

## Expression Cloning of a Rat Hepatic Reduced Glutathione Transporter with Canalicular Characteristics

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### Abstract

Using the *Xenopus* oocyte expression system, we have previously identified an ~4-kb fraction of mRNA from rat liver that expresses sulfobromophthalein-glutathione (BSP-GSH)-insensitive reduced glutathione (GSH) transport (Fernandez-Checa, J., J. R. Yi, C. Garcia-Ruiz, Z. Knezic, S. Tahara, and N. Kaplowitz. 1993. *J. Biol. Chem.* 268:2324–2328). Starting with a cDNA library constructed from this fraction, we have now isolated a single clone that expresses GSH transporter activity. The cDNA for the rat canalicular GSH transporter (RcGshT) is 4.05 kb with an open reading frame of 2,505 nucleotides encoding for a polypeptide of 835 amino acids (95,785 daltons). No identifiable homologies were found in searching various databases. An ~96-kD protein is generated in *in vitro* translation of cRNA for RcGshT. Northern blot analysis reveals a single 4-kb transcript in liver, kidney, intestine, lung, and brain. The abundance of mRNA for RcGshT in rat liver increased 3, 6, and 12 h after a single dose of phenobarbital. Insensitivity to BSP-GSH and induction by phenobarbital, unique characteristics of canalicular GSH secretion, suggest that RcGshT encodes for the canalicular GSH transporter. (*J. Clin. Invest.* 1994. 93:1841–1845.) Key words: glutathione • transport • organic anions • phenobarbital • induction

### Introduction

The liver plays a key role in interorgan reduced glutathione (GSH)<sup>1</sup> homeostasis, releasing GSH at the sinusoidal pole into plasma and at the canalicular pole into bile (1). Efflux at both poles appears to be carrier mediated by low affinity systems that exhibit multispecificity in hepatic uptake and biliary secretion of certain organic anions (1–3). Furthermore, canalicular GSH secretion is an important determinant of bile acid-inde-

pendent bile flow (4). Physiologic differences in the transport of GSH at the two poles of the hepatocyte have suggested the existence of two distinct transport systems, possibly encoded by distinct genes. The canalicular transporter activity is inhibited by sulfobromophthalein (BSP) but not by BSP-GSH (2, 5), and is induced by phenobarbital (5), whereas the sinusoidal transporter is inhibited by BSP-GSH (2, 3), and is not induced by phenobarbital (5). Recently, we have identified two distinct size class mRNAs from rat liver that express GSH transport in *Xenopus laevis* oocytes: ~2.5-kb mRNA that encodes for a BSP-GSH-inhibitable GSH transporter (putative sinusoidal) and ~4-kb mRNA that encodes for a GSH transporter insensitive to BSP-GSH (putative canalicular) (6). We now report for the first time the cloning and molecular characterization of an hepatic GSH transporter with characteristics of the canalicular transporter.

### Methods

*RNA isolation, oocyte preparation, and transport.* Isolation of poly(A)<sup>+</sup> RNA from rat liver (male Sprague Dawley rats, 200–250 g), size fractionation, and microinjection of mRNA and cRNA into stage 5 and 6 defolliculated *X. laevis* oocytes were performed as previously described (6). GSH uptake and efflux were determined as previously described using [<sup>35</sup>S]GSH and unlabeled GSH (6) 2 or 3 d after injection of mRNA, cRNA, or water. For uptake, 10 mM extracellular GSH with or without 5 mM BSP-GSH was used, and for efflux, oocytes were injected with 66 nl per oocyte containing either 16 nmol GSH ± 4 nmol BSP-GSH versus water alone. These are the identical conditions as in our previous report (6). Verification of the molecular form of the radiolabel transported was performed by HPLC (6). As in our previous studies (6), no evidence of breakdown products of [<sup>35</sup>S]GSH was seen in HPLC of cells or medium in either uptake or efflux experiments. [<sup>35</sup>S]GSH was obtained from DuPont-New England Nuclear (Boston, MA) (145 Ci/mmol).

*cDNA library construction and clone isolation.* The 3.5–4.0-kb mRNA size fraction that conferred peak stimulation of GSH uptake/efflux (BSP-GSH insensitive) in *Xenopus* oocytes was used to construct a directional cDNA library in the expression vector, pcDNA1 (Invitrogen, San Diego, CA). Briefly, size-fractionated mRNA was used as a template for cDNA construction using reverse transcriptase and a synthetic oligo(dT)-NotI primer. The conversion of RNA-cDNA hybrid into double-stranded cDNA was done by using RNaseH in combination with DNA polymerase 1 and *Escherichia coli* DNA ligase. BstXI adaptors were then added to the double-stranded cDNA at the 5' end, and the resulting cDNA was subsequently ligated to the previously BstXI- and NotI-digested plasmid vector, pcDNA1, which was then electroporated into MC 1060/p3 cells. The cDNA inserts in this vector are flanked by T7 and SP6 RNA polymerase promoters at their 5' and 3' ends, respectively, allowing production of both sense and

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1. Abbreviations used in this paper: BSP, sulfobromophthalein; GSH, reduced glutathione; RcGshT, rat canalicular GSH transporter.

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antisense cRNA transcription. The library consisted of  $\sim 5 \times 10^6$  colonies.

For screening, a pool of plasmid DNA was prepared from the library (200  $\mu$ l) and aliquots (1  $\mu$ l) were electroporated into *E. coli*. The transformants were divided in four groups. Plasmid DNA from each group of colonies was prepared by an alkali-SDS lysis method using a Mag DNA purification kit (Promega Biotec, Madison, WI). Plasmid DNA was linearized with NotI and in vitro transcribed with T7 RNA polymerase in the presence of the GpppG cap, using a protocol supplied with the Riboprobe transcription system (Promega Biotec). The complementary RNA (cRNA) from the same pool of colonies was injected into oocytes. This procedure was repeated four times (1- $\mu$ l aliquots of the total plasmid pool) until a pool that induced the uptake of [ $^{35}$ S]GSH 10-fold over background was identified and sequentially subdivided into four groups of colonies twice, each time using cRNA to express activity. At that point, 500 colonies remained; an individual plasmid pool was prepared from each colony (1–2  $\mu$ g DNA in 50  $\mu$ l H<sub>2</sub>O each). 5- $\mu$ l aliquots of each individual pool were mixed into two groups of pools representing 250 colonies. cRNA prepared from each was used to express activity in oocytes. From the plasmid DNA of 250 colonies shown to contain the rat canalicular GSH transporter (RcGshT) activity, 5 pools of 50 individual plasmids (5- $\mu$ l aliquots) were mixed and the one expressing GSH transport was identified. 5 pools of 10 individual plasmids from these 50 were then mixed and screened. The individual 10 plasmids from the positive pool were then screened and the single positive one identified.

**Northern blot analysis of the cDNA clone.** Poly(A)<sup>+</sup> RNA (5  $\mu$ g) prepared from various rat tissues was separated on a 1% agarose gel with 6.7% formaldehyde, blotted onto a nylon membrane by capillary transfer, then hybridized to a 1.5-kb cDNA derived from a subcloned insert (BamHI-XbaI fragment) corresponding to nucleotide 1132–2623 labeled with  $^{32}$ P by random priming (Primer-It II Kit; Stratagene, La Jolla, CA). Hybridization was at 42°C overnight in 50% formamide, 6 $\times$  SSPE (0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 2% SDS containing 100  $\mu$ g/ml salmon sperm DNA and 10<sup>7</sup> dpm labeled insert. Blots were washed twice for 10 min in 2 $\times$  SSC, 0.1% SDS at room temperature, twice for 30 min in 0.1 $\times$  SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.5% SDS at 65°C, then twice for 30 min in 0.1 $\times$  SSC at room temperature, and autoradiographed for 3 d onto Hyperfilm<sup>R</sup> (Amersham Corp., Arlington Heights, IL) using image-intensifying screens.

**In vitro translation of RcGshT cRNA.** In vitro translation of cloned RcGshT cRNA was performed by using a rabbit reticulocyte system (Promega Biotec) and [ $^{35}$ S]methionine (DuPont-New England Nuclear) (1200 Ci/mmol) according to the instructions of the manufacturer. Translated protein was run in SDS-PAGE and autoradiographed as above.

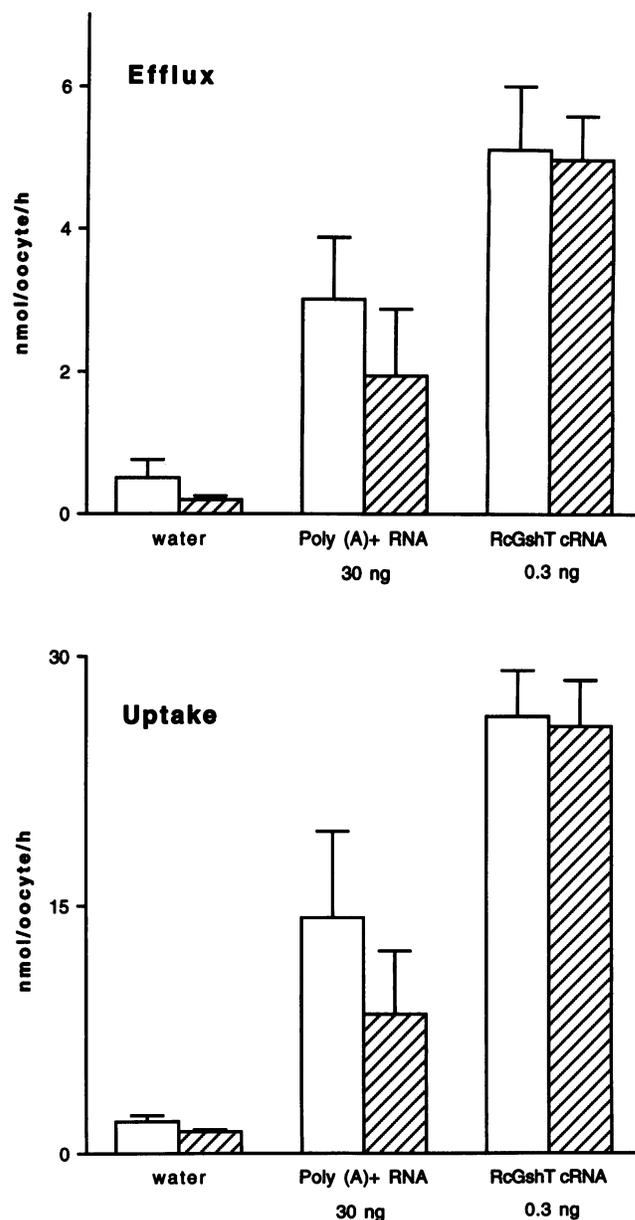
**Effect of phenobarbital on RcGshT mRNA.** Fed male Sprague-Dawley rats (250 g) were injected with a single dose of phenobarbital (80 mg/kg in saline i.p.) or equal volume of saline 3, 6, or 12 h before isolation of total liver RNA. 20  $\mu$ g of each total RNA was run on agarose gel electrophoresis, transferred to nylon membrane, and hybridized with a  $^{32}$ P-labeled RcGshT DNA fragment, as described above. The same membrane was rehybridized with  $^{32}$ P-labeled rat  $\beta$ -actin cDNA (Clontech, Palo Alto, CA). Autoradiography and densitometry (2222-020 Ultra Scan XL laser densitometer with software 3.0; LKB, Piscataway, NJ) were used to quantitate relative RNA. Phenobarbital-induced transcript densities were normalized to saline controls to estimate fold increase.

**Sequence analysis of the cDNA.** The insert of cloned cDNA was digested with different restriction enzymes and the resulting fragments of cDNA were subcloned into the plasmid vector pGEM-f11(–) (Promega Biotec). The nucleotide sequence from each of the subcloned fragments was determined by dideoxy chain termination using the Sequenase DNA sequence kit (U.S. Biochem. Corp., Cleveland, OH). Both strands of the clone were sequenced using SP6 and T7 sequencing primers. cDNA-specific oligonucleotide primers were also used to

complete the sequencing analysis. Comparison of RcGshT amino acid and nucleotide sequences were performed with GCG software (University of Wisconsin Genetics Computer Group Program Package) to search GenBank, EMBL, Swiss Prot, and PROSITE data bases.

## Results and Discussion

The previously identified 3.5–4-kb mRNA size fraction from rat liver that expressed BSP-GSH-insensitive transport of GSH



**Figure 1.** GSH transport in *X. laevis* oocytes. Oocytes were injected with RcGshT cRNA (0.3 ng), poly(A)<sup>+</sup> RNA (30 ng), or water (33 nl). 2 d later, GSH efflux (top) or uptake (bottom) were determined. For efflux, oocytes were loaded with [ $^{35}$ S]GSH (16 nmol) with or without BSP-GSH (4 nmol), and efflux was immediately determined. For uptake, oocytes were exposed to medium containing [ $^{35}$ S]GSH (10 mM) with or without BSP-GSH (5 mM). Results of experiments with three different oocyte preparations are shown as mean  $\pm$  SD, with open bars representing the absence and hatched bars the presence of BSP-GSH.

(6) was used to construct a cDNA library. After screening multiple subdivisions, a single clone (RcGshT) was isolated that expressed GSH efflux and uptake in oocytes (Fig. 1). When corrected for equivalent amount of RNA injected into oocytes, the cRNA of the individual clone exhibited > 100-fold enrichment in transport activity compared with total rat liver poly(A)<sup>+</sup> RNA whether or not the values for water controls are first subtracted. As expected, BSP-GSH did not *cis*-inhibit uptake or efflux of GSH after expression of RcGshT, but partially inhibited after expression of total poly(A)<sup>+</sup> RNA due to the coexpression of the putative sinusoidal GSH transporter.

The DNA sequence and deduced amino acid sequence of RcGshT are shown in Fig. 2. The cDNA insert consists of 4,050 nucleotides with an open reading frame of 2,505 nucleotides, predicting a polypeptide of 835 amino acids with a molecular mass of 95,785 daltons. The start codon was identified according to the model of Kozak (7). Kyte-Doolittle analysis (8) was consistent with an integral membrane protein with 10 possible membrane-spanning domains, 4 of which are strongly hydrophobic (amino acids 102–120, 126–148, 168–187, 386–404), and an additional 6 that are of appropriate length but with weaker hydrophobicity (193–211, 258–278, 324–346, 475–493, 550–568, 683–704). There is no cleavable signal sequence indicating that the NH<sub>2</sub> terminus is likely to be cytoplasmic. Five possible N-linked glycosylation sites (amino acids 10, 55, 466, 573, and 638) are present as well as multiple potential phosphorylation sites (protein kinase A [PKA] site at amino acid 282; tyrosine kinase site at 679; PKC sites at 198, 255, 315, 326, 429, 498, 536, 592, 644, 758, and 791; and casein kinase II sites at 24, 57, 156, 227, 411, 684, and 787). No significant amino acid or nucleotide sequence homology (defined as > 10%) could be identified in searching current data bases of nucleotide and amino acid sequences.

The prediction that RcGshT encodes for an integral membrane protein with multiple membrane-spanning domains suggests that this is the transporter polypeptide. However, a role for this gene product in activating a latent endogenous oocyte, as well as rat liver, transporter is impossible to exclude entirely at this time. This is a general reservation that can be made with nearly all the membrane transporters cloned to date.

*In vitro* translation using a rabbit reticulocyte system revealed a polypeptide of ~ 96 kD on SDS-PAGE (Fig. 3), in close agreement with the predicted molecular mass calculated from the open reading frame. A single hybridizable transcript of ~ 4.0 kb was identified on Northern blot analysis of rat liver (Fig. 4). Analysis of other organs revealed a similar single hybridization signal in brain, kidney, intestine, and lung, and no signal in heart (Fig. 4). The localization in the kidney suggests that this gene may be responsible for the known apical secretion of tubular epithelial GSH (9), which is key element of the intrarenal gamma-glutamyl cycle (9). Little is known about apical GSH transport by intestinal or lung epithelial cells; however, in the case of lung, it is of interest that alveolar fluid GSH achieves near millimolar concentrations (10). The high levels of transcripts in the brain is somewhat surprising. Although a blood-brain barrier transport of GSH has been suggested (11, 12), it has also been reported that astrocytes efflux GSH (13). Further work will be required to identify the cell type in the brain in which this transporter mRNA is expressed.

The identification of RcGshT as encoding for the canalicular transporter is suggested on the basis of its insensitivity to

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-110 CCCATATCCCGCTAAAAACTCATAATCCCAAAAAAGATAAATATAAAAAATATCACTGAG -51
-50 TCAATCAATAAATAATTTGGCATACAGGACACATCCAGAGGAATATTTATGAGCAGAGA 9
-15 M S T E 4
10 AACAAATGAAATTCATTAATAGTGAATGGGCAATCAACTAAAACAGCCGATATC 29
5 T I E I F N N S D E W L K H A L S 64
70 GAAAGGAGAAATCCGCATTACTACATGGTTAACTCCCTGATATCCCTTATAGAAATATA 129
135 T K D I F L R P D L R P L D R I Y 144
130 TCCATATGCAATCCGATACCATGAAAGGGTATATAACGGCCAGGAAATTTATATATA 189
45 A Y A F D Y H E K E N I T D A E I Y Y K 64
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65 F L C I Y A F E N H E Y L K D F G I S M 84
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85 F S P Q R N I N K H I D A L Q T K L Q L 104
310 ACTTCCCGGTATGATGACTATTCAGTATTTATTCGATGGGCAATGTCAGATTTGGGGCT 169
105 L P G M H T I Q L F I V N V R L G L 129
370 AAAAATATCGATACCGAATGCAATGTTCTATCAATTTATTAACATTTGTGAGGATGAT 424
125 K I S I T Q C N W S I T L L T I V R M I 144
430 AGTGTAAAGATAAAGCCAGGCATATTAAGACTTAAAGCAATATTCAGAAGATAA 489
145 V L R V K R R R H I L N S L M D N S E D N 164
490 TCGGCTAAGCATACAATCATTTGGTAAATTTGCTGGTTATAGTGCAGTTAACCAAT 549
165 R L N I T I I W L L L V I V Q L T T I 184
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610 AATCAATATTTTCAATGCGTTAGAGAACAATCTACTTATCCACCTTGAATAAGCGTTA 669
205 I I F Q Q V R E Q S T L S P L N K A Y 224
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225 S D S I L E H E L R P F A L 244
730 TATTTCGATCCCTGAAAGCGGTTATGACGACCAAGAACCGGATTTGCCCATAT 789
245 I G D S L E A V D I T T R N A A N G D I I 264
790 TTCTCTTTTGGCGTGCAGGGATGCGAGGCCGCTTTTTCNAAGAGGGTTCCCTGGG 849
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850 GGGGCTAAGAAAGATAAAATTTCTCATATCTTTCTGGGGGATCCCGCTGGGGGAA 909
285 G A K K D K I L I S F S W G I P A R G K 964
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345 P F G G Q K F K I S 364
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365 F S W G I P A R G I L T V N E K Q V Y I 384
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425 K S I Y S L R R I F G K I G Y Y R C I E 444
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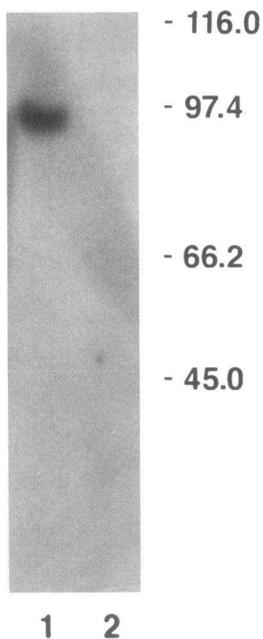
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605 H Y L S D E H L A G K K E L Y D F T P E 624
1870 AAGCATTTCAAGGGCCATCGATATTTGATAGATCAAAAATAAAAATGATATCAAGAC 1929
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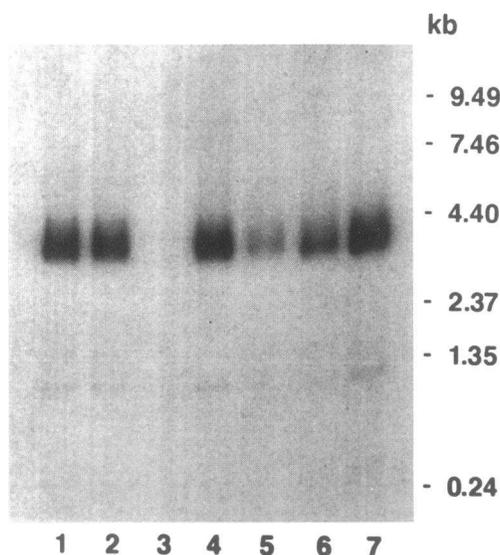
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Figure 2. Nucleotide and deduced amino acid sequence of RcGshT. The first nucleotide and amino acid residue of the start site are designated as position 1 and the termination codon follows the last amino acid residue. Polyadenylation signal sequence is underlined. These sequence data have been submitted to EMBL/GenBank under accession number U06845.

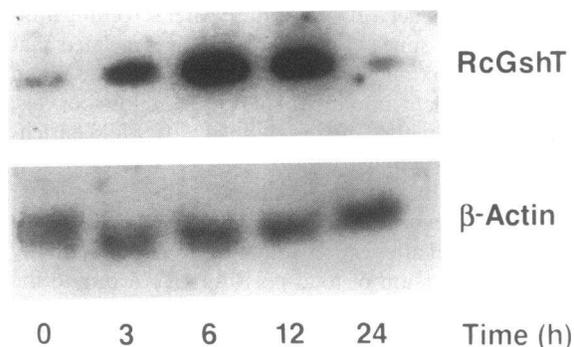


**Figure 3.** In vitro translation of RcGshT cRNA in the rabbit reticulocyte lysate system. Shown is an autoradiograph of the reaction mixture after SDS-polyacrylamide (8%) gel electrophoresis. The locations of protein standards are shown at the right (kD).

BSP-GSH. However, to add further support to the identity of RcGshT as the canalicular GSH transporter, we examined the effect of phenobarbital. Phenobarbital treatment in vivo is known to induce GSH secretion into bile (14) and increase the  $V_{max}$  for GSH transport in canalicular-enriched membrane vesicles (5) without affecting sinusoidal GSH efflux in the intact



**Figure 4.** Northern blot analysis of rat tissue distribution of RcGshT mRNA. 5  $\mu$ g poly(A)<sup>+</sup> RNA isolated from rat brain (lane 1), male liver (lane 2), heart (lane 3), kidney (lane 4), lung (lane 5), small intestine (lane 6), and female liver (lane 7) were subjected to electrophoresis on 6.7% formaldehyde/1% agarose gel, blotted onto a nylon membrane, and hybridized with <sup>32</sup>P-labeled 1.5-kb insert of a subclone of RcGshT corresponding to nucleotides 1132–2623. Blots were subsequently washed under high stringency (see Methods). Autoradiograph was obtained after a 3-d exposure. The scale at the right indicates position of standard markers (kb).



**Figure 5.** Time course of changes in RcGshT mRNA in rat liver after phenobarbital treatment. Rats were given phenobarbital (80 mg/kg i.p.) and killed at 0, 3, 6, 12, and 24 h. Total RNA (20  $\mu$ g) from each liver was loaded per lane, subjected to electrophoresis, and blotted as described in Methods. The blots were hybridized with <sup>32</sup>P-RcGshT 1.5-kb cDNA fragment and rehybridized with the <sup>32</sup>P-actin probe.

liver or GSH transport in sinusoidal-enriched membrane vesicles (5, 14). Indeed, an increase in mRNA was observed at 3, 6, and 12 h (peak, 6 h) after a single dose of phenobarbital that subsequently declined to baseline at 24 h; the peak increase was at 6 h (Fig. 5). An eight- to ninefold increase was found 6 h after phenobarbital compared with saline treatment determined in two pairs of rats. The mechanism for this effect of phenobarbital and the level of regulation (transcription vs. mRNA stabilization) remain to be determined.

In summary, this is the first report of successful cDNA cloning of a GSH transporter. Using the *Xenopus* oocyte expression system, we have isolated a 4.05-kb cDNA (RcGshT) from a rat liver library encoding for a polypeptide of 835 amino acids that mediates GSH transport. Insensitivity to BSP-GSH and induction by phenobarbital provide strong support for its identification as the canalicular GSH transporter. Immunohistochemistry using polyclonal antibodies to synthetic peptides is planned to identify the precise cellular and membrane domain localization of RcGshT gene product in liver and other organs. The lack of sequence homology with known transporters indicates that it represents a new family of transporter polypeptides. Its wide organ distribution suggests that it plays a role in GSH turnover (efflux) in many cell types.

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