Transport of many organic anions across the bile canalicular membrane is mediated by the canalicular multispecific organic anion transporter (cMOAT). Previously, we cloned cDNA that may encode cMOAT from Sprague-Dawley rat liver (Ito, K., Suzuki, H., Hirohashi, T., Kume, K., Shimizu, T., and Sugiyama, Y. (1997) Am. J. Physiol. 272, G16–G22). In the present study, the function of this cloned cDNA was investigated by examining the ATP-dependent uptake of S-(2,4-dinitrophenyl)-glutathione (DNP-SG) into membrane vesicles isolated from an NIH/3T3 cell line transfected with an expression vector containing the cloned cDNA. Although the membrane vesicles from the control NIH/3T3 cells exhibited endogenous activity in transporting DNP-SG and leukotriene C4 in an ATP-dependent manner, the transfection of cMOAT cDNA resulted in a significant increase in the transport activity for these ligands. The uptake of DNP-SG into membrane vesicles was osmotically sensitive and was stimulated to some extent by other nucleotides triphosphates (GTP, UTP, and CTP) but not by AMP or ADP. The Km and Vmax values for the uptake of DNP-SG by the membrane vesicles were 0.175 ± 0.031 μM and 11.0 ± 0.73 pmol/min/mg protein, respectively, for the transfected rat cMOAT and 0.141 ± 0.036 μM and 3.51 ± 0.39 pmol/min/mg protein, respectively, for the endogenous transporter expressed on control NIH/3T3 cells. These results suggest that the product of the previously cloned cDNA has cMOAT activity being able to transport organic anions in an ATP-dependent manner. Alternatively, it is possible that the cDNA product encodes an activator of endogenous transporter since the previously cloned cDNA has cMOAT activity being able to transport organic anions in an ATP-dependent manner. It is well established that the biliary excretion of organic anions is mediated by the canalicular multispecific organic anion transporter (cMOAT)1 (1–4). It is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible that cMOAT can accept many glutathione conjugates as a substrate (1–4), it is possible
that this human homologue of rat cMOAT has the function of reducing the intracellular concentration of this antitumor drug, thus conferring drug resistance.

Functional analysis, however, remains to be performed to finally show that the previously cloned cDNA actually encodes a protein with cMOAT activity. In the present study, we established an NIH/3T3 cell line transfected with an expression vector containing cMOAT cDNA and examined the transport activity using membrane vesicles isolated from these transfected cells.

EXPERIMENTAL PROCEDURES

Materials—[14,15,19,20-3H]LTC4 (128 Ci/nmol) was purchased from Amersham International Ltd (Buckinghamshire, UK). Unlabeled and 3H-labeled DNP-SG (50.0 μCi/nmol) were synthesized enzymatically using [2-glycine-3H]glutathione (NEN Life Science Products), 1-chloro-2,4-dinitrobenzene, and glutathione S-transferase (Sigma) as described previously (8), and the purity (>90%) was checked by thin layer chromatography. pCXN2 mammalian expression vector (31) was supplied by Dr. J. Miyazaki, Osaka University.

Preparation of the Transfected Cell Line—cMOAT cDNA with the shortest 3'-UTR length in pBluescript II SK (−) vector described previously (22) was excised with EcoRI and then inserted into the EcoRI site in the pCXN2 vector. The NIH/3T3 cells, transfected with pCXN2 vector by Lipofectin (Life Technologies), was maintained in the presence of 800 g/ml G418 (Geneticin, Life Technologies) to obtain the colonies. We identified five colonies and determined the expression of cMOAT in each of these using Northern blot analysis. We prepared membrane vesicles from one clone whose cMOAT expression was highest.

Transport Studies—Membrane vesicles were prepared from 2 × 106 of the control and transfected NIH/3T3 cells as described previously (32) and were frozen in liquid nitrogen and stored at −100 °C until use. Protein concentrations were determined by the Lowry method. In addition, the orientation of membrane vesicles was determined by examining the nucleotide pyrophosphatase accessibility (33). Functional analysis, however, remains to be performed to finally show that the previously cloned cDNA actually encodes a protein with cMOAT activity. In the present study, we established an NIH/3T3 cell line transfected with an expression vector containing cMOAT cDNA and examined the transport activity using membrane vesicles isolated from these transfected cells.

RESULTS

Northern Blot Analysis of the Expression of Rat cMOAT—The expression of rat cMOAT in the cells cultured for 10 weeks after transfection was confirmed by Northern blot analysis. As shown in Fig. 1A, rat cMOAT probe hybridized to the NIH/3T3 cells transfected with a vector containing rat cMOAT cDNA, but not to those transfected with the vector. The length of the transcript in cMOAT-transfected NIH/3T3 cells was comparable with the shortest band observed in SD rat liver (22).

Uptake of [3H]DNP-SG and [3H]LTC4 into Membrane Vesicles—The enrichment of leucine amino peptidase was 8.1- and 6.6-fold in plasma membrane vesicles from cMOAT-transfected and vector-transfected cells relative to the cell homogenate, respectively. The sideness of the membrane vesicles was also comparable between these cells; 36 and 34% of the membrane vesicles were inside-out for cMOAT-transfected and vector-transfected NIH/3T3 cells, respectively. Fig. 2 shows the time profiles for the uptake of [3H]DNP-SG and [3H]LTC4 by membrane vesicles. Although the membrane vesicles from the control cells exhibited the ability to transport [3H]DNP-SG and [3H]LTC4 in an ATP-dependent manner, the stimulating effect of ATP was greater in the cMOAT-transfected NIH/3T3 cells (Fig. 2A and B). The clearance for the uptake of [3H]DNP-SG into cMOAT-transfected cells was 9.85 ± 0.42 μl/min/mg of protein (mean ± S.E.; n = 3), which is significantly (p < 0.05) higher than that observed in NIH/3T3 cells without any plasmid (3.15 ± 0.075 μl/min/mg of protein; n = 3) or vector-transfected NIH/3T3 cells (2.66 ± 0.22 μl/min/mg of protein; n = 3). The clearance for the uptake of [3H]DNP-SG into membrane vesicles isolated from NIH/3T3 cells not transfected with any plasmid (3.15 ± 0.075 μl/min/mg of protein; n = 3) was not significantly different from that observed for the vector-transfected NIH/3T3 cells (2.66 ± 0.22 μl/min/mg of protein; n = 3). Moreover, we found that the expression of rat cMOAT in the transfected cells was reduced during storage in liquid N2; after thawing, expression of the transcript in the transfected cells fell below the limit of detection. In accordance with the reduced expression of transfected cMOAT, the uptake of DNP-SG into membrane vesicles from thawed cells was significantly (p < 0.05) reduced to 3.13 ± 0.22 μl/min/mg of protein (n = 3), a figure not significantly different from that observed in parental and vector-transfected NIH/3T3 cells.

In addition, the uptake of [3H]DNP-SG into membrane vesicles from cMOAT-transfected and vector-transfected cells was reduced by increasing the sucrose concentration in the medium. The y-intercept for relationship between the amount of uptake of DNP-SG and [3H]LTC4 into membrane vesicles from transfected and vector-transfected cells was compared with the reciprocal of the sucrose concentration in the medium. The y-intercept for relationship between the amount of DNP-SG associated with the vesicles versus the reciprocal of the sucrose concentration in the medium was almost 0. GTP, CTP, and UTP stimulated the uptake of [3H]DNP-SG into membrane vesicles isolated from cMOAT-transfected cells to 38.3, 41.2, and 44.4% of that observed in the presence of ATP. In the same manner, the uptake of [3H]DNP-SG into membrane vesicles from vector-transfected cells in the presence of GTP, CTP, and UTP was 55.6, 54.4, and 56.6% of that observed in the presence of ATP. In contrast, no effect of ADP or AMP was observed for both membrane vesicle preparations.
FIG. 2. Time profiles for the uptake of [3H]DNP-SG and [3H]LTC4 into membrane vesicles. Membrane vesicles (10 μg) prepared from cMOAT-transfected (●) or vector-transfected NIH/3T3 cell lines (□) were incubated at 37°C in 20 μl of medium (10 mM Tris-HCl, 250 mM sucrose, 10 mM MgCl2, pH 7.4) containing 1 μM [3H]DNP-SG (panel A) or 10 μM [3H]LTC4 (panel B) with (closed symbols) or without (open symbols) ATP regenerating system (5 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine phosphokinase). Each point and vertical bar represent the mean ± S.E. of triplicate determinations in a typical experiment.

FIG. 3. Concentration-dependence of [3H]DNP-SG uptake by membrane vesicles. Membrane vesicles were incubated at 37°C with 50 mM [3H]DNP-SG in 20 μl of medium (10 mM Tris-HCl, 250 mM sucrose, 10 mM MgCl2, pH 7.4) containing varying concentrations of DNP-SG for 10 min, during which linearity is observed. The closed triangle (▲) represents the uptake mediated by the transfected rat cMOAT calculated by subtracting the endogenous uptake by vector-transfected NIH/3T3 vesicles (●) from that of cMOAT-transfected NIH/3T3 vesicles (□). Each point and vertical/horizontal bar represents the mean ± S.E. of triplicate determinations in a typical experiment.

DISCUSSION

In the present study, we examined the function of the product of the recently cloned rat cDNA, whose expression is defective in EHBR and TR (20–22), by examining the transport of typical substrates for cMOAT in the cDNA transfected cells. Since 1) the uptake of [3H]DNP-SG into membrane vesicles from the cMOAT-transfected cells was stimulated to a greater extent by ATP compared with that from the vector-transfected cells (Fig. 2A) and 2) since the expression of cMOAT in the cMOAT-transfected cells was confirmed by Northern blot analysis, it was concluded that rat cMOAT activity is associated with the expression of an mRNA level. Uptake of DNP-SG and LTC4 into membrane vesicles isolated from the control NIH/3T3 cells was stimulated by ATP, suggesting the presence of endogenous ABC transporters for these glutathione conjugates (Fig. 2). To estimate the function of rat cMOAT, therefore, the uptake in control cells should be subtracted from that in cMOAT-transfected NIH/3T3 cells. Since the uptake of DNP-SG into membrane vesicles was comparable between the parent and vector-transfected NIH/3T3 cells (see “Results”), the vector introduction may not affect the expression of endogenous transporters.

The uptake of [3H]DNP-SG was osmotically sensitive (see “Results”), suggesting that a large part of the accumulation by these transporters can be accounted for by transport into the intravesicular space, but not by binding to the vesicle surface. GTP, CTP, and UTP could also enhance the uptake of DNP-SG by cMOAT to some extent (see “Results”), which was consistent with the hMRP (35). Vanadate was effective in reducing the ATP-stimulated uptake of DNP-SG, which was consistent with the observations in CMVs (8) and in hMRP (32).

Kinetic analysis revealed that the Km of rat cMOAT was 0.175 μM (see “Results”), which was more than 10-fold lower than that reported for the uptake of DNP-SG by CMVs; using rat CMVs, Kobayashi et al. (8) reported a Km of 4 μM for DNP-SG although the Km reported by Akerboom was 70 μM (36). The Km value determined in the present study is similar to that found for ATP-dependent uptake of DNP-SG into plasma membrane vesicles from murine leukemia cells (L1210; Km = 0.63 μM) (37). Comparison of the previous reports revealed the presence of a 10-fold difference in the Km value for DNP-SG between hMRP-overexpressing tumor cells (GLC4/ADR, Km = 30 μM) (32) and hMRP-transfected HeLa cells (3.6 μM) (38). Although we do not have a good explanation to account for the discrepancy in the Km values between the cMOAT-transfected mouse (NIH/3T3) cells and rat hepatocytes, one hypothesis involves considering the difference in the “atmosphere” of the protein molecule due to the difference in lipid composition between the two animal species. Alterna-
tively, it is also plausible that the cDNA product encodes an activator of endogenous transporter since the \( K_v \) value of DNP-SG was comparable between the vector- and cMOAT-dependent K\(_v\)-channel activity (20–22) and low (\( K_v = 450 \mu M \)) affinity systems, the former being inhibited by LTC\(_4\) with a \( K_v \) value of 0.20 \( \mu M \). Northern blot analysis using a mouse MRP probe suggested that the expression of MRP in mouse MRP was amplified from BALB/c mouse liver and lung, respectively, using degenerated PCR primers described previously (26). The sequence between forward and reverse primers is listed in this figure. Asterisks represent the consensus sequence. Dots represent the amino acids homologous to each other.

FIG. 4. Alignment of the nucleotide and deduced amino acid sequence of the cDNA probe for mouse cMOAT and mouse MRP. Carboxyl-terminal ABC region of the mouse cMOAT and mouse MRP were amplified from BALB/c mouse liver and lung, respectively, using degenerated PCR primers described previously (26). The sequence between forward and reverse primers is listed in this figure. Asterisks represent the consensus sequence. Dots represent the amino acids homologous to each other.

In conclusion, we have shown that the product of the previ-ously cloned cDNA (20–22) has the ability to transport glutathione conjugates in an ATP-dependent manner, which is the most important characteristic of cMOAT. Together with the previous finding that the expression of the cloned cDNA 1) is predominately observed in the liver among all the tissues examined (20, 22), 2) is almost exclusively observed on the bile canalicular membrane (20, 21), and 3) is hereditarily defective in both allelic mutant rat strains (EHBR and TR\(^{-}\)) (20–22) and humans (28), this leads us to conclude that the defective expression of this transporter is the pathogenesis for the jaundice in the Dubin-Johnson syndrome found in humans.

Note Added in Proof—After submission of this manuscript, Madon et al. (1997) FEBS Lett. 406, 75–78, communicated the accelerated efflux of DNP-SG from oocytes and COS-7 cells expressing rat cMOAT.

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