb upstream from the leader sequence for $mRNA_{II}$ on the GGT gene. The small difference in size between the two amplified products is fully accounted for by the length of the cDNA sequence between oligomers 1 and 2. Therefore, the $mRNA_{II}$-specific leader sequence lies in a single exon in the GGT gene. This genomic organization is confirmed by the DNA amplifications between primers 1 and 4 (1–4) and 2 and 4 (2–4) which give rise to the expected 3.4-kb and 3.2-kb DNA fragments recognized by oligomers 2 and 4 (Figure 2A,B). A 60-bp fragment was amplified from oligonucleotides 3 and 5 (3–5) and detected by hybridization to oligomer 4 (Figure 2B). The size of this fragment corresponds exactly to the length of the cDNA, thus meaning that the 59-bp $mRNA_{III}$-specific sequence is

not interrupted by an intron in the gene. The 1-kb fragment revealed by oligomer 4 (Figure 2B, lane 1–4) but not by oligomer 2 (Figure 2A, lane 1–4) cannot correspond to a GGT sequence. This nonspecific hybridization results from the use of oligomer 4 as a primer in the amplification experiment and as a probe in the hybridization.

All the 5′ untranslated sequences which have been reported to date for the GGT $mRNA_I$, $mRNA_{II}$, and $mRNA_{III}$ can now be mapped on the GGT gene on a 6.1-kb fragment upstream from the translation start site (ATG) (Figure 3). We have already reported that the $mRNA_I$ transcription start site (promoter I) is located 790 bp upstream from the start codon and 1.8 kb downstream the $mRNA_{II}$-specific sequences (Kurauchi et al., 1991). The 59 nucleotides specific for the $mRNA_{III}$ are located 3.2 kb upstream from the leader exon for the $mRNA_{II}$ (Figure 3).

The restriction sites of the 3.4-kb DNA sequence used in the study are mapped in Figure 3. The 600-bp *Pvu*II–*Hin*dIII fragment was subcloned into the plasmid pGEM-3Z; the recombinant clone (pGEM-3-1) contains the first 72 bases of the $mRNA_{II}$ as well as a 528-nucleotide flanking sequence (Figure 3).

Mapping of the Transcription Initiation Site for $mRNA_{II}$ on the GGT Gene. The *Bgl*II-*Hin*dIII genomic DNA fragment which spans the putative $mRNA_{II}$ start site and ends at base +72 in the $mRNA_{II}$ sequence was labeled at its 5′ end on the complementary strand and hybridized to kidney poly(A)$^+$ RNA. The heteroduplex was digested with S_1 nuclease, and the lengths of the protected fragments were determined on an acrylamide gel by comparison with a sequence ladder (Figure 4, lanes GATC). Two clusters of labeled DNA fragments which extend 72 to 79 (C) and 95 to 102 (B) nucleotides from the labeled *Hin*dIII site were protected from the digestion (Figure 4, lane 1). The presence of a cluster of bands around each start site is often observed in S_1 nuclease protection experiments; it can be attributed to a slight heterogeneity in the DNA protection resulting from an instability of the duplex around the start site. The specificity of that assay is shown by the presence of the intact *Bgl*II–*Hin*dIII probe (A) and the absence of any protected fragment in the reaction performed without poly(A)$^+$ RNA (Figure 4, lane 2). The predominant band in each cluster identifies each of the two transcription start sites which are 26 bases apart on the gene. The major start site (+1) identified by the 76-bp fragment coincides with the 5′ end of the major cDNA product extended on the $mRNA_{II}$ in the primer extension experiment depicted previously (Chobert et al., 1990).

DNA Sequence. The genomic *Pvu*II–*Hin*dIII fragment subcloned into pGEM-3Z (pGEM-3-1) (Figure 3) extends from base –528 upstream from the major initiation site to base +72 in the leader exon for $mRNA_{II}$ which ends at the base +138 downstream from the major initiation site (Figure 5). The major transcription start site (+1) is flanked by the sequence CTTGCTGCT (–12 to –4) which is usually located immediately upstream from the cap site (Lewin, 1980). The TAAT motif (–26 to –23) could play the role of the canonical TATA box. In fact, the dinucleotide CA which is located 29 bases upstream from the TATA box in many eukaryotic genes (Bucher & Trifonov, 1986) is found at the position –55. A CAAT element and three inverse complements (ATTG), which are known to be the target of transcription factors (Graves et al., 1986), map at positions –488 to –485, –317 to –314, –312 to –308, and –245 to –242 (Figure 5). The motifs TTAGTCA (–47 to –41), CCCGCC (–80 to –75), CCCCAGGC (–350 to –343), and CCTTAAATGG (–386 to –377) correspond

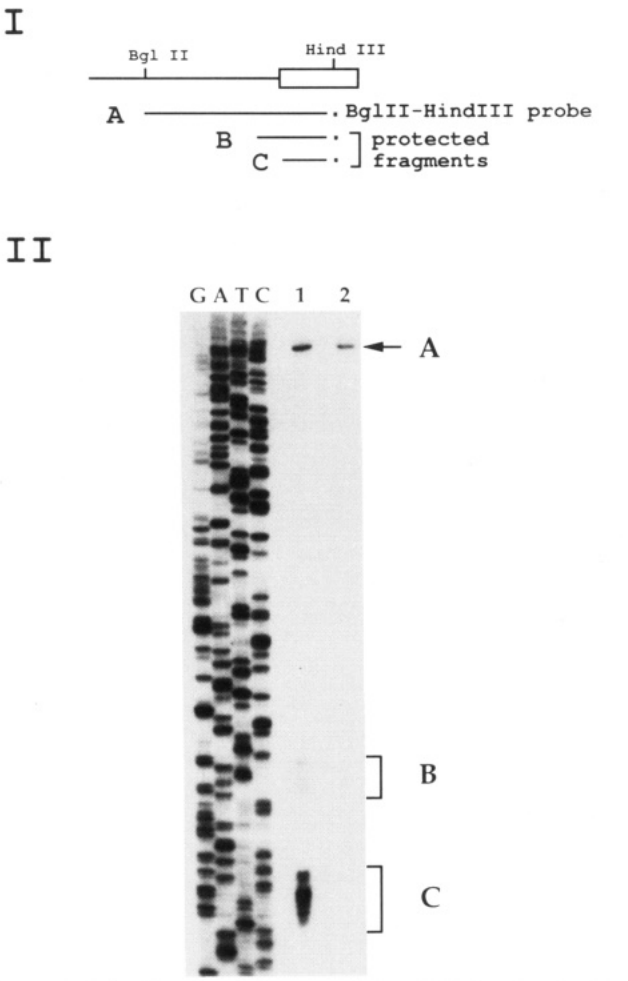

FIGURE 4: Identification of the transcription initiation sites by S1 nuclease mapping analysis. (I) Schematic representation of the experiment: the relevant region of the gene encompassing the leader exon for $mRNA_{II}$ (open box) and the 5′ flanking region are diagrammed at the top. The *Bgl*II–*Hin*dIII-labeled fragment (A) was hybridized to 20 μg of kidney poly(A)$^+$ RNA, and the heteroduplex was digested with S_1 nuclease. B and C are the major protected fragments which are seen on the autoradiography. (II) Gel analysis of the DNA protected fragments: GATC, unrelated sequence ladder; lane 1, fragments resistant to S_1 nuclease digestion; lane 2, undigested probe. The fractionation was obtained by electrophoresis on a 6% acrylamide, 7 M urea sequencing gel.

exactly to the consensus sequences for the AP-1 (Hirai & Yaniv, 1989), SP-1 (Dynan & Tjian, 1985), and AP-2 transcription factors (Mitchell et al., 1987) and for the c-*fos* serum-responsive element (SRE) (Rivera et al., 1990), respectively. The sequence GGACANNNTGTCT from –224 to –208 can be closely aligned to the consensus DNA sequence (GGTACANNNTGTTCT) recognized by the glucocorticoid receptor (Beato, 1989).

Promoter Activity of GGT Sequences Upstream from the Transcription Start Site for the GGT $mRNA_{II}$. The promoter activity of the sequence flanking the GGT $mRNA_{II}$ start site was examined by transfecting several GGT-CAT recombinant plasmids into the LLC-PK1 pig kidney cell line. The sequence from nucleotide –528 to nucleotide +72 downstream from the major start site drives the expression of a significant CAT activity which represents 5% of the activity driven by the β-galactosidase plasmid in LLC-PK1. Deletion of the sequence between positions –528 to –322 induces a 50% decrease in the promoter activity (Figure 6). A further deletion from base –322 to base –114 restores the initial promoter activity. The shortest construct (–30 to +72) which lacks the consensus

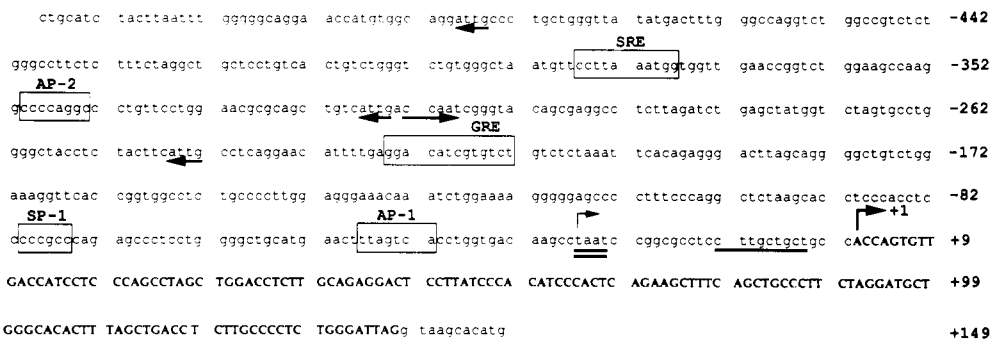

FIGURE 5: Nucleotide sequence flanking the $mRNA_{II}$ leader exon on the GGT gene. The numbering indicates the nucleotide position relative to the major transcription start which is designated +1. Nucleotides present in the mature $mRNA_{II}$ are printed in boldface upper-case letters, and nucleotides in the flanking region are in lower-case letters. Sequences similar to know regulatory elements are boxed. The TAAT sequence is underlined twice. The consensus sequence flanking the cap site is underlined once. The putative CAAT elements are underlined with arrows.

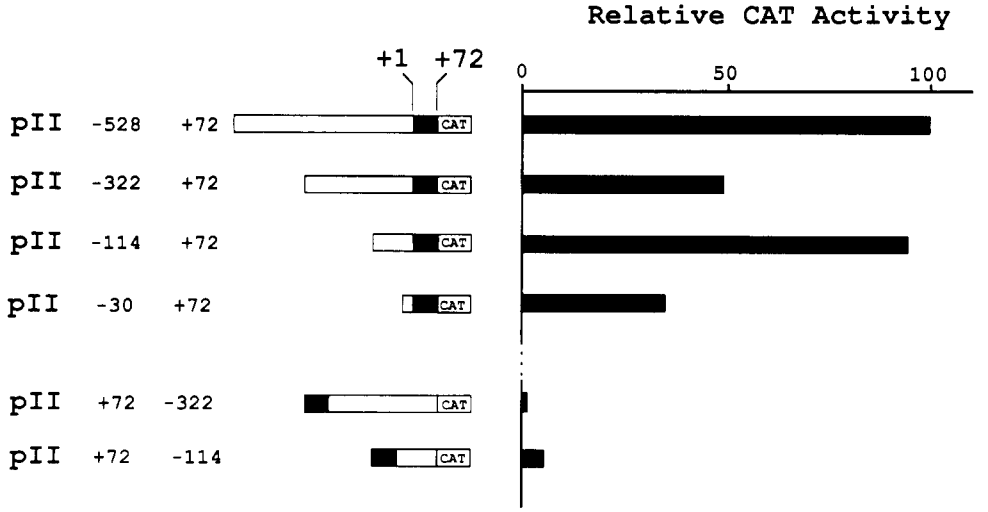

FIGURE 6: Expression of GGT-chloramphenicol acetyltransferase (CAT) constructs in transient transfection assays in LLC-PK1 cells. Relative CAT activity of the GGT-CAT constructs used to drive the CAT activity. The predominant transcription start site is indicated (+1), and the black box represents the leader sequence from +1 to +72. Each construct is designated by the position of the most 5′ nucleotide. Constructs cloned in the opposite orientation are pII +72 −322 and pII +72 −114. All the CAT values have been corrected for the β-galactosidase activity; each is the mean of five experiments normalized to the value obtained for the longest construct (100%) in the same experiment.

Sp-1 binding site displays a reduced promoter activity as compared with the −114 to +72 construct which can be viewed as having the basal promoter activity. The CAT vectors without any GGT sequence (not shown) or with a GGT sequence ligated to the CAT gene in the opposite orientation (pII +72 −322; pII +72 −114) do not exhibit a significant promoter activity (Figure 6).

GGT mRNA Expression in Tissues and Cell Lines. The tissue or cell specificity of the expression of the GGT $mRNA_{II}$ was investigated by Northern blot analysis of poly-$(A)^+$ RNA samples. Hybridization to the cRNA-12 probe (Figure 1), which recognizes the GGT $mRNA_{II}$, reveals the expression of this 2.2-kb mRNA only in the kidney and in the epididymis (Figure 7A). A second hybridization of this blot to the cDNA probe 139A (Figure 1), which recognizes various GGT mRNA species, reveals a complex pattern for GGT mRNA expression in different tissues and cell lines (Figure 7B). Four different bands (2.2, 2.4, 2.5, and 2.6 kb) are identified (Figure 7B). A 2.2-kb signal was found mainly in the kidney and in H5 hepatoma cells and also, at a lower level, in the epididymis, the HTC cells, the small intestine and in the lung on a longer exposure (data not shown). This 2.2-kb GGT signal was further analyzed by hybridization to the cRNA-17 probe (Figure 1) which identifies the 2.2-kb $mRNA_I$ only in the kidney (Figure 7C). Therefore, the strong 2.2-kb signal in the H5 sample cannot be accounted for by the expression of the GGT $mRNA_I$ or $mRNA_{II}$; it corresponds to a new mRNA species ($mRNA_V$) which had not been identified before. The synthesis of the GGT $mRNA_V$ could also explain the 2.2-kb band which appears in the HTC hepatoma cells and in the mammary gland since no specific sequences for $mRNA_I$ or $mRNA_{II}$ were amplified in these samples by the polymerase chain reaction technique (Darbouy et al., 1991).

The 2.4-kb GGT mRNA, identified as $mRNA_{III}$ in the fetal liver and in the mammary gland, is also expressed in the epididymis (Figure 7B) and in the lung on a long exposure (data not shown); the 2.5-kb mRNA ($mRNA_{IV}$) is detected in the HTC hepatoma cell and in the small intestine as reported (Darbouy et al., 1991). Finally, we observe the expression of a new and larger mRNA species of 2.6-kb ($mRNA_{VI}$) in the poorly differentiated H5 hepatoma cell line which was not found in any of the other samples. Therefore, it appears now that six different mRNA species are transcribed from the rat GGT gene.

DISCUSSION

In the rat, a single-copy gene encodes several GGT transcripts which are know to differ in their 5′ untranslated regions (Chobert et al., 1990; Griffiths et al., 1989; Darbouy et al., 1991). The GGT $mRNA_I$ is transcribed from a proximal promoter (promoter I) that we cloned previously (Kurauchi et al., 1991). We report here the characterization and the

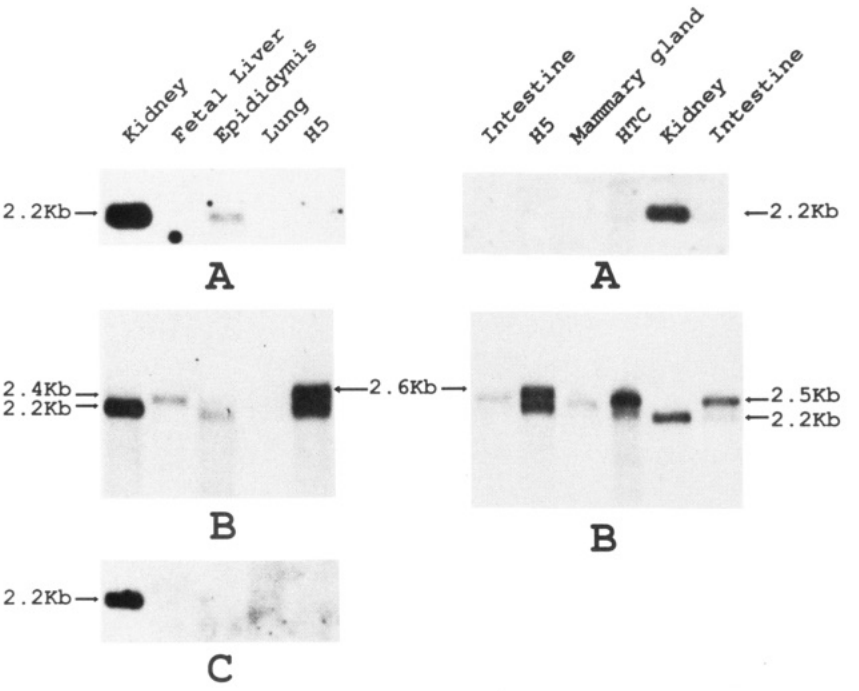

FIGURE 7: Northern blot analysis of poly(A)$^+$ RNA. Poly(A)$^+$ RNAs (500 ng for the kidney sample, 2 μg for the epididymis and H5, and 5 μg for the other samples) were blotted onto a nylon membrane and hybridized successively to the cRNA-12 (A), cDNA 139A (B), and cRNA-17 (C) probes (see Figure 1). Autoradiography was performed with two intensifying screens at −80 °C for 2 days (A and C) and 12 h (B).

expression of a more distal promoter (promoter II) coding for the $mRNA_{II}$ which maps 2 kb upstream from the GGT promoter I and 3.2 kb downstream from a 59-bp sequence which is transcribed in the GGT $mRNA_{III}$ (Griffiths et al., 1989); as a result, we firmly demonstrate that the $mRNA_{III}$ species is encoded by a third promoter (promoter III) upstream on the gene, which has not been cloned to date.

The GGT promoter II displays the two main characteristics of many eukaryotic genes, a TATA-like sequence (TAAT) 26-bp upstream from the major start site and several CAAT sequences. In addition, several consensus sequences known to bind transcription factors are present within the 528-bp fragment located upstream from the major transcription start site. The GGT-CAT constructs which extend up to the nucleotide −528 from the $mRNA_{II}$ predominant start site exhibit a significant promoter activity in LLC-PK1 cells. The promoter activity is inhibited by 50% following the deletion of the sequence from nucleotide −528 to nucleotide −322 and reactivated by a further deletion up to nucleotide −114. It reveals that the expression of this promoter is controlled by the interplay of positive and negative elements within 528 bp upstream from the major start site. Unfortunately, we did not succeed in cloning sequences further upstream; all the clones which were obtained exhibit DNA rearrangement. The presence of unstable DNA sequences in this region of the GGT gene could explain why we have been unable to isolate the 5′ end of this gene from a genomic library (Kurauchi et al., 1991).

Among the tissues and cell lines investigated, the promoter II activity was detected only in the epididymis and in the kidney in which the accumulation of the $mRNA_{II}$ is restricted to the straight part of the proximal tubule (Chobert et al., 1990). Two GGT mRNAs (2.2 kb and 2.3 kb) have been reported previously in the epididymis (Hinton et al., 1991); we demonstrate now that the 2.2-kb GGT signal is accounted for by the expression of the $mRNA_{II}$. Thus, promoter II is expressed in the epididymis whereas the proximal promoter I is silent in this organ. The expression of the highly tissue-specific GGT promoter II in the epididymis and in the kidney could be related to the common embryological origin of the urinary and genital systems (Hinton & Turner, 1988).

In this work, we also identify two new mRNA species ($mRNA_V$ and $mRNA_{VI}$) which are highly expressed in the H5 cells, a cell line established from a chemically-induced primary hepatoma (Deschatrette & Weiss, 1974). These two transcripts cannot be transcribed from promoters I and II since they do not hybridize to the $mRNA_I$ and $mRNA_{II}$ leader sequences. The mechanisms involved in the deviation of the GGT gene expression in the HTC and H5 hepatoma cell lines as compared with normal or fetal liver is not clear at present. The $mRNA_{IV}$, $mRNA_V$, and $mRNA_{VI}$ can be derived from a primary transcript initiated at promoter III which is then spliced differentially in the hepatoma cells as compared with the normal liver where it gives rise to the $mRNA_{III}$. Alternatively, these mRNAs could be transcribed from another promoter(s) not yet identified. The identification on the GGT gene of three independant promoters coding for the $mRNA_I$, $mRNA_{II}$ and $mRNA_{III}$, respectively, provides an explanation for the synthesis of these multiple mRNAs from a single gene.

Alternative promoters have been identified in several eukaryotic genes, and their use affords a flexibility in the control of the expression of these genes (Schibler & Sierra, 1987; Matsuguchi et al., 1990). First, a promoter can be functional only at a particular developmental stage (Ueno et al., 1988; Benyajati et al., 1983) or in a given tissue (Shapiro et al., 1991). Gene transcription from multiple promoters also allows a posttranscriptional control by generating transcripts with alternate untranslated sequences which can affect the stability (Kozak, 1988) or the translational efficiency of the mRNA (Leibold et al., 1988). The role of these multiple, untranslated 5′ ends upstream of an identical open reading frame has not been investigated yet. At the gene level, the regulation of the multiple GGT promoters occurs through tissue-specific transacting factors. The GGT promoters I and II could appear as invaluable tools in the identification of the

proteins which are involved in gene regulation during the development of the urogenital system.

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REFERENCES

Agrawal, Y. P., & Vanha-Perttula, T. (1988) *Biol. Reprod. 38*. 996–1000.
An, G., Hidaka, K., & Siminovitch, L. (1982) *Mol. Cell. Biol. 2*, 1628–1632.
Barouki, R., Finidori, J., Chobert, M. N., Aggerbeck, M., Laperche, Y., & Hanoune, J. (1984) *J. Biol. Chem. 259*, 7970–7974.
Beato, M. (1989) *Cell 56*, 335–344.
Benyajati, C., Spoerel, N., Haymerle, H., & Ashburner, M. (1983) *Cell 33*, 125–133.
Berk, A. J., & Sharp, P. A. (1977) *Cell 12*, 721–732.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bucher, P., & Trifonov, E. N. (1986) *Nucleic Acids Res. 14*, 10009–10026.
Cameron, R., Kellen, J., Kolin, A., Malkin, A., & Farber, E. (1978) *Cancer Res. 38*, 823–829.
Chirgwin, J. M., Przybyla, A. E., McDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.
Chobert, M. N., Bernard, O., Bulle, F., Lemonnier, A., Guellaen, G., & Alagille, D. (1989) *J. Hepatol. 8*, 22–25.
Chobert, M. N., Lahuna, O., Lebargy, F., Kurauchi, O., Darbouy, M., Bernaudin, J. F., Guellaën, G., Barouki, R., & Laperche, Y. (1990) *J. Biol. Chem. 265*, 2352–2357.
Coon, H. G., & Weiss, M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 852–859.
Darbouy, M., Chobert, M. N., Lahuna, O., Okamoto, T., Bonvalet, J. P., Farman, N., & Laperche, Y. (1991) *Am. J. Physiol. 261*, C1130–C1137.
Deschatrette, J., & Weiss, M. C. (1974) *Biochimie 56*, 1603–1611.
Dynan, W. S., & Tjian, R. (1985) *Nature 316*, 774–778.
Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6777–6781.
Graves, B. J., Johnson, P. F., & McKnight, S. L. (1986) *Cell 44*, 565–576.
Griffiths, S. A., & Manson, M. (1989) *Cancer Lett. 46*, 69–74.
Hinton, B. T., & Turner, T. T. (1988) *News Physiol. Sci. 3*, 28–31.
Hinton, B. T., Palladino, M. A., Mattmueller, D. R., Bard, D., & Good, K. (1991) *Mol. Reprod. Dev. 28*, 40–46.
Hirai, S. I., & Yaniv, M. (1989) *New Biol. 1*, 181–191.
Kozak, M. (1988) *J. Cell Biol. 107*, 1–7.
Kurauchi, O., Lahuna, O., Darbouy, M., Aggerbeck, M., Chobert, M. N., & Laperche, Y. (1991) *Biochemistry 30*, 1618–1623.
Leibold, E. A., & Munro, H. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2171–2175.
Lewin, B. (1980) in *Gene Expression: Eucaryotic Chromosomes*, Vol. II, pp 822, Wiley-Interscience, New York.
Matsuguchi, T., Takahashi, K., Ikejiri, K., Ueno, T., Endo, H., & Yamamoto, M. (1990) *Biochim. Biophys. Acta 1048*, 165–170.
Meister, A. (1984) *Hepatology 4*, 739–742.
Mitchell, P. J., Wang, C., & Tjian, R. (1987) *Cell 50*, 847–861.
Pawlak, A., Lahuna, O., Bulle, F., Suzuki, A., Ferry, N., Siegrist, S., Chikhi, N., Chobert, M. N., Guellaën, G., & Laperche, Y. (1988) *J. Biol. Chem. 263*, 9913–9916.
Rajagopalan, S., Park, J. H., Patel, P. D., Lebovitz, R. M., & Lieberman, M. W. (1990) *J. Biol. Chem. 265*, 11721–11725.
Richards, W. L., Tsukada, Y., & Potter, V. R. (1982) *Cancer Res. 42*, 1374–1383.
Rivera, M. V., Sheng, M., & Greenberg, M. E. (1990) *Gene Dev. 4*, 255–268.
Schibler, V., & Sierra, F. (1987) *Annu. Rev. Genet. 21*, 237–257.
Shapiro, L. H., Ashmun, R. A., Roberts, W. M., & Look, T. (1991) *J. Biol. Chem. 266*, 11999–12007.
Stevens, J., Hayden, P., & Taylor, G. (1986) *J. Biol. Chem. 261*, 3325–3332.
Tate, S. S., & Meister A. (1981) *Mol. Cell. Biochem. 39*, 357–368.
Ueno, T., Takahashi, K., Matsuguchi, T., Endo, H., & Yamamoto, M. (1988) *Biochem. Biophys. Acta 950*, 411–419.
Wapnir, R. A., Mancusi, V. J., & Goldstein, L. A. (1982) *Experientia (Basel) 38*, 647–648.
Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Wilaub, G., & Chasun, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1373–1376.

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