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) is a key enzyme in glutathione metabolism (1–3). It is expressed in many epithelial tissues, 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 p21ras-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 mM Na2SeO35H2O (SFFD) and was supplemented with insulin (5 mg/ml), PGE1 (25 mg/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
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. 1A. The lengths of the unique 5′-sequence are 94 bp for III, 95 bp for IV, respectively. The β-galactosidase ribonuclease probe was obtained from Ambion, Inc. (Austin, TX). The plasmids were linearized and then transected with either T3 or T7 polymerase (Stratagene Inc., La Jolla, CA) in the presence of [α-32P]UTP to obtain the uniformly labeled antisense strand.

Oligonucleotide 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 fibroblasts (FVB) kidneys and selected by oligo(dT)-cellulose type III (Collaborative Research Inc., Bedford, MA).

Ribonuclease protection assays were performed with the RNA 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 × 105 cpn of a [α-32P]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 type II and IV, the amount of RNA in each lane was determined by scanning the 6.0-kb XhoI fragment containing 346 bp of the GGT type II promoter region was sequenced, and a clone with the sense orientation was selected for microsequencing.

Expression of GGT Promoter II in Kidney—Gel mobility shift analyses were performed with a double-stranded oligonucleotide (5′-ATGTCCTAGTGCGCTGGGTTACCC) 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 IIAP2 binding site (5′-GATCGAACCTGACCGGCGCTCGGCTCGG) (8) or the sense orientation. The double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP, and 1 × 105 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 AMBIOS Radioanalytic Imaging System and the AMBIOS QuantProbe Software version 4.01 (AMBIOS, Inc.).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by oligonucleotide in vitro mutagenesis as described by Kunkel, with the Muta-Gen Phagemed 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′-GCAAATAAGGGTTAAGCTTCTAGATCTATAGTTGATA) or P1 (5′-GCAATAAAGGGTTAAGCTTCTAGATCTATAGTTGATA); P2rs (5′-TCTGGTAAATTTAGAGCTATTAGCATTATTTGTTA) or P2 (5′-TCTGGTAAATTTAGAGCTATTAGCATTATTTGTTA). The underlined sequences correspond to the AatII (GAGCTC) or NotI (GGCGGC) restriction sites. The sequences deleted in each construct were PII-P1 and PII-P1rs (−310 to −318), PII-P2 and PII-P2rs (−283 to −297), PII-P3 (−229 to −220), PII-P4 (−219 to −210), PII-F5 (−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, AatII in primers P1rs, P2rs, and P8 and NotI 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.

Luciferase Reporter Constructions—The promoterless plasmid pEF-CAT I (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 PstI digestion of the 6.6-kb XhoI clone (7); the PstI ends were flushed with T4 polymerase, and the fragment was cloned into the XhoI site of pFCAT I. PIII-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 amplification of sequences of appropriate size using PII-346 as a template. The PCR amplifications were performed as described previously (13). PII-95 was obtained by PCR with the oligonucleotides 5′-(CTCTGGAGAAGGGTCGCTTGCTCG) and 3′-(GGCGGCCTCTCGAGAAGGTCACTAA), and PII-95 was obtained by PCR with the oligonucleotides 5′-(CTCTGGAGAAGGGTCGCTTGCTCG) and 3′-(GGCGGCCTCTCGAGAAGGTCACTAA) or 3′-(GGCGGCCTCTCGAGAAGGTCACTAA) and 5′-(GGCGGCCTCTCGAGAAGGTCACTAA).

The PCR products were cloned into the pBluescript vector (Novagen, Madison, WI), digested with XhoI, and cloned into the XhoI site of pFCAT I. The 116-bp fragment was obtained by PCR using PIII-346 as a template, and using the oligonucleotides 5′-(5′-CTCTGGAGAAGGGTCGCTTGCTCG) and 3′ -(5′-GGCGGCCTCTCGAGAAGGTCACTAA) or 5′-(5′-CTCTGGAGAAGGGTCGCTTGCTCG) and 3′-(GGCGGCCTCTCGAGAAGGTCACTAA) or 5′-(GGCGGCCTCTCGAGAAGGTCACTAA) and 3′-(GGCGGCCTCTCGAGAAGGTCACTAA).

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 PCRTIMII (Invitrogen, Inc.) and released after digestion with HindIII and NotI (7). This probe was end-labeled with a T4 polynucleotide kinase (Promega) and [γ-32P]ATP, followed by digestion with HindIII to release the 3′ end of the GGT promoter. Footprint assays were performed with the Core Footprinting System (Promega) or using 1 or 2 footprints units of purified human AP2 protein (Promega) and 1 × 105 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.
and F1 progeny were analyzed by Southern blot of tail DNA. Briefly, 10 μg of genomic DNA were digested with ScaI, 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 EcoRI-HindIII 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′-GAAGTAAAAAGCAGAAAGTAAAT, 3′-GCCAACAGTGCAGATTCAGGAGTGTAAACCCACCGAGCGACGAGCT, and 3′-GAATACTCCCTCAGGACCGGCAAC were designed to amplify the transgene across the SV40 introns present in the β-galactosidase reporter vector pNASS8. 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 BamHI, 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 MgCl2, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(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-rasβ21-12 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).

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 than a 746-bp promoter region in C1.1 cells. 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.

FIG. 1. Quantitation of GGT mRNAs in the MPT cell line by ribonuclease protection assays. A, structure of the riboprobes used in the ribonuclease assay. 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.
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 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 (TTAGTGACC) (−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 GGT promoter region upstream of the major start site of type II RNA, cloned upstream of the CAT reporter gene. PII-JFcat 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.

II 5′ region, both transgenic and nontransgenic genomic DNAs yield a band of about 9 kb which corresponds to the endogenous GGT gene. Complete SacI digestion of the integrated transgene results in a 2.4-kb SacI-SacI 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.

We obtained four female founder mice that were positive for the transgene (F0-1, F0-1, F0-52, and F0-46) (Fig. 3B). 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 F1 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. 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. 4A). 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-
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. 5A). 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. 5C). 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. 6A) (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. 2B). 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. 6B); 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-85) by cloning this 116-bp sequence upstream of the −95-bp region (PII-95/116) (Fig. 7A). This construct corresponds to an internal deletion of the region −230 to −95 (Fig. 7A). Transient transfection experiments in MPT cells showed approximately a 7-fold increase in CAT activity with PII-95/116 relative to PII-95 (Fig. 7B); 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
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 (2338 to 2294) 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 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 × 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-
Expression of GGT Promoter II in Kidney

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 (GGGTCCG).

DISCUSSION

In this study we report the isolation and establishment of MPT cells in culture from the kidneys of GGT1-rasV12 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 been examined 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-isofoms 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 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. 6A). 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 overlap 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. 6A).

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. 6A) (33). The SRE is followed immediately by a consensus binding sequence for Nkx-2.5, a murine homeobox homologue of Drosophila tinman (Fig. 6A) (−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. 6A). 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 overlap 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. 6A).
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 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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