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Functional Re-evaluation of the Putative Glutathione Transporters, RcGshT and RsGshT

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Transport systems that mediate glutathione (GSH) efflux from hepatocytes into blood plasma and bile have been characterized extensively in sinusoidal and canalicular membrane vesicles, and recent reports describe two candidate GSH transport proteins: the rat sinusoidal GSH transporter (RsGshT) and rat canalicular GSH transporter (RcGshT). However, studies in our laboratory have been unable to confirm the function of these gene products. *Xenopus laevis* oocytes injected with either rat liver mRNA, the cRNA for RcGshT or the cRNA for RsGshT did not transport GSH at a higher rate than water-injected oocytes, when measured either as ^{3}H-GSH uptake or efflux, at low or high GSH concentrations, or in the presence or absence of acivicin to inhibit γ-glutamyltransferase activity. In contrast, transport of ^{3}H-taurocholate was markedly accelerated in oocytes injected with rat liver mRNA or the cRNA for the Na^+-taurocholate cotransporting polypeptide (Ntcp), confirming the integrity of the mRNA and the viability of the oocytes. Northern blot analysis failed to detect an RcGshT transcript in rat liver total RNA or rat liver mRNA. Of significance, the RcGshT and RsGshT cDNA sequences are similar to those found in the *Escherichia coli* K-12 genome, indicating possible cloning artifacts. Further studies are needed to resolve this discrepancy, and to isolate and characterize hepatic GSH transport proteins.

INTRODUCTION

Most of the glutathione (GSH)[b] synthesized within hepatocytes, a major site of synthesis of this tripeptide [1-3], is subsequently transported across the apical (canalicular) membrane into bile, and across the basolateral (sinusoidal) plasma membrane into blood for delivery to other tissues. GSH transport into bile functions as a driving force for bile secretion [4, 5] and plays a role in hepatic detoxification [6, 7], whereas transport into blood is important in supplying GSH and its constituent amino acids to extrahepatic cells. [2, 8-11].

Three hepatic GSH efflux mechanisms have been described. Low-affinity, high capacity systems are present in canalicular and sinusoidal membranes with K_m of ~16 and ~6 mM, respectively [8, 12]. In addition, a high-affintiy, low capacity system is present in the canalicular membrane with a K_m of ~0.2 mM [12]. The low affinity systems are selective for GSH, wheras the high affinity system may function to transport glutathione *S*-conjugate into bile [12]. Both canalicular and sinusoidal GSH transporters are electrogenic carriers [12-14].

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[b] *Abbreviations:* GSH, glutathione; RsGshT, sinusoidal GSH transport protein; RcGshT, canalicualr GSH transport protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; BSP-SG, bromosulfophthalein-glutathione conjugate; BLAST, basic local alignment search tool.

Recent reports described possible cDNA clones for the low-affinity sinusoidal and canalicular GSH transporters [15, 16]. These proteins were termed RcGshT and RsGshT for rat canalicular and sinusoidal GSH transporters, respectively [15, 16]. The RsGshT clone of ~2.8 kb encodes a protein of approximately 40 kD, whereas the RcGshT clone of 4.0 kb translates into a 96 kD protein [15, 16]. Distinctions between the canalicular and sinusoidal transporters include differential sensitivity to transport inhibitors: bromosulfophthalein-glutathione conjugate (BSP-SG), methionine, and cystathionine selectively inhibit sinusoidal GSH transport without affecting canalicular transport [17-21], and phenobarbital leads to an increase of canalicular transport alone [22, 23]. RcGshT and RsGshT transport GSH bidirectionally; both proteins are able to uptake and efflux GSH [8]. However, under physiological conditions they are thought to transport GSH down its concentration gradient: i.e., efflux [8].

The present study re-evaluates the functional role of these two putative GSH transporters using the *Xenopus laevis* oocytes expression system under the same conditions as in the original reports. However, our results are not in accordance with the previous reports.

MATERIALS AND METHODS

Materials

[Glycine-2-^{3}H]GSH, [^{3}H(G)]taurocholic acid, and [^{32}P]dCTP were purchased from New England Nuclear, Boston, MA. Chemicals were obtained from Sigma, St. Louis, MO, or J.T. Baker, Phillipsburg, NJ. The cDNA for rat canalicular (RcGshT) and the cRNA for the sinusoidal (RsGshT) GSH transporters were provided by Dr. Neil Kaplowitz, Department of Medicine, University of Southern California School of Medicine. The cDNA for the rat sodium-bile acid cotransporting polypeptide (Ntcp) was provided by Dr. Peter J. Meier, Division of Clinical Pharmacology, Department of Medicine, University Hospital, Switzerland.

Animals

Mature *Xenopus laevis* were purchased from Nasco, Fort Atkinson, WI. Animals were maintained under a constant light cycle at a room temperature of 18°C. Male Sprague-Dawley rats weighing 200-250 g were obtained from Charles River Laboratories (Kingston, NY).

Isolation of rat liver mRNA

Total RNA from 4-5 g of rat liver was prepared using the guanidinium thiocyanate/cesium chloride method [24]. mRNA was purified by chromatography with oligo(dT)-cellulose using an mRNA purification kit (Pharmacia Biotech, Piscataway, NJ). The OD_{260} was used to calculate RNA concentration, and the OD_{280} was used as an index of protein contamination. The mRNA was also examined by formaldehyde-agarose gel electrophoresis. cRNAs for RcGshT and Ntcp were synthesized using a mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Austin, TX).

Oocytes isolation and microinjection

Isolation of *Xenopus laevis* oocytes was performed as previously described [25, 26]. Frogs were anesthetized by immersion for 15 min in ice-cold water containing 0.3 percent tricaine. Oocytes were removed and washed with OR-2 solution [(in mM) 82.5 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES-Tris (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5] and incubated at room temperature with gentle shaking for 90 min in OR-2 solution containing 2 mg/ml of collagenase (Sigma, type IA). Oocytes were transferred

to fresh collagenase solution after the first 45 min of incubation. Collagenase was removed by extensive washing in OR-2 solution at room temperature. Stage V and VI oocytes were selected and incubated at 18°C in modified Barth's solution [(in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, and 20 HEPES-Tris, pH 7.5], supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). After 3-4 hr or overnight incubation, healthy oocytes were injected with 50 nl of rat liver mRNA (1 μg/μl) or cRNA (RcGshT, RsGshT, or Ntcp). Control oocytes were injected with a corresponding volume of sterile water. Glass micropipettes with a tip diameter of 20-40 μm were pulled on a Sutter Instrument model P8 pipette puller (Novato, CA). A Drummond (Broomall, PA) microinjector attached to a Brinkmann (Westbury, NY) MM33 micromanipulator was used for microinjection. Injected oocytes were cultured for 3 days at 18°C with a daily change of modified Barth's culture medium. Healthy oocytes with a clean brown animal half and a distinct equator line were selected for experiments.

GSH transport in oocytes

GSH uptake into oocytes injected with either water, rat liver mRNA, or cRNA was measured in modified Barth's solution. Oocytes were pretreated with 0.5 mM acivicin for 30 min at room temperature to inhibit γ-glutamyltransferase activity [25]. From 6-8 oocytes were incubated at 25°C for 1 hr in 100 μl of modified Barth's solution in the presence 1 μCi of [^{3}H]GSH (10 mM GSH) plus 10 mM dithiothreitol. The reaction was stopped by adding 2.5 ml of ice-cold modified Barth's solution, and oocytes were washed three times with 2.5 ml of ice-cold modified Barth's solution. Two oocytes were placed in a polypropylene scintillation vial containing 0.2 ml of 10 percent sodium dodecyl sulfate, and were lysed by vigorous mixing. After addition of 5 ml of Opti-Fluor (Packard Instruments, Downers Grove, IL), samples were counted in a Packard model 4530 scintillation spectrometer.

To determine GSH efflux, oocytes were re-injected with 50 nl of a 110 mM stock solution of [^{3}H]GSH. Because the intracellular water space of stage V and VI oocytes is approximately 0.5 μl/oocyte [26], this maneuver raised the intracellular GSH concentration by approximately 10 mM (5 nmol/oocyte). The [^{3}H]GSH injection solution also contained 1-2 mM dithiothreitol to prevent GSH oxidation. After the second injection, oocytes were preincubated for 30 min at room temperature in modified Barth's solution, and were washed twice with 2.5 ml of modified Barth's solution to remove extracellular radioisotope. Two oocytes were combined in 200 μl of modified Barth's solution, and efflux was measured for 1 h at 25°C. The radioactivity in both the culture medium and oocytes was measured at predetermined time intervals.

Taurocholate uptake into oocytes

Oocytes were injected with 50 ng of rat liver mRNA or 0.5 ng of Ntcp cRNA. After 3-4 days at 18°C, uptake of 10 μM [^{3}H]taurocholate was determined for 1 hr at 25°C in 100 μl of uptake solution (modified Barth's solution). Uptake was terminated by addition of ice-cold stop solution, which consisted of modified Barth's solution plus 1 mM unlabeled taurocholate [27].

Sequencing

RcGshT cDNA was provided in PGEM-11 (zf) vector. It was digested with restriction endonucleases Hind III and Xba I to give the expected 2.5 kb size fragment of RcGshT cDNA (open reading frame) and the PGEM-11 (zf) vector. The RcGshT cDNA was sequenced using Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster, CA) with T7 primer.

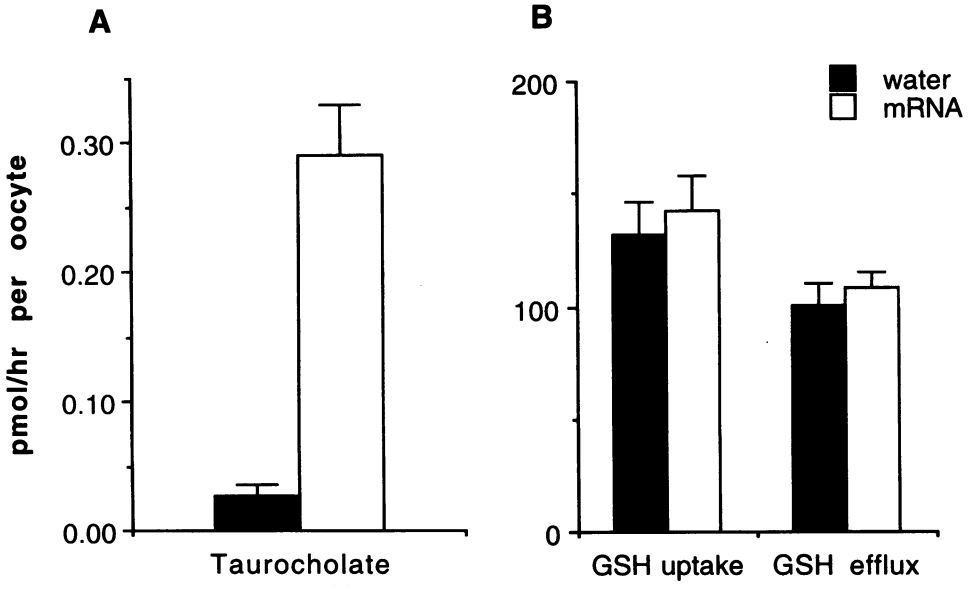

Figure 1. Taurocholate and GSH transport in *Xenopus laevis* oocytes injected with rat liver mRNA. Oocytes were injected with 50 ng (50 nl) of rat liver mRNA or 50 nl of water. Uptake studies were conducted 3 days post-injection. **A:** 1 hr taurocholate uptake values were determined in modified Barth's solution at 25°C in the presence of 10 μM [^{3}H]taurocholic acid (2.1 μCi). **B:** 10 mM [^{3}H]GSH (1 μCi) uptake was determined in 100 μl of modified Barth's solution at 25°C for 1 hr in the presence 10 mM dithiothreitol. Oocytes were pretreated with 0.5 mM acivicin to inhibit γ-glutamyltransferase activity. For GSH efflux, oocytes were injected with 50 nl of [^{3}H]GSH (5 nmol/oocyte) to raise the GSH levels. Oocytes were preincubated in modified Barth's solution at room temperature for 30 min and washed with modified Barth's solution, before measuring efflux for 1 hr at 25°C. Values are means ± SD of 4-8 different oocytes preparations.

Northern blot analysis

Total rat liver RNA, mRNA, and cRNAs were electrophoresed on 6.7 percent formaldehyde/1.2 percent agarose gel and transferred to Maximum Strength Nytran membrane (Schleicher and Schuell, Keene, NH). Hybridization was carried out at 42°C overnight in 50 percent formamide, 5x Denhardt's solution, 5x saline-sodium phosphate-EDTA buffer, 0.15 percent sodium dodecyl sulfate and 100 μg/ml salmon sperm DNA. The hybridization probes were a 1.0 kb fragment of RcGshT cDNA (XbaI-BamHI) and a 0.9 kb fragment of Ntcp cDNA, each labeled with [^{32}P]dCTP by the nick-translation method (GIBCO BRL, Grand Island, NY). Following hybridization, the blot was washed in 2x saline-sodium citrate buffer, 0.1 percent sodium dodecyl sulfate at room temperature for 3 washes of 5 min each, followed by 0.1x saline-sodium citrate buffer, 0.1 percent sodium dodecyl sulfate at 65°C for two washes of 30 min each and exposed to Kodak X-AR5 film (Kodak, Rochester, NY).

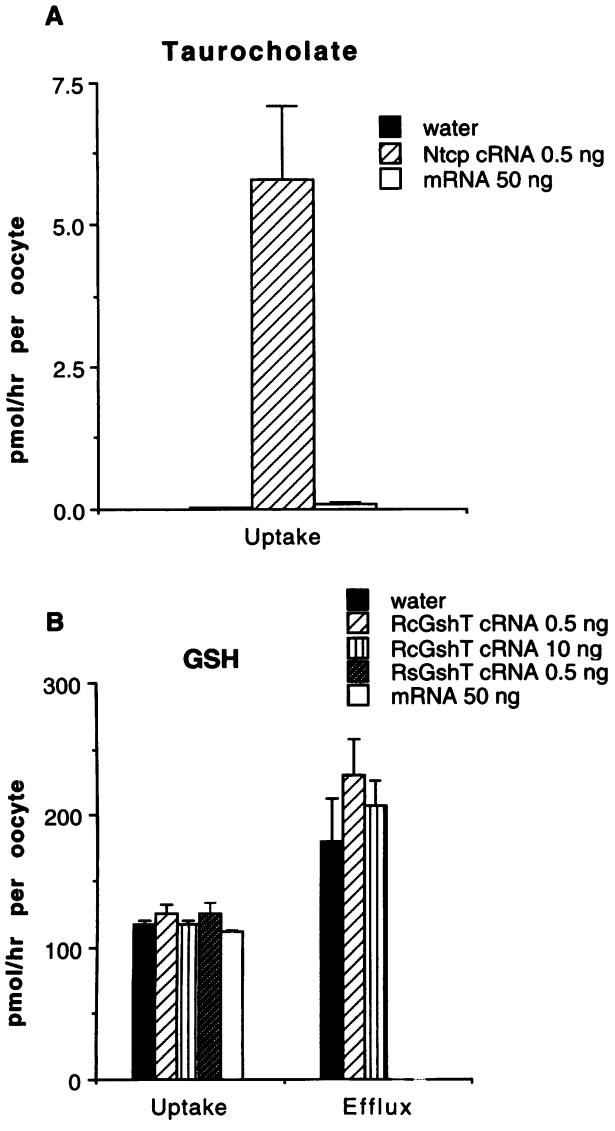

Figure 2. Expression of RcGshT, RsGshT and Ntcp cRNA in oocytes. Oocytes were injected with either water, RcGshT (0.5 ng or 10 ng/oocyte), RsGshT (0.5 ng or 10 ng/oocyte) or Ntcp cRNA (0.5 ng or 10 ng/oocyte), and cultured for 3 days.

A: Taurocholate uptake was measured at 25°C for 1 hr in the presence of 10 μM [^{3}H]taurocholic acid in 100 μl of modified Barth's solution.

B: GSH uptake in oocytes was measured in 100 μl of modified Barth's solution containing [^{3}H]GSH (10 mM, along with 10 mM dithiothreitol and 0.25 mM acivicin) for 1 hr at 25°C. To measure GSH efflux, the oocytes were reinjected with [^{3}H]GSH (5 nmol/oocyte), incubated for 30 min at room temperature in modified Barth's solution, washed twice in 2.5 ml of modified Barth's solution, and efflux was measured for 1 hr at 25°C in 200 μl of the same solution. GSH uptake in oocytes was measured in 100 μl of modified Barth's solution containing [^{3}H]GSH (10 mM, plus 10 mM dithiothreitol and 0.25 mM acivicin) for 1 hr at 25°C. Values are means ± SD of 3 experiments, each performed in quadruplicate. In the latter experiment, GSH efflux in oocytes injected with RsGshT cRNA or rat liver mRNA was not measured.

RESULTS

Xenopus laevis oocytes injected with rat liver mRNA demonstrated a markedly enhanced transport of ^{3}H-taurocholate (Figure 1A), confirming previous findings [27], and demonstrating the integrity of the mRNA and viability of the oocytes. In contrast, parallel experiments that examined GSH uptake and efflux demonstrated only a small (not significant) enhancement of ^{3}H-GSH transport in mRNA-injected oocytes (Figure 1B). These results differ from previous reports [15, 16, 28]. Of importance, the basal (i.e., endogenous) GSH transport rate in oocytes was high, ~130 pmol/oocyte/h, in agreement with our previous findings [25]. This high endogenous GSH transport rate may have masked any transport induced by injecting rat liver mRNA. GSH transport was also measured in oocytes that were not pretreated acivicin, and at higher (20 mM) and lower (2.5 mM) GSH concentrations, but once again there was no difference between water- and mRNA-injected oocytes (data not shown). The reason for the discrepancy between the present findings and those from previous studies [15, 16, 28] is not known.

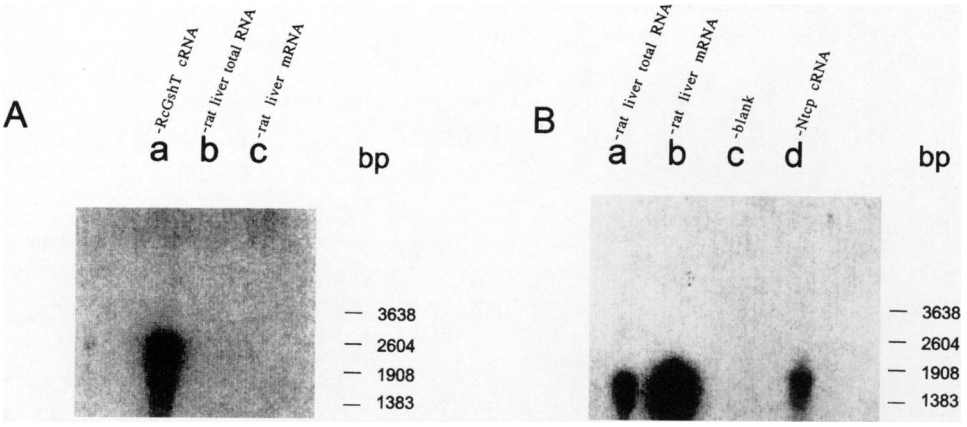

Figure 3. Northern blot analysis. Section A: hybridized with a ^{32}P-labeled 1.0 kb RcGshT cDNA fragment (XbaI-BamHI). **Section B:** hybridized with a 0.9 kb ^{32}P-labeled EcoR I fragment of Ntcp cDNA. **A:** RcGshT cRNA (4.5 ng; lane a), total rat liver RNA (16.5 μg; lane b), rat liver mRNA (8.5 μg; lane c). **B:** Total rat liver RNA (16.5 μg; lane a), rat liver mRNA (6 μg; lane b), Ntcp cRNA (4.5 ng; lane d), lane c is blank.

To address this issue, additional studies examined GSH transport in oocytes injected with the cRNAs for RcGshT and RsGshT (Figure 2). Expression of Ntcp cRNA was used as a positive control for these studies. Oocytes injected with 0.5 ng of Ntcp cRNA demonstrated a high rate of taurocholate transport, as expected [27]. However, oocytes injected with the cRNAs for RcGshT and RsGshT did not transport GSH at a higher rate than water-injected oocytes (Figure 2). Increasing the amount of cRNA injected to 10 ng/oocyte did not stimulate GSH uptake or efflux (Figure 2). In the latter experiment, GSH efflux in oocytes injected with RsGshT cRNA or rat liver mRNA was not measured.

Northern blot analysis using a 1 kb RcGshT cDNA probe failed to detect a transcript in 16.5 μg of rat liver total RNA or 8.5 μg of rat liver mRNA (Figure 3A), indicating that the transcript is either absent, or present in very low amounts in rat liver. The probe did react with 4.5 ng of the cRNA for RcGshT (lane "a" of Figure 3A). In contrast to RcGshT, a 1.7-kb Ntcp transcript was detected using 16.5 μg of rat liver total RNA and 6.0 μg of mRNA (Figure 3B), as expected [27].

When a partial sequence of RcGshT cDNA was obtained and compared with other published sequences (BLAST search), the results revealed that it was essentially identical with a sequence found in the *E. coli* K-12 genome (Table 1). Interestingly, the published RcGshT sequence is slightly different from our sequence; however, a high degree of homology also exists between the published RcGshT cDNA sequence and the *E. coli* K-12 genome (Table 1).

Furthermore, the published sequence for RsGshT cDNA also has a high degree of homology with the *E. coli* K-12 genome, but the homologous region is different from that of RcGshT (BLAST search; data not shown). The observed high degree of similarity with the bacterial DNA indicates that RcGshT and RsGshT are possible cloning artifacts.

Table 1. Comparison of a partial sequence obtained from RcGshT plasmid, with the published sequence of RcGshT and with the *E. coli* K12 genome. Differecnces between the *E. coli* K12 and the published RcGshT are denoted by underlining, and between and *E. coli* K12 and the provided RcGshT cDNA by the asterisk (only one base pair difference was noted for the latter).

Provided RcGshT:		**********	
E. coli K-12:	3064	TGAAAAAATTGAAGAGGCAAGGATTTGTATAGACAAAT	3101
Published RcGshT:	2323	TGGAGAAAATGAAGAGCCAAGGATTTGTATAGCCAAAT	2360
Provided RcGshT:		**********	
E. coli K-12:	3102	CACTACAACTCGAACCCAGAAGACGAAAAGCAGTTGTG	3139
Published RcGshT:	2361	CGCTACAACGCGAACCCAGAAGCCGAAAAGCAGGTTGT	2398
Provided RcGshT:		**********	
E. coli K-12:	3140	ATAAAAGAATGTGTAGATATGTATGTGCCTAACCCGCT	3177
Published RcGshT:	2399	GATAGAAGATGTGTAGATATGTATGTGCCTAACCCGCC	2436
		*	
Provided RcGshT:	222	CAAAAACCACATTAAACTCTACTATAAAGAAACTGAGA	185
E. coli K-12:	3178	CAAAAACAACATTAAACTCTACTATAAAGAAACTGAGA	3215
Published RcGshT:	2437	CAAAAACAACATTAAACTCTACTATAAAGAAACTGAGA	2474
Provided RcGshT:	184	GTGAAAGCCATCGAGTTATAATTGACAACATTTTGAAA	147
E. coli K-12:	3216	GTGAAAGCCATCGAGTTATAATTGACAACATTTTGAAA	3253
Published RcGshT:	2475	GTGAAAGCCATCGAGTTATAATTGACAACATTTTGAAA	2512
Provided RcGshT:	146	TTAAAGCAGCTGACGAGAATTTGTATGCGATAAATTTT	109
E. coli K-12:	3254	TTAAAGCAGCTGACGAGAATTTGTATGCGATAAATTTT	3291
Published RcGshT:	2513	TTAAAGCAGCTGACGAGAATTTGTATGCGAAAATTTTT	2550
Provided RcGshT:	108	TATTGACGCAATTTTATTATTTAGATGTATGCAACTTG	71
E. coli K-12:	3292	TATTGACGCAATTTTATTATTTAGATGTATGCAACTTG	3329
Published RcGshT:	2551	ATTGACGCAATTTTATTATTTAGATGTATGCAACTTG	2587
Provided RcGshT:	70	TTATTTAAACAACAGAGATAAAAATATCTAAGATATTC	33
E. coli K-12:	3330	TTATTTAAACAACAGAGATAAAAATATCTAAGATATTC	3367
Published RcGshT:	2588	TTATTTAAACAACAGAGAGATAAAAATTAACTAACTCT	2625
Provided RcGshT:	32	ACCTTATTGCAAGATATTTAAAATGCTCTAGA	1
E. coli K-12:	3368	ACCTTATTGCAAGATATTTAAAATGCTCTAGA	3399
Published RcGshT:	2626	AGAGGGAAATTTAAGCTAGCGATGGCCTAAAG	2657

DISCUSSION

The present findings demonstrate that *Xenopus laevis* oocytes fail to exhibit enhanced GSH transport when injected with rat liver mRNA. This is most likely explained by the fact that oocytes have a high rate of endogenous GSH transport [25], which hinders detection of heterologously expressed GSH transporters from rat liver mRNA. Previous studies in our laboratory reveal that the endogenous GSH efflux mechanisms in oocytes is sensitive to cellular ATP depletion, with an apparent K_m of ~5 mM [25]. In contrast to GSH, the endogenous transport of the bile acid taurocholate is low in oocytes, and injection of rat liver mRNA markedly increases taurocholate transport when compared to water-injected controls [27] (Figure 1).

The present findings also do not support a role for RcGshT and RsGshT in mediating GSH transport. Injection of the cRNA for RcGshT or RsGshT failed to stimulate GSH transport. GSH transport was measured both in the efflux and uptake modes, in the presence and absence of acivicin, and in the presence of varying GSH concentrations (2.5, 10 and 20 mM), but no difference in transport between water- and cRNA-injected oocytes was detected. This contrasts with previous reports indicating avid GSH transport by these putative GSH transporters [15, 16, 29]. Previous studies also demonstrated an apparent concentrative (i.e., uphill) transport of GSH by RcGshT and RsGshT [15, 16, 29]. The reported rates of GSH uptake of ~28-40 nmol/oocyte/hr [15, 16, 29] correspond to intracellular GSH concentrations of approximately 56-80 mM, as compared to 10 mM GSH in the culture medium. This concentrative GSH transport is inconsistent with the present knowledge of electrogenic and bidirectional GSH transport down its concentration gradient [8, 13, 14, 29-31].

Furthermore, Northern blot analysis did not detect an RcGshT transcript in either rat liver total RNA or rat liver mRNA, suggesting that the transcript is either absent or present below detection level. However, after sequencing a portion of the RcGshT cDNA and comparing it with other published sequences, the result closely matched with a sequence found in the *E. coli* K-12 genome. Interestingly, the published sequence for the sinusoidal transporter, RsGshT, [16] also has high sequence identity with the *E. coli* K-12 genome, although with a different portion of the genome. The RcGshT and RsGshT cDNA sequences were published prior to the publication of the homologous sequences found in the *E. coli* K-12 genome. This high degree of sequence identity is not likely to exist between bacterial and mammalian systems and indicates a possible cloning artifact. Lu et al. [32] recently indicated that these putative genes for GSH transporters may be cloning artifacts, in agreement with our present findings.

In conclusion, highly homologous sequences to RcGshT and RsGshT are found in the *E. coli* genome. The function of these gene products in *E. coli* is not known. The present findings demonstrate that RcGshT and RsGshT are not involved in GSH transport. Additional studies are needed to resolve the discrepancy between the present findings and those of Yi et al. [15, 16], and to isolate and characterize mammalian canalicular and sinusoidal GSH transporters.

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