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A 346-Base Pair Region of the Mouse γ-Glutamyl Transpeptidase Type II Promoter Contains Sufficient Cis-acting Elements for Kidney-restricted Expression in Transgenic Mice*

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Antonia R. Sepulveda‡§, Shiu L. Huang‡, Russell M. Lebovitz‡§, and Michael W. Lieberman‡§¶

From the Departments of ‡Pathology and §Cell Biology, Baylor College of Medicine, Houston, Texas 77030

The mouse γ-glutamyl transpeptidase (GGT) gene encodes seven distinct mRNAs that are transcribed from seven separate promoters. Type II mRNA is the most abundant in kidney. We have developed a cell line with features of renal proximal tubular cells which expresses GGT mRNA types with a pattern similar to that of mouse kidney. Because a 346-bp sequence from the type II promoter directed the highest level of CAT activity in these cells, this region was used to drive the expression of a β-galactosidase reporter gene in transgenic mice. Two transgenic mouse lines expressed β-galactosidase limited to the renal proximal tubules. Site-directed deletions within this 346-bp promoter region demonstrated that cis-elements containing the consensus binding sites for AP2, a glucocorticoid response element (GRE)-like element, and the initiator region were required for transcriptional activity and were not additive. Purified AP2 bound and footprinted the AP2 consensus region, making it likely that transcription from the GGT type II promoter is regulated in part by AP2. These data suggest that transcription of the type II promoter requires multiple protein DNA interactions involving at least an AP2 element, and probably a GRE-like element and the initiator region.

γ-glutamyl transpeptidase (GGT)[1] is a key enzyme in glutathione metabolism (1–3). It is expressed in many epithelial cells, but the highest levels are found in kidney, small intestine, pancreas, fetal liver, and other organs, which have secretory or absorptive function (1, 3). In kidney GGT expression is restricted to proximal tubules, where the γ-glutamyl cycle plays an important role in the recycling of GSH (1, 3). Renal GGT is primarily associated with the apical surface of the proximal tubule with its active site in the extracellular milieu. GGT activity in proximal tubules results in reabsorption of greater than 99.9% of the tubular glutathione (as the constituent amino acids) and thus functions in cysteine reabsorption (1, 4).

We have previously identified and characterized the structure of six different GGT mRNAs in mouse kidney (5). The GGT mRNA species differ in their 5′-untranslated sequences but share a common coding region (5, 6). The different GGT mRNAs are expressed from separate promoters that are present in the 10-kb 5′-flanking region of the GGT gene (7). We have studied the relative abundance of the GGT mRNAs in kidney and found that type II mRNA is the most abundant, representing approximately 45% of the total, while the five remaining GGT mRNAs are present at lower levels (7). Different GGT RNAs are expressed in a tissue restricted-pattern, and in general one type is present in only a few different tissues (3). For example, type III is expressed only in fetal liver and type IV is also detected in epididymis and in embryonic cells derived from the endoderm of the yolk sac (3, 8).

Although GGT is expressed in a relatively ubiquitous manner, the restricted pattern of expression of individual GGT mRNAs has led to the hypothesis that the different promoters are tissue-restricted. In contrast to other GGT mRNAs, significant expression of type II is limited to the kidney where it is also the most abundant GGT mRNA (7). Our previous studies also demonstrated that the type II GGT promoter conferred significantly higher levels of CAT activity in transient transfections in mouse proximal tubular cells than in fibroblasts, indicating that those cis-acting elements were sufficient to direct the expression of the reporter gene in a cell specific manner (7). We therefore examined the type II promoter to determine if it contains sufficient cis-acting elements to direct kidney restricted expression of a β-galactosidase reporter gene *in vivo* by generating transgenic mice.

In addition we have performed deletion analyses of the 346-bp type II promoter to determine the cis-acting binding sequences that are responsible for transcriptional activity in mouse proximal tubular (MPT) cells. We obtained a series of 5′ truncations and short sequence deletions by site-directed mutagenesis and tested them by transient transfection assays of the CAT reporter gene. Selected sequences containing critical elements required for promoter activity were tested for their ability to activate a minimal GGT promoter region and heterologous minimal promoters.

EXPERIMENTAL PROCEDURES

Cell Culture—The *ras*-transformed MPT cell line was established from the kidneys of p21^{ras}-transgenic mice line 499 (9). The culture medium contained a 1:1 mixture of Dulbecco modified Eagle's medium and Ham's F12 medium (Life Technologies, Inc.) with 10 mM Hepes buffer, sodium bicarbonate at 1.1 mg/ml, 10 nM $Na_2SeO_3 5H_2O$ (SFFD) and was supplemented with insulin (5 mg/ml), PGE_1 (25 ng/ml), triiodothyronine (5×10^{-11} M), hydrocortisone (5×10^{-8} M), and transferrin (5 mg/ml) (10). Whole kidneys were minced and suspended in 1 mg/ml collagenase in SFFD. Cells were harvested and plated in 25-mm plates. The cells were maintained as mixed cell cultures in medium

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) L17331 and L17336.

¶ To whom correspondence and reprint requests should be addressed. Tel.: 713-798-6501; Fax: 713-798-6001; E-mail: mikel@bcm.tmc.edu.

[1] The abbreviations used are: GGT, γ-glutamyl transpeptidase; CAT, chloramphenicol acetyltransferase; MPT, mouse proximal tubular; FVB, Friend leukemia virus strain B mice; GRE, glucocorticoid response element; RT, reverse transcription; PCR, polymerase chain reaction; G3PDH, glucose-3-phosphate dehydrogenase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; SRE, serum response element; Luc, luciferase; bp, base pair(s); kb, kilobase pair(s).

supplemented with 7% fetal calf serum and passed every 3–4 days.

Preparation of Probes for Ribonuclease Protection Assays—The antisense RNA probes used for the quantification of the GGT mRNAs II and IV were obtained by *in vitro* transcription of the GGT cDNAs previously reported (5), and their structures are represented in Fig. 1*A*. The lengths of their unique 5′ sequences are 94 and 99 bp for probes II and IV, respectively. The β-galactosidase ribonuclease probe was obtained from Ambion, Inc. (Austin, TX). The plasmids were linearized and then transcribed with either T3 or T7 polymerase (Stratagene Inc., La Jolla, CA) in the presence of [α-^{32}P]UTP to obtain the uniformly labeled antisense strand.

Ribonuclease Protection Assays—Total RNA was isolated from the organs of transgenic mice using the acid-phenol guanidine procedure (11). Poly(A)$^+$ RNA was obtained from MPT cells and adult Friend leukemia virus strain B mice (FVB) kidneys and selected by oligo(dT)-cellulose type III (Collaborative Research Inc., Bedford, MA).

Ribonuclease protection assays were performed with the RPA II ribonuclease protection kit (Ambion, Inc.). Briefly, poly(A)$^+$ RNA from MPT cells or total RNA from FVB or transgenic mouse tissues was hybridized for 18 h at 45 °C with 1×10^5 cpm of a [α-^{32}P]UTP labeled RNA probe. Ribonuclease digestion of the hybridized probe and sample RNA was performed at 30 °C for 30 min, with 0.1 unit of ribonuclease A and 20 units of ribonuclease T1. The protected RNA fragments were separated on 6% polyacrylamide, 7 M urea gels. For quantitation of GGT mRNA types in MPT cells, the number of counts in each band was determined with the AMBIS Radioanalytic Imaging System and the AMBIS QuantProbe Software version 4.01 (AMBIS, Inc., San Diego, CA).

GGT-CAT Plasmid Constructions—The promoterless plasmid pJFCAT1 (12) was used to subclone the 5′-flanking regions of GGT in front of CAT. PII-2.7 is a 2.7-kb fragment that results from *Pst*I digestion of the 6.0-kb *Xho*I clone (7); the *Pst*I ends were flushed with T4 polymerase, and the fragment was cloned into the *Xho*I site of pJFCAT1. PII-346 (−346 to +70 bp) and PII-746 (−746 to +70) have been described (7) and were previously named PII-416 and PII-816, respectively. Since convenient restriction sites were absent in some regions, constructs PII-230 (−230 to +70) and PII-95 (−95 to +70) were obtained by amplifying sequences of appropriate size using PII-346 as a template. The PCR amplifications were performed as described previously (13). PII-230 was obtained by PCR with the oligonucleotides 5′ (5′-CTCGAGAAGGGTTCACCGGTGGCCTCTGC) and 3′ (5′-GCCGCCCTCGAGGCAAGAGGTCAGCTAA), and PII-95 was obtained by PCR with the oligonucleotides 5′ (5′-CTCGAGGTCACAAGCCTGACGCTGCGCC) and 3′ (5′-GCCGCCCTCGAGGCAAGAGGTCAGCTAA). The PCR products were cloned into the pT7Blue vector (Novagen, Madison, WI), digested with *Xho*I, and subcloned into the *Xho*I site of pJFCAT1.

The 116-bp fragment was obtained by PCR using PII-346 as a template, and using the oligonucleotides 5′ (5′-AAGGATCCGATCTAAGCTATGGTCTAGTG) and 3′ (5′-AAGGATCCAGATCTTCCAGACAGCCCCTGCTAAG), subcloned into the pT7Blue vector, excised with *Bam*HI, and cloned upstream of PII-95 (7). The double-stranded oligonucleotides p6a (5′-GATCACTCGAGCCCCTTAGAGGGAACCAAATCTGGAAAGTGGGGA) and p6b (5′-GATCACTCGAGGGAACCAAATCTGGA) were cloned into the *Bgl*II site of the enhancerless CAT reporter vector (pSV40 CAT) (Promega, Madison, WI), to obtain pSV-6a and pSV-6b, respectively. Single copies of each double-stranded oligonucleotide upstream of CAT were obtained after screening by restriction digestion with *Xho*I and confirmation of the sequence by DNA sequencing. The sense orientations were used.

Luciferase Reporter Constructs—The TATA-Luc minimal promoter reporter containing −58 bp of the cardiac α-actin promoter was kindly provided by R. Schwartz (Baylor College of Medicine) (14). The double-stranded oligonucleotide p6a (see above) was cloned upstream of the α-actin minimal promoter into a *Sac*I site in the polylinker of the vector. A construct containing seven copies of the double strand oligonucleotide was used in transient transfection assays.

DNA Footprint and Gel Mobility Shift Analysis—A 416-bp DNA fragment containing 346 bp of the GGT type II promoter region was cloned into the vector PCRTMII (Invitrogen, Inc.) and released after digestion with *Hin*dIII and *Not*I (7). This probe was end-labeled with a T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP, followed by digestion with *Bam*HI to release the 3′ end of the GGT promoter. Footprint assays were performed with the Core Footprinting System (Promega) using 1 or 2 footprint units of purified human AP2 protein (Promega) and 1×10^5 cpm of labeled probe. The probe was incubated with the protein extract for 10 min, followed by digestion with 0.15 unit of RQ1 RNase-free DNase (Promega). The products were resolved on 6% polyacrylamide, 7 M urea sequencing gels.

Gel mobility shift analyses were performed with a double-stranded oligonucleotide (5′-ATGGTCTAGTGCCTGGGGTACCCC) containing the AP2 consensus binding site (GCCTGGGG) present at −326 to −319 bp in the GGT type II promoter (7). Control oligonucleotides included the human metallothionein AP2 binding site (5′-GATCGAACTGACCGCCCGCGGCCCGT) (58); an oligonucleotide containing a mutated human metallothionein AP2 binding site (5′-GATCGAACTGAAATGTAGATGCCCGT); and an oligonucleotide containing the binding site for AP1 (5′-CGCTTGATGAGTCAGCCGGAA) (24). The double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP, and 1×10^5 cpm were incubated with 1 μg of purified human AP2 (Promega), using the gel shift assay system (Promega). The products were resolved in 4% nondenaturing acrylamide gels. Quantitation of free and bound probe was performed with the AMBIS Radioanalytic Imaging System and the AMBIS QuantProbe Software version 4.01 (AMBIS, Inc.).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by oligonucleotide *in vitro* mutagenesis as described by Kunkel, with the Muta-Gene Phagemid *In Vitro* Mutagenesis Version 2 (Bio-Rad). The template for mutagenesis was the single-stranded DNA pJFCAT-346 (PII-346), previously described (7). The oligonucleotides used for mutagenesis were: P1rs (AP2) (5′-GCAATAAAGTAGGGGTAGACGTCTAGACCATAGCTTAGA) or P1 (5′-GCAATAAAGTAGGGGTATAGACCATAGCTTAGA), P2rs (GRE) (5′-TCTGTGAATTTAGAGACGACGTCTAGACCATAGCTTAGA) or P2 (5′-TCTGTGAATTTAGAGACTAGACCATAGCTTAGA), P3 (5′-CTAAGGGGCAGGCCACGCGGCCGCTTCCAGACAGCCCCTGCT), P4 (5′-TTGGTTCCCTCTAAGGGGGCGGCCGCCGGTGAACCCTTCCAGAC), P5 (5′-CTTTCCAGATTTGGTTCCGCGGCCGCCAGAGGCCACCGGTGAACC), P6 (5′-GGCTCCCCCACTTTCCAGGCGGCCGCCTCTAAGGGGCAGAGGC), P7 (5′-ACTAAGAGCCTGGGAAAGCGGCCGCCATTTCCAGATTTGGT), and P8 (INR)(5′-CATCTGAAGGCTTCTGACGTCGAGTCCTCTGTAAGA). The underlined sequences correspond to the *Aat*II (GACGTC) or *Not*I (GCGGCCGC) restriction sites. The sequences deleted in each construct were PII-P1 and PII-P1rs (−310 to −318), PII-P2 and PII-P2rs (−283 to −273), PII-P3 (−229 to −220), PII-P4 (−219 to −210), PII-P5 (−209 to −200), PII-P6 (−199 to −190), PII-P7 (−179 to −170) and PII-P8 (−9 to +10). Positive clones were screened by digestion with the restriction enzyme specific for the sequence present within the mutated primers, *Aat*II in primers P1rs, P2rs, and P8 and *Not*I in P3, P4, P5, P6, and P7. The oligonucleotides P1 and P2 have complete deletions of the base pairs (−327 to −320) and (−294 to −284), and do not contain a restriction enzyme recognition site; these clones were screened by sequencing.

Sequence Analyses—Sequence analyses of the type II promoter region were performed with the Quest program from Intelligenetics, using the release 6.0 of the Transcription Factors Data Base (15), and with the Transfac data base (16).

Cell Transfections—Approximately 1×10^6 MPT cells in 100-mm plates were transfected using the calcium phosphate co-precipitation method (17). Precipitates contained 20 μg of the GGT-CAT construct and 500 ng of plasmid pCMVβ (CLONTECH, Palo Alto, CA), which expresses β-galactosidase. The β-galactosidase activity was used for correction of the transfection efficiency. Five hours after addition of the DNA to MPT cells a glycerol shock was performed. The cells were harvested and extracts obtained at 48 h post-transfection by freezing and thawing. CAT assays were performed from at least three independent transfections, as described elsewhere (7).

Transfection of luciferase reporter genes was performed using LipofectAMINE (Life Technologies, Inc.) with a DNA/LipofectAMINE ratio of 1/5 (w/v). 0.5×10^6 cells were plated onto 60-mm plates and transfected with a total of 2 μg of DNA. After 48 h of post-transfection, cell extracts were obtained using 200 μl of lysis buffer solution (Promega). Thirty microliters were used to determine luciferase activity using a standard protocol (Promega).

Generation of Transgenic Mice—To obtain transgenic mice, 346 bp of the GGT type II promoter were cloned upstream of the β-galactosidase reporter gene of the plasmid pNASSβ (CLONTECH). The type II promoter 416-bp fragment containing the 346-bp 5′-flanking region was excised with *Xho*I from the pT7Blue vector (7) and subcloned into the *Xho*I site of the β-galactosidase reporter vector pNASSβ to yield pII-346/β-galactosidase. Clones containing the 416- bp insert were sequenced, and a clone with the sense orientation was selected for microinjection. A 4.3-kb fragment that resulted from digestion of the pII-346/β-galactosidase with *Eco*RI and *Hin*dIII was used for microinjection of fertilized eggs. Injections and implantations were performed using standard protocols (18).

Screening of Transgenic Mice—The transgenes from founder animals

and F_1 progeny were analyzed by Southern blot of tail DNA. Briefly, 10 μg of genomic DNA were digested with *Sac*I, electrophoresed through a 0.8% agarose gel, and transferred to Zeta-Probe (Bio-Rad) nylon membranes. The DNA probe used was the 4.3-kb *Eco*RI-*Hin*dIII fragment that was also used for microinjections. Labeling was performed using a random primer labeling kit (Boehringer Mannheim). Hybridization and washing conditions were used as recommended by the Zeta-Probe membrane manufacturer.

Reverse Transcription-PCR (RT-PCR)—The oligonucleotides 5′ (5′-GAACTGAAAAACCAGAAAGTTAACT), 3′a (5′-TCCCAGTCACGAGTTGTAAAACGACG), and 3′b (5′-CAATGCCTCCCAGACCGGCAAC) were designed to amplify the transgene across the SV40 introns present in the β-galactosidase reporter vector pNASSβ. RT-PCR was performed with the Access RT-PCR System (Promega). A sample of total RNA extracted from transgenic mouse tissues was first digested with *Bam*HI, which cleaves a site that is present in the intronic region of SV40, to eliminate residual genomic DNAs in the RNA preparations, and was followed by treatment of the sample with 3 units of DNase RQ1 (Promega). One microgram of total RNA was used in each RT-PCR reaction. The integrity of the RNAs was confirmed by amplification of the correct product of G3PDH (CLONTECH). The reaction products were electrophoresed in 2.5% agarose gels and visualized with ethidium bromide staining.

β-Galactosidase and GGT Histochemistry—Mouse tissues were sampled, and 8-μm thick frozen sections were prepared. The tissues were fixed in 1% glutaraldehyde for 5 min and stained at 30 °C overnight to 24 h in a solution containing 100 mM sodium phosphate, pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Tissue sections were counterstained with neutral red. β-Galactosidase activity in tissues was identified by its ability to convert X-gal into a water-insoluble blue product. To demonstrate GGT in tissue sections histochemistry was performed on frozen sections fixed with methanol (100%) for 5 min and stained as described previously (19).

RESULTS

Establishment and Characterization of the MPT Cell Line—We have previously demonstrated that five separate 5′-flanking regions of the GGT gene have promoter activity in mouse kidney C1.1 cells (7, 20). However, ribonuclease protection assays performed to determine the steady state levels of the most abundant GGT mRNA types in kidney (types II and IV) did not show significant expression of these messages in C1.1 cells (Fig. 1 and data not shown). We therefore developed a cell line that would reproduce the pattern of GGT expression *in vivo*.

To establish a mouse proximal tubular cell line, we took advantage of the fact that a line of transgenic mice carrying a rat GGT(I)-*ras*$^{Val\text{-}12}$ develops proximal tubular hyperplasia (9). We were able to establish an MPT cell line from the kidneys of GGT-*ras* transgenic mice. After 30 passages, the cells maintained a polygonal epithelial morphology, formed cell islands, and, when grown to confluency, formed occasional domes. Histochemical staining for GGT in the MPT cell population was positive and most accentuated in the domes after confluency was reached (data not shown). Northern blot analyses showed that they expressed GGT RNA at a level that is greater than 10% of that present in total kidney RNA (21). This level is approximately 3-fold higher than the level expressed by C1.1 cells. Since types II and IV are the most abundant GGT mRNA types in kidney, we used ribonuclease protection assays (RPA) to perform a quantitative analysis of these GGT mRNA types in established MPT cells (7).

The structural features of the GGT cDNAs, with a common 3′ segment and a unique 5′-flanking region with a DNA sequence that is type-specific, allows a determination of the relative abundance of each GGT transcript relative to total GGT RNA in a cell population by ribonuclease protection (7). Using this approach we found that the type II mRNA represents approximately 45% and the type IV approximately 25% of the total GGT mRNA in MPT cells (Fig. 1). These results agree well with those from studies in which we quantified GGT mRNAs in the kidney and found 45% of the GGT mRNA was type II and 33% was type IV (7).

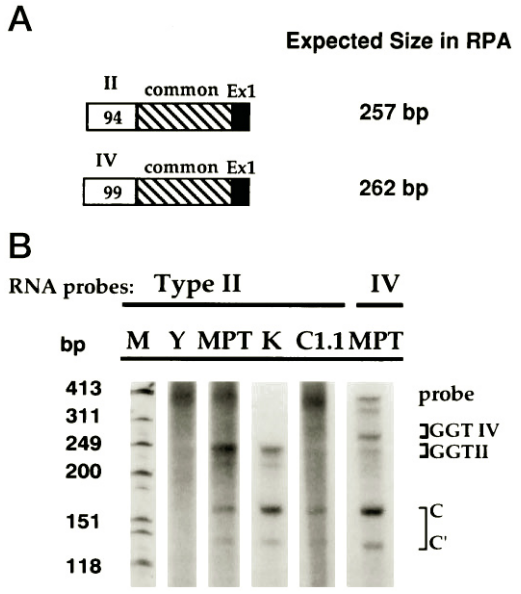

FIG. 1. **Quantitation of GGT mRNAs in the MPT cell line by ribonuclease protection assays.** *A*, structure of the riboprobes used in the ribonuclease assays. *Ex1* represents 25 bp of the first coding exon. The common untranslated exon is indicated and is 138 bp in length. The common region and the Ex1 region are identical in probes II and IV and protect the 163-bp (*B*, *band C*) region of all GGT mRNAs. The lengths of the unique untranslated regions of types II and IV are indicated in the *white boxes* (94 and 99 bp, respectively). Probe II is protected by 257-bp type II mRNAs and a 163-bp fragment (which corresponds to the common region) for all other GGT mRNAs, including the type IV. Probe IV is protected by 262-bp type IV mRNAs and the 163-bp fragment (which corresponds to the common region) for all other GGT mRNAs, including the type II. *B*, ribonuclease protection of yeast (*Y*) RNA and 5 μg of poly(A)$^+$ RNA from MPT cells, mouse kidney (*K*), and C1.1 cells. All the RNA samples were hybridized to the type II specific probe. MPT mRNA was also hybridized with the riboprobe specific for GGT type IV mRNA. The fragments GGT II and GGT IV represent protection of the RNA probe by the corresponding type of GGT mRNA. All the other GGT mRNA types protect only the common region (fragments *C* (163 bp) and *C′* (138 bp)). The C′ band results from utilization of an alternative splice acceptor site near the first coding exon, as described previously (7). The total GGT mRNA was estimated from the sum of the counts present in the 163-bp band and specific band(s) within each lane. For each specific RNA (*e.g.* type IV), its percentage of the total GGT was determined from the amount of the long fragment protected (counts in 262 bp) divided by the total (counts in 262 + 163 bp). *Lane M* is a size marker; *probe* indicates undigested free probe.

Deletion Analyses of the GGT Type II Promoter—In previous experiments we found that in transient transfection assays of CAT reporter constructs a 346-bp 5′ fragment directed higher CAT activity than a 746-bp promoter region in C1.1 cells (7). In addition, no significant CAT activity was found in NIH-3T3 cells with these constructs, indicating that cis-elements present in the 346-bp fragment were sufficient for kidney cell specificity. We have evaluated a series of GGT (II) constructs for CAT activity in MPT cells. A construct containing additional 5′-flanking sequences (PII-2.7) (Fig. 2) displayed lower level of CAT activity (~60%) relative to PII-346. Sequential 5′ truncations of the 346-bp region were tested next. Removal of 50 bp (−346 to −296) (PII-296) resulted in a decline in transcription to approximately 30% of PII-346. This 50-bp region contains an AP2 consensus site (GCCTGGGG) (−326 to −319) (22) which is a candidate for an activator of GGT transcription (see below). Removal of the 5′ 116 bp of PII-346 (PII-230) results in a reduction in CAT activity to about 25–30% of PII-346. This finding indicates that putative activating cis-elements are present within this 116-bp region. Also, within this region

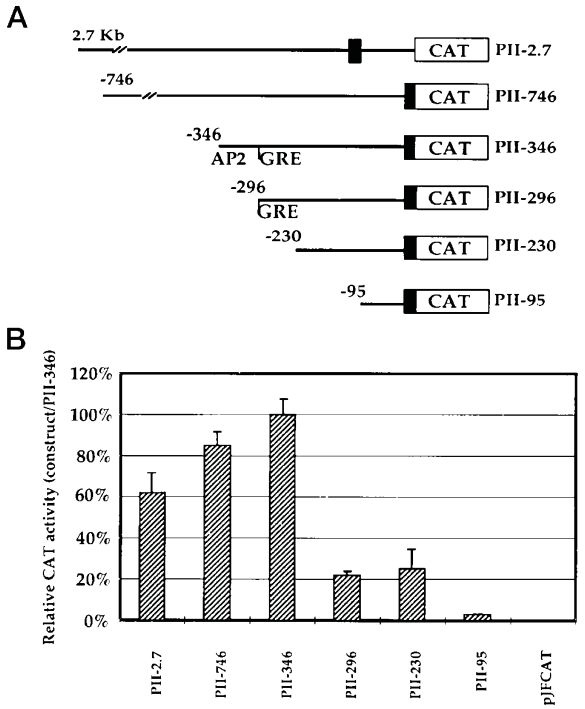

FIG. 2. **Deletion analyses of the GGT type II promoter by transient transfection assays into MPT cells.** *A*, schematic representation of the GGT-CAT reporter constructs. PII-2.7 contains 2.7 kb of the type II promoter region. The type II unique untranslated region is represented by *solid boxes*. In constructs PII-746, PII-346, PII-296, PII-230, and PII-95, the *arabic numerals* indicate the length of the type II promoter region upstream of the major start site of type II RNA, cloned upstream of the CAT reporter gene. *B*, pJFCAT is the promoterless cloning vector. CAT activity of the different constructs is shown as percent of the construct that showed highest activity (PII-346). The *bars* represent the average, and *error bars* represent the standard deviation of at least three separate transfections. The values were normalized for transfection efficiency using the β-galactosidase activity in the same transfection.

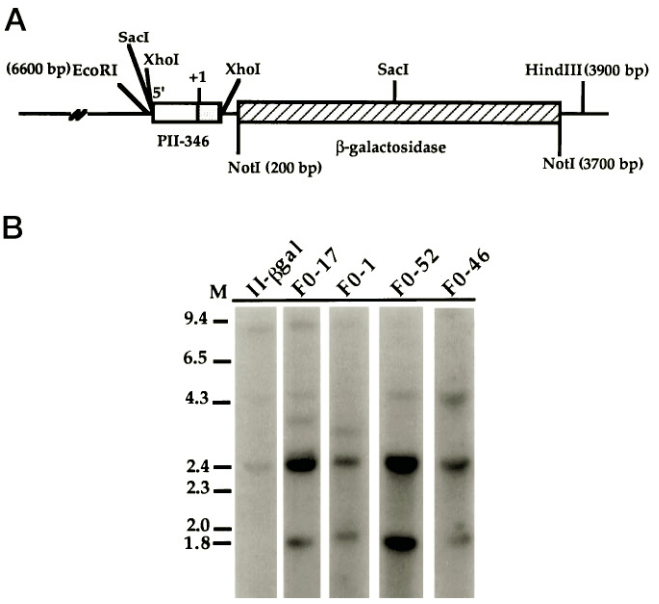

FIG. 3. **Structure of the pII-346/β-galactosidase transgene and screening of transgenic mice by Southern blot analysis.** *A*, the 416 bp of the type II 5′-flanking region containing the 346-bp promoter were cloned into the *Xho*I site of the reporter vector. *B*, Southern blot analyses of tail DNA demonstrating four positive founders (F0-17, F0-1, F0-52, and F0-46). *II-βgal* corresponds to one genomic copy equivalent of the plasmid containing the transgene mixed with genomic DNA from a control nontransgenic mouse, in the same amount present in the lanes of founder mice. Complete *Sac*I digestion of the integrated transgene results in a 2.4-kb *Sac*I-*Sac*I fragment, which includes the type II 5′ region and part of the β-galactosidase cDNA, and a fragment greater than 1.8 kb in length, which includes part of the β-galactosidase cDNA and a segment of mouse genomic DNA of varying size in the various founders. Tandem integrations of the transgene which are head to tail result in a 1.8-kb band.

there is an imperfect glucocorticoid receptor consensus binding site (GRE) (GACATCATGTC) (−294 to −284) (23). The GRE-like site and the AP2 site do not appear to be additive because deletion of the AP2 site alone (PII-296) results in the same level of activity of PII-230, in which both the AP2 consensus and the GRE-like sequence are removed. Deletion of 249 bp (PII-95) results in a marked decrease in transcription with essentially no activity remaining with this construct (Fig. 2). Within the region deleted in PII-95 there are several consensus binding sites, including putative binding sites for AP1 (TTAGTCACC) (−105 to −97) (24), SP1 and AP2 (TCCCCCGCCCA) (−141 to −132) (22, 24), and cAMP response element binding factor/activating transcription factor (TGACGTCA) (−62 to −55) (25) transcription factors.

Characterization of Transgenic Mice Carrying the pII-346/β-Galactosidase Construct—Of the constructs we tested PII-346 has the highest CAT activity in transient transfection assays in MPT cells (Fig. 2). Thus we used this region to construct a fusion reporter gene by cloning it upstream of the bacterial β-galactosidase gene (Fig. 3*A*). The pII-346/β-galactosidase construct was transfected into the MPT cell line by transient transfections. Transfected cells stained positively for β-galactosidase (data not shown). The same construct was used to generate transgenic mice by microinjection (see "Experimental Procedures").

Putative transgenic mice were screened by Southern blotting of genomic DNA digested with *Sac*I (Fig. 3*B*). The hybridizing probe was the same *Eco*RI-*Hin*dIII fragment that we used for microinjection. Because this probe contains 416 bp of the type II 5′ region, both transgenic and nontransgenic genomic DNAs yield a band of ~9 kb which corresponds to the endogenous GGT gene. Complete *Sac*I digestion of the integrated transgene results in a 2.4-kb *Sac*I-*Sac*I fragment which includes the type II 5′ region and part of the β-galactosidase cDNA and a fragment greater than 1.8 kb in length, which includes part of the 3′ end of the β-galactosidase cDNA and a segment of mouse genomic DNA of varying size. Tandem integrations which are head to tail also result in a 1.8-kb band (Fig. 3, *A* and *B*).

We obtained four female founder mice that were positive for the transgene (F0-17, F0-1, F0-52, and F0-46) (Fig. 3*B*). The transgenes integrated as multiple copies in a tandem head to tail orientation in the four animals, as demonstrated by the presence of a 1.8-kb band present in all lanes. The mouse F0-17 yielded F_1 progeny, while the other females did not after multiple attempts.

Analyses of the Expression of the Transgene in Mouse Tissues—We stained sections of kidney, liver, small intestine, spleen, uterus, adrenal gland, lung, heart, and brain for β-galactosidase. Only kidney sections stained positively for β-galactosidase. Expression of β-galactosidase was found only in a population of renal cortical tubules and not in glomeruli, interstitium or in the medullary portions of the kidney (Fig. 4*A*). Both founder F0-1 and mice of line 17 were positive while nontransgenic FVB mice were negative. We took advantage of a histochemical stain for GGT which is specific for proximal tubules to localize β-galactosidase expression (19, 26). Serial sections were performed, and adjacent levels were stained for GGT and β-galactosidase. No β-galactosidase-positive tubules were GGT-negative, while β-galactosidase-positive tubules were also GGT-positive (Fig. 4, *B* and *C*), demonstrating that 346 bp of the type II promoter directed expression of the trans-

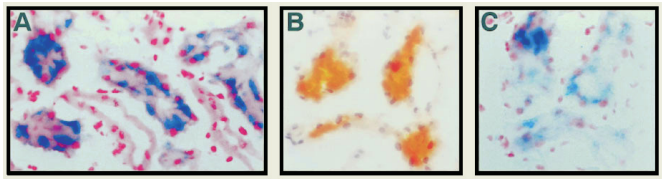

FIG. 4. **Demonstration of expression of the pII-346/β-galactosidase transgene in kidney by histochemistry for β-galactosidase and localization to renal proximal tubules.** *A*, β-galactosidase histochemical reaction in frozen sections of kidney of mouse F0-1. Note the cytoplasmic blue reaction product in positive renal tubules. Histochemical reactions for GGT (*B*) and β-galactosidase (*C*) were performed on serial sections. Note that in *B* the same tubules are stained by the reaction for GGT and β-galactosidase. All photographs were taken at × 400 magnification.

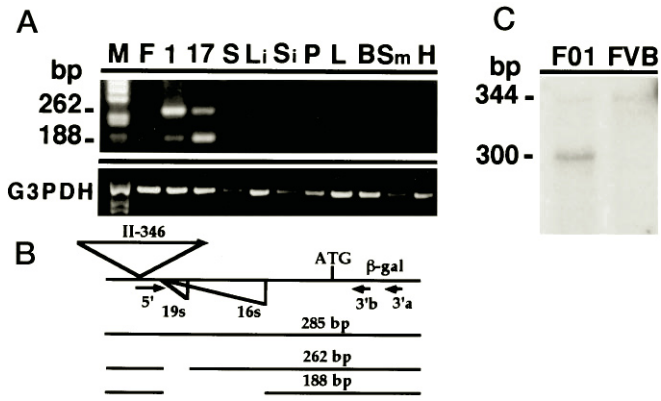

FIG. 5. **Demonstration of expression of the pII-346/β-galactosidase transgene in transgenic mouse tissues by RT-PCR and ribonuclease protection.** *A*, RT-PCR of FVB-negative control mouse kidney RNA (*F*), kidney RNA from F0-1 (*1*), and kidney RNA from the transgenic line 17 (*17*). The remaining lanes are RT-PCR reactions performed with RNA from tissues of line 17 mice and represent spleen (*S*), liver (*Li*), small intestine (*Si*), pancreas (*P*), lung (*L*), brain (*B*), skeletal muscle (*Sm*), and heart (*H*). The *bottom panel* represents the RT-PCR products of G3PDH which were run in parallel with the RNA samples used for amplifications with primers specific for β-galactosidase. *B*, representation of the structure of the transgene in the region between the cloning site of the type II promoter 346 bp (*II-346*) and the translation start site (*ATG*) of the β-galactosidase gene. Primers 3′b and 5′ were used for amplification of the RT-PCR product. These primers yield a 262-bp fragment when the vector SV40 19 s splice acceptor site is used (*19s*) and 188 bp when the SV40 16 s splice acceptor site is utilized. *C*, ribonuclease protection of total kidney RNA from transgenic mouse F0-1. The 344-bp fragment represents the protected band, and the 330-bp fragment represents free undigested probe.

gene to the correct cell population in kidney.

To confirm the histochemistry results, we used RT-PCR and ribonuclease protection to demonstrate the expression of β-galactosidase in transgenic mouse tissues. Total RNAs from kidney, spleen, liver, small intestine, pancreas, lung, brain, skeletal muscle, and heart were examined. RT-PCR was designed to identify the transgene by the presence of the two spliced RNA variants that are originated through utilization of two separate splice acceptor sites present in the vector pNASSβ, resulting in two bands of 262 and 188 bp in length (Fig. 5, *A* and *B*) (27). Only the kidneys of F0-1 and mice of line 17 were positive (Fig. 5*A*). To demonstrate the integrity of RNA samples used in the RT-PCR reactions, parallel amplifications with oligonucleotide primers specific for G3PDH were performed. The expected G3PDH reaction product was present in all RNAs tested. In addition, ribonuclease protection using a β-galactosidase specific probe was performed with RNA from F0-1 and line 17 kidneys. We were able to demonstrate a protected 300-bp band with kidney RNA from mouse F0-1 but not with RNAs from negative control FVB mice (Fig. 5*C*). We did not observe a specific ribonuclease protected band in line 17, probably as a result of low levels of expression.

Contribution of Cis-elements within the Type II 346-bp Promoter Region to Transcriptional Regulation—As an initial attempt to identify key cis-acting elements of the type II promoter, we performed site-directed mutagenesis of sequences that match or have high identity with the binding sites for known transcription factors. Analysis of 5′ truncations of the 346-bp region of promoter II demonstrated that deletion of the 116-bp (−346 to −230) region (PII-230) resulted in a 70–75% decline in transcription of the CAT reporter gene in MPT cells (Fig. 2, *A* and *B*). Sequence analyses revealed consensus binding sites for AP2 (−326 to −319) and a GRE-like sequence (−284 to −271) within these 116 bp (Fig. 6*A*) (22, 28). Similarly, deletion of the 5′ 50-bp (PII-296) that includes the AP2 consensus binding site resulted in a 75–80% decrease in transcription activity of the CAT reporter construct (Fig. 2*B*). There are two possible explanations for the results of the transfection data. First it is possible that the GRE is not required for transcription activation. The second explanation is that the GRE is important for transcription but it does not have an additive effect with AP2. Because the GRE-like site is conserved in mouse and rat, we decided to investigate the second possibility by testing whether a mutation of the GRE-like site alone would affect transcription of the GGT promoter. To demonstrate that the putative binding sequences for AP2 and GRE are involved in the regulation of the type II promoter, we performed site-directed mutagenesis and tested two types of deletion mutations of the AP2 and GRE-like sites (Fig. 6, *A* and *B*). In one type the consensus binding sequence was simply removed, and in the other, the site was partially replaced by a restriction enzyme site that could be used for screening purposes. Both yielded similar results, indicating that utilization of the restriction enzyme site did not affect the level of transcription. Both deletions of the AP2 site (PII-P1rs and PII-P1) resulted in an approximately 80% drop in transcription (Fig. 6*B*); this is the same extent of transcriptional decrease that was observed when the AP2 consensus site was removed in the 5′ truncation PII-296 (Fig. 2). Deletions of the GRE-like element (PII-P2rs and PII-P2) resulted in a drop in CAT activity of 65 and 82%, respectively. Taken together, these findings indicate that the AP2 and GRE-like elements do not appear to act synergistically, although both appear to be required for transcription activation, because their individual deletions affected transcription to a similar extent.

To demonstrate the role of the 116-bp (−346 to −230) region in increasing transcription from the type II promoter, we tested its ability to activate a minimal type II promoter (PII-95) by cloning this 116-bp sequence upstream of the −95-bp region (PII-95/116) (Fig. 7*A*). This construct corresponds to an internal deletion of the region −230 to −95 (Fig. 7*A*). Transient transfection experiments in MPT cells showed approximately a 7-fold increase in CAT activity with PII-95/116 relative to PII-95 (Fig. 7*B*); the overall activity, however, was only 18% of the maximal promoter. These data suggest that, although the cis-elements present in the 116-bp region can activate the transcription of type II promoter, additional elements present within the −230 to −95 region may be required for full promoter activity.

AP2 Binds to the GGT AP2 Binding Site and Footprints a Region of the 5′-Flanking Region of the Type II Promoter—To determine whether the putative AP2 binding site actually binds AP2, we performed gel shift assays. This analysis showed that purified AP2 is able to bind an oligonucleotide that contains the AP2 consensus sequence present in the (−310 to −318) region of the GGT type II promoter (Fig. 8). This binding

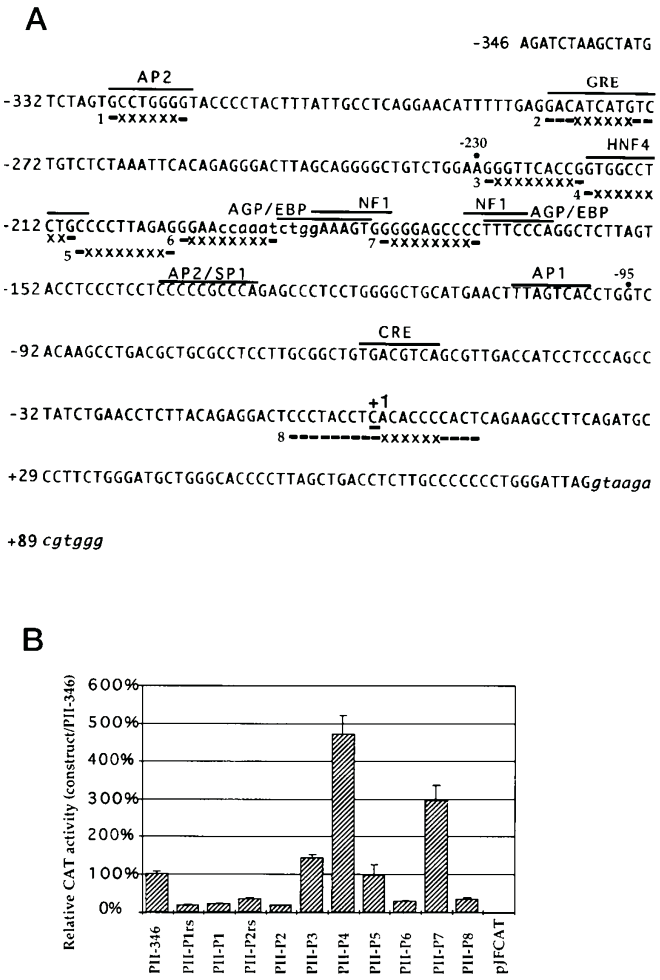

FIG. 6. **Deletion analyses of the GGT type II promoter by transient transfection assays into MPT cells.** *A*, sequence of 440 bp of the GGT 5′-flanking region containing the 346-bp promoter. *Overlined* sequences indicate consensus binding sites of known transcription factors. The bases *underlined* by *-xx* symbols correspond to sequences deleted by site-directed mutagenesis, and the *x* indicates the bases that were replaced by sequences of a restriction enzyme site (*Aat*II or *Not*I). The *lowercase letters* correspond to a SRE. The lowercase letters in *italic* represent the first intronic bases at the type II splice donor site. The major transcription start site is indicated by *+1*. *B*, graphic representation of transient transfection assays of the type II promoter constructs. See *A* for individual deletions. CAT activity is represented in the *y* axis relative to the PII-346 construct. *rs* indicates the deletions that incorporate a restriction site, partially replacing the deleted bases. *pJFCAT* is the promoterless cloning vector. The *bars* represent the average, and *error bars* represent the S.D. of at least three separate transfections.

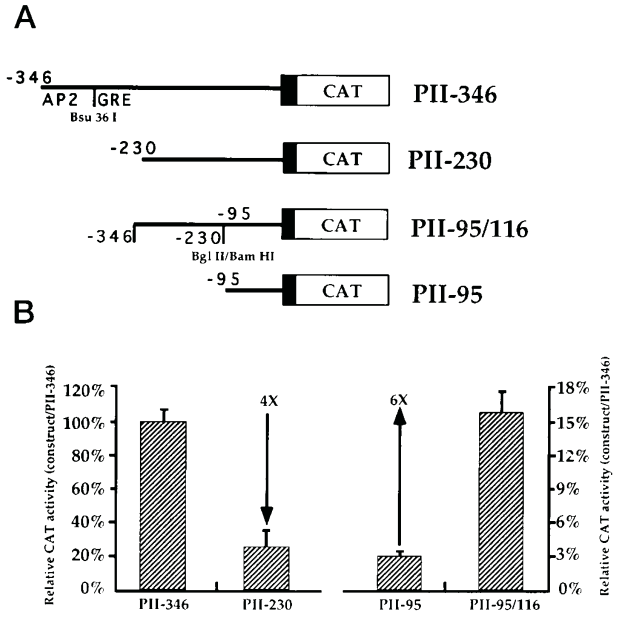

FIG. 7. **Deletion analyses of the GGT type II promoter by transient transfection assays into MPT cells.** *A*, schematic representation of GGT type II promoter constructs PII-346, PII-230, PII-95/116, and PII-95. The −346 to −230 bases were cloned upstream of the 95-bp region in construct PII-95, resulting in PII-95/116. *B*, on the right side is a graphic representation of transient transfection assays of the above constructs into MPT cells. CAT activity is represented in the *y* axis relative to PII-346. On the *left side*, for comparison, are the results of transfection assays of PII-346 and PII-230 into MPT cells. The *bars* represent the average, and *error bars* represent the S.D. of at least three separate transfections.

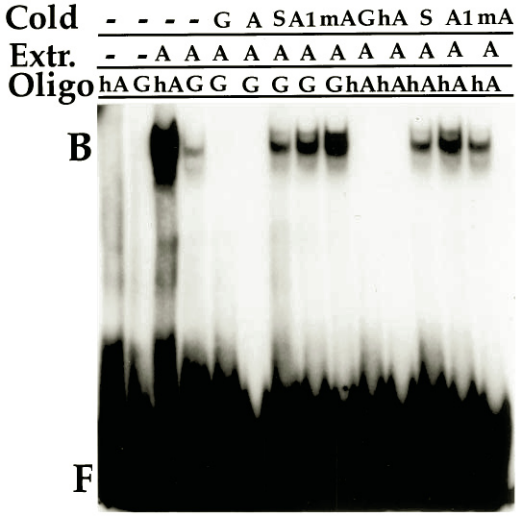

FIG. 8. **Gel mobility shift analysis: recombinant AP2 protein specifically binds an oligonucleotide containing the GGT-AP2 binding sequence.** The oligonucleotide containing the human metallothionein AP2 or GGT-AP2 binding sites are indicated as *hA* and *G*, respectively. Each labeled oligonucleotide (*Oligo*) was incubated with purified AP2 (*A*) extract (*Extr.*). The competitor in each reaction is indicated in the *upper line* as *Cold* and consisted of double-stranded, unlabeled oligonucleotides containing the GGT-AP2 site (*G*), human metallothionein AP2 site (*A*), SP1 site (*S*), AP1 site (*A1*), and a mutated human metallothionein AP2 site (*mA*). *B* indicates the band generated by bound AP2 and *F* indicates free oligonucleotide.

ability is sequence-specific, since it was abolished in the presence of a cold competitor oligonucleotide containing the GGT AP2 consensus sequence but not by an oligonucleotide with a mutation in the AP2 binding site nor by an oligonucleotide containing the sequence of the human metallothionein AP1 binding site (Fig. 8). The affinity of the purified AP2 protein for the GGT AP2 sequence was 10-fold less than for the metallothionein AP2 consensus. This was determined by the ratio of bound to free counts (Fig. 8).

Footprint analysis with an end-labeled DNA probe spanning the GGT AP2 binding sequence revealed that a 45-bp region (−338 to −294) is protected by human AP2 (Fig. 9). The protected 45-bp region includes the GGT AP2 binding site.

Putative Regulatory Cis-elements within the Type II Promoter −230 to −95 Region—We performed the initial sequence analysis of the type II promoter with the Transcription Factors Data base release 6.0 and searched for consensus sequence matches with a calculated intrinsic probability of random occurrence of $<2.0 \times 10^{-4}$. This approach resulted in a relative paucity of putative binding sites within the −230- to −95-bp region. We, therefore, performed site-directed deletion muta-

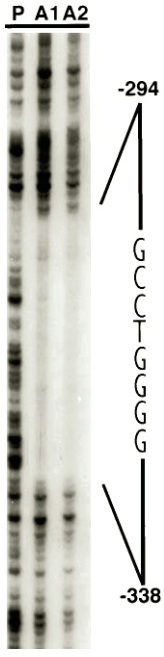

FIG. 9. **Footprint analysis: human AP2 determines a 45-bp footprint in a region of the type II promoter that includes an AP2 binding sequence.** *P* indicates the labeled GGT probe incubated in the absence of purified human AP2 protein and digested with DNase I. *A1* and *A2* represent reactions in which the GGT probe was incubated with 1 (A1) or 2 (A2) footprint units of purified human AP2. The protected sequence extends from bases −294 to −338 bp of the GGT type II promoter and includes the AP2 consensus sequence (GGGGTCCG).

tions of a series of individual random sequences to pinpoint possible cis-acting sequences. We obtained five constructs: PII-P3, PII-P4, PII-P5, PII-P6, and PII-P7 (Fig. 6, *A* and *B*). The results of transient transfections into MPT cells revealed that two deletions (PII-P4 (−219 to −210) and PII-P7 (−179 to −170)) resulted in approximately 5- and 3-fold transcriptional increases, respectively (Fig. 6, *A* and *B*). Two sequences do not appear to affect transcription (PII-P3 (−229 to −220) and PII-P5 (−209 to −200)). One deletion resulted in a 71% drop in CAT activity (PII-P6 (−199 to −190)). Because the type II promoter is a TATA-less promoter that displays partial sequence identity with the terminal deoxynucleotidyltransferase initiator and matches a general consensus for initiators around the transcription start site (29, 30), we performed site-directed mutagenesis in the region that overlaps the major start site (−9 to +10) (PII-P8) (Fig. 6*A*). Transient transfection assays showed that these bases appear to be required for activity of the promoter, because deletion of this region results in 80% drop in CAT activity, relative to the promoter region PII-346 (Fig. 6*B*). Other cis-elements that match the consensus binding sites for putative transcription regulators are present within the −230- to −95-bp region, including a CREB/ATF consensus sequence (TGACGTCA; −62 to −55) (31), and consensus binding sites for AP1 (−105 to −97) (24), SP1 (−141 to −132) (32), and AP2 (−141 to −132) (22) (Fig. 6*A*).

Additional sequence analyses were performed in an attempt to identify putative transcription factor binding sites that overlap the sequences that were deleted in constructs PII-P6, PII-P4, and PII-P7. Using the TESS Transfac data base we identified a putative serum response element (SRE) overlapping PII-P6 (Fig. 6*A*) (33). The SRE is followed immediately by a consensus binding sequence for Nkx-2.5, a murine homeobox homologue of *Drosophila tinman* (Fig. 6*A*) (−186 to −180) (14). Because transcription activity and specificity of transcription activators may be achieved through interactions between a limited and specific set of proteins, these sites appeared to represent good targets for transcription regulation. We therefore tested if a region containing the SRE/Nkx binding site had the ability to activate a minimal heterologous promoter. Single copies of double-stranded oligonucleotides containing the sequences (p6a: −167 to −200) or (p6b: −175 to −192) of the type II promoter were cloned upstream of the CAT reporter gene in the enhancerless pSV40-CAT vector. Transfection studies revealed no significant transcriptional activation with these constructs relative to the vector pSV40-CAT alone (data not shown). To determine whether the lack of enhancing activity was related to this particular vector, we cloned a fragment containing seven multimerized copies of the double-stranded oligonucleotide p6a upstream of a minimal promoter driving the luciferase reporter gene in the TATA-Luc vector (14) and failed to observe increased expression (data not shown).

Sequences matching the consensus binding site for the ubiquitous transcription factor NF1 (34) are present in a palindrome that extends through a region that overlaps the sequences deleted in PII-P6 and PII-P7. This finding suggests a complex regulation of the GGT promoter through this region, since the PII-P7 deletion results in a transcriptional activation (Fig. 6*A*). Interestingly, sequences matching a consensus binding site for α1-acid glycoprotein/enhancer binding protein, a member of C/EBP family of transcription factors (35), partially overlaps the NF1 sequences with a similar palindromic organization. In addition, the sequence deleted in PII-P4, which results in transcriptional increase and therefore predicts a site for down-regulation, matches the binding site for the liver-enriched transcription factor HNF4 (36) in 10 out of 11 bp (Fig. 6*A*).

DISCUSSION

In this study we report the isolation and establishment of MPT cells in culture from the kidneys of GGT(I)-*ras*$^{Val\text{-}12}$ transgenic mice. These cells are useful to study the regulation of the GGT gene in kidney because, unlike other kidney cell lines, they express GGT in a pattern that parallels the expression of GGT *in vivo* (Fig. 1). MPT cells make substantial GGT mRNA and, as in the kidney, GGT type II mRNA is the most abundant of the GGT mRNAs.

Characterization of promoters of genes that are expressed in kidney is in its early stages (37). Even less is known about transcriptional regulation in specific segments of the nephron in the differentiated kidney. Examples of promoters of genes normally expressed in kidney that have began to be analyzed include erythropoietin (38) and renin (39, 40). Many genes have been shown to be expressed in proximal renal tubules, including the enzymes of metabolic pathways such as phosphoenolpyruvate carboxykinase (41, 42), argininosuccinate lyase (43), and aldolase B (44); channel and channel-associated proteins such as the α- and β-isoforms of (Na^+K^+)-ATPase (45, 46); and the angiotensin type II receptor, which is an important regulator of proximal tubule salt water reabsorption and angiotensinogen (47).

A major limitation to the study of mechanisms of kidney and in particular renal proximal tubule-specific gene transcription has been the lack of promoters that show expression restricted to the kidney. The GGT gene is expressed in many epithelial cells including those of the proximal renal tubules; however, of the six different GGT mRNAs that are expressed in kidney, type II is the most abundant. Further, type II mRNA is not found in other visceral organs (7). Thus the type II promoter region is a good candidate to study mechanisms of transcriptional regulation in kidney.

Our data demonstrate that a 346-bp region immediately 5′ of the transcription start of promoter II shows maximal promoter activity. Inclusion of as much as 2.7-kb does not augment

expression. Our experiments with transgenic mice demonstrate that this region contains sufficient information to direct transcription to the proximal convoluted tubules and is not promiscuously expressed in other tissues. Although we have established only one line of mice carrying our β-galactosidase construct, we do not believe the results are explained by site-specific integration. First, the possibility is extremely unlikely on a chance basis, and second, we have obtained similar results in a second founder (Fig. 3).

To identify the cis-acting elements that are required for the transcription of GGT type II in kidney cells (MPT) we performed a series of 5′ truncations and site-directed deletions. Deletion of 116 bp from PII-346 (construct PII-230), resulted in a drop in transcription to 25–30%, which indicates that important regulatory elements are present in this region. A consensus binding site for the transcription factor AP2 and a GRE-like site are present within this region (22, 24). The PII-296 construct, in which the AP2 site was removed, showed a significant drop in transcription to about 25% of the activity seen with PII-346 (Fig. 2). Further, purified AP2 is able to specifically bind an oligonucleotide containing the GGT AP2 binding sequence. In addition, purified AP2 determines a footprint in the region of the type II promoter that includes the AP2 binding site. These findings strongly support a role of AP2 in the activation of the GGT type II promoter. Construct PII-95, with only 95 base pairs upstream of the major transcription start site, showed negligible activity. Although addition of the 116 bp containing the AP2 and GRE-like element to a minimal GGT promoter resulted in 6-fold activation, the CAT activity of this construct was about 5-fold less than that of the 346-bp promoter, indicating that other elements in the promoter are necessary for full activity. In addition, these two elements did not appear to have additive activity. These results indicate that cis-elements present within −230 to −95 bp are required for maximal activity of the type II promoter. An alternative explanation is that the deletions affected the spacing of elements relative to the transcription basal machinery. Changes in the relative positions of factor binding sites often decreases enhancer function as well as specificity. A suggested explanation for this result is that there are architectural factors that have no transcriptional activity on their own but can act synergistically with other transcriptional factors, through the assembly of higher order nucleoprotein complexes. A good example of the required three-dimensional enhancer complex is the binding of four factors to the T cell receptor α enhancer *in vitro* (48–51).

Transient transfections of site-directed mutations within the −230- to 95-bp region identified two sequences that do not appear to affect transcription, one deletion, which resulted in a 71% drop in CAT activity (PII-P6), and two deletions (PII-P4 and PII-P7), which resulted in approximately 5- and 3-fold transcriptional increases, respectively (Fig. 6). These findings show that GGT type II promoter, like other promoters, contains both positive and negative cis-acting elements (44, 52, 53).

Several reports indicate that the tissue specific expression of genes is achieved through complex interactions that involve numerous cis-acting regions that exert positive and negative effects on promoter activity. This type of regulation is exemplified by the human erythropoietin receptor gene (54) and the skeletal α-actin gene (55).

Regarding the mechanisms that direct kidney specificity of the GGT type II promoter, it is possible that non-DNA-binding factors, such as coactivators or adapters, can determine tissue specificity. Examples of this type of regulation include the B cell-specific coactivator OCA-B (56) and the tissue-specific coactivator DCoH (57). It is possible that negative regulatory elements function in tissues other than kidney. For example, the consensus binding sequence for the liver enriched factor HNF4 has a negative effect on the transcription of type II promoter and may mediate the repression of this promoter in liver cells. It is also possible that there are tissue-specific elements in the GGT promoter that were not revealed by the mutagenesis analysis.

The combination of transient transfection assays in the novel kidney MPT cell line and expression of a pII-346/β-galactosidase reporter gene in transgenic mice demonstrates that 346 bp of the mouse type II GGT promoter are sufficient to confer specific tissue expression of GGT type II to proximal tubular renal cells. Future dissection of the protein factors that bind to regulatory sites in the promoter and of their cooperative interactions should help elucidate the regulation of transcription in proximal renal tubules.

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