ATP-dependent transport of bile acids is a key determinant of bile flow in mammalian liver and is associated with cholesterol excretion, gallstone formation, and numerous inherited and acquired hepatobiliary diseases. Secretory vesicles and a vacuole enriched fraction purified from Saccharomyces cerevisiae also exhibit ATP-dependent bile acid transport. ATP-dependent transport of bile acids by the vacuolar fraction was independent of the vacuolar proton ATPase, responded to changes in the osmotically sensitive intravesicular space, and was the vacuolar proton ATPase, responded to changes in bile acids by the vacuolar fraction was independent of pendent bile acid transport. ATP-dependent transport of bile acids is a major determinant of bile flow. Purified rat canalicular membrane vesicles (CMV) exhibit ATP-dependent ATP-dependent bile acid transport was abolished when the BAT1 coding region was deleted from the genome and restored upon reintroduction of the gene. The deduced amino acid sequence predicts that Bat1p is an ABC-type protein 1661 amino acids in length, similar to mammalian cMOAT/cMRP1 and MRP1 transporters, yeast Ycf1p, and two yeast proteins of unknown function. Information obtained from the yeast BAT1 gene may aid identification of the gene encoding the mammalian bile acid transporter.

Bile acids (BA) are the main product of cholesterol catabolism in mammals. These amphipathic organic detergents are synthesized in the liver and excreted in bile into the intestine, where they are required for lipid emulsification prior to absorption. Over 90% of BA in the intestine undergoes an efficient and regulated process of enterohepatic circulation, in which BAs are taken up from the portal circulation and ultimately secreted into the canaliculus. Transport across the canalicular membrane is rate-limiting in transfer of BA from blood to bile (1–3) and is a major determinant of bile flow. Purified rat canalicular membrane vesicles (CMV) exhibit ATP-dependent BA transport (4–6), and a similar activity has been characterized in the microvillous membrane of the human trophoblast (7).

Several reports suggest that cCAM 105 (cell adhesion molecule), an abundant canalicular membrane protein, represents a BA transporter/ecto-ATPase (8). However, the ATP-dependent activity in CMV is directly energized by ATP hydrolysis, requires an inside-out vesicle configuration (4), and is sensitive to vanadate (9), whereas ecto-ATPase activity is vanadate-insensitive, and BA transport is unaffected in mutants lacking ATPase activity (10). Additionally, ecto-ATPase-free CMV exhibit ATP-dependent taurocholate uptake (11).

ATP-dependent transport activity in the canalicular membrane is essential for translocation of many bile components from the hepatocyte into the canaliculus. Molecular characterization has indicated that ATP-binding cassette (ABC)-type proteins are responsible for these activities. ABC-type proteins are a large family of integral membrane proteins that effect ATP-dependent membrane translocation of an enormous variety of substrates and are present in organisms ranging from bacteria to man (12). Members of this superfamily typically contain one or two highly conserved nucleotide binding domains (NBD), which are paired with hydrophobic regions capable of spanning the membrane multiple times. ABC-type proteins that have been identified in the canalicular membrane include MDR1, which transports hydrophobic drugs into bile (13); MDR3, a phospholipid flipase (14–16); and cMOAT/cMRP1, which is associated with transport of glutathione, glucuronide, and sulfate conjugates across the canalicular membrane. These transporters are not abundant in liver, and the genes encoding these proteins were initially identified in other tissues, or because of their similarity with other ABC-type proteins. MDR1 is highly expressed in multidrug-resistant cell lines, and MDR2 was first identified due to its high degree of homology with MDR1. Similarly, cMOAT/cMRP1 was isolated because it is similar in function and sequence to MRP1 (20), another ABC-type protein involved in multiple drug resistance. However, a mammalian homolog of the gene or genes that encode the ATP-dependent BA transporter has not been identified.

Heterologous expression of MDR1 (21), MDR2 (15), and MRP1 (22) in yeast has been useful in the study of mammalian ABC-type proteins. Secretory vesicles purified from transgenic sec1–1 mutant yeast provide an abundant source of right-side-out membrane vesicles that are ideal for biochemical characterization of transport activity. These vesicles also contain ATP-dependent transport activities for BA and glutathione conjugates, which are similar to activities present in the bile canaliculus (23). ATP-dependent transport of glutathione conjugates is mediated by Ycf1p (24), an ABC-type protein that
exhibits considerable amino acid similarity with the mammalian glutathione conjugate transporters MRPI and cMOAT/cMRPI. Yef1p is sorted predominantly to the yeast vacuole, which is an acidic compartment, rich in catalytic enzymes similar to mammalian lysosomes, that plays an important role in storage and homeostasis of calcium, phosphate, and amino acids (26). We have shown that a vacuole-enriched fraction from yeast also contains an ATP-dependent BA transport activity and report the cloning and characterization of the Bat1 gene (bile acid transporter), which encodes a ABC-type protein mediating ATP-dependent BA transport in yeast.

**EXPERIMENTAL PROCEDURES**

**Materials—**[H]Glutathione, [14C]Chenodeoxycholic acid, [3H]Cholic acid, [3H]Glycocholic acid, [3H]Tauroursodeoxycholic acid were obtained from DuPont NEN; Zymolase 100T was from Seikagaku; bafilomycin A1 was from Wako Chemicals; and Difco yeast media were from VWR. All other reagents were purchased from Sigma. [3H]2-Dinitrophenyl glutathione (DNPSG) was synthesized and purified as described (27).

**Yeast Strains and Media—**Synthetic growth and YEPD media were prepared from standard techniques. The yeast strains JYW35 (MAT a::HIS3, leu2–3, 112, his3–200, trpl–3901, lys2–801, sue2–29, Mel3-12::his3) (28), NY3 (MAT a::URA3, ura3–2, 112, his3–Δ200) (30), SF383–5A (MAT a, leu2–3, 112, ura3–52, ade6), and SF383–5Amp1–Δ8 (MAT a, leu2–3, 112, ura3–52, ade6, tfp1–Δ8; LEU2) (31) have been described. QL1 was derived from SF383–5Amp1–Δ8 (MAT a, leu2–3, 112, ura3–52, ade6, tfp1–Δ8; LEU2, ura3::metalase) (29). DOY2 (MAT a, leu2–3, 112, trp1–901, ypl112–c::HIS3, ydh3::URA3) was the progeny of mating of JYW35 and DOY2 (MAT a, ura3::HIS3, leu2–3, 112, his3–200, ydh5::URA3). DOY2, DOY10 (MAT a, ura3::HIS3, leu2–3, 112, his3–200, QTR1::URA3) and DOY11 (MAT a, ura3::HIS3, leu2–3, 112, his3–200, bat1Δ1::URA3) and DOY12 (MAT a, ura3::HIS3, leu2–3, 112, his3–200, bat1Δ1::URA3) were derived from RS12. DOY2 (MAT a, ura3::HIS3, leu2–3, 112, his3–200, QTR1::URA3) was derived from NY3.

**Preparation of Organellar and Membrane Fractions—**Secretory vesicles were prepared from temperature-sensitive sec–1 strains as described previously (23). Vacuoles were prepared as described (32) with the following modifications. Buffers A, B, and C were supplemented with the protease inhibitors as follows: peptatin A (2 μg/ml), leupeptin (2 μg/ml), aprotinin (2 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). Glycerol was added to vacuoles in buffer C to a final concentration of 5% (v/v) before freezing at −80 °C. Protease inhibitor concentrations were determined by the method of Lowry et al. (33) on trichloroacetic acid-precipitated samples. Enzyme assays for marker enzymes were carried out as described (32). As has been reported (14, 16), vacuoles prepared by the Fixoll flotation procedure are highly enriched in vacuolar markers-α-mannosidase and alkaline phosphatase (20- and 15-fold increase in specific activity relative to total homogenate, respectively). There is little or no contamination (less than 5% of the specific activity observed with the total homogenate) with enzyme markers for mitochondria (succinate dehydrogenase), endoplasmic reticulum (cytchrome c reductase), or cyttoplasm (glucose-6-phosphate dehydrogenase). Total membranes were prepared from yeast grown in SG. Yeast cells were disrupted by bead beating in five volumes of 20 mM MES/Tris, pH 8.0, 50 mM KC1, 1 mM EDTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 2 μM peptatin A, and 2 μM leupeptin. Cell debris was pelleted by centrifugation at 250 × g for 10 min. Total membranes were pelleted by centrifugation at 100,000 × g, resuspended in disruption buffer, and frozen at 80 °C.

**Transport Assays—**ATP-dependent transport of [3H]DNPSG and BA was measured by the transport suppression filter assay, transport by secretory vesicles was determined as described previously (23). Vacuolar transport was measured as follows; radiolabeled substrate was added to 50 μl of 2 × reaction buffer (20 mM MES/Tris, pH 8.0, 8.0 μM ATP, 20 mM MgCl2, 10 mM creatine phosphate, 10 μM creatine kinase) and mixed with 50 μl of vacuolar vesicles (20–30 μg of protein) prewarmed to 30 °C. At the specified time points, 15–25–μl aliquots of the solution were diluted to 1 ml with cold wash buffer (10 mM MES/Tris, pH 8.0, 5 μM MgCl2, 20 mM KCl) and filtered under mild vacuum through glass fiber filters (25-mm GF/C, Whatman). The filter disks were washed with 5 ml of wash buffer, dried, and the retained counts determined by liquid scintillation.

**Gene Disruptions—**Disruption constructs were generated by PCR using chimeric oligonucleotide primer pairs (30), which consisted of 12–15 nucleotides homologous to sequences flanking the URA3 gene insert in the Yep24 plasmid (34) on the 3′ end linked with 40–45 nucleotides homologous to yeast genomic sequences flanking the gene of interest. PCR amplification of a Yep24 template using these primers generated DNA fragments consisting of the URA3 gene flanked by short stretches of homology to the gene of interest. Transformation of the RS12 yeast strain with these PCR products resulted in the replacement of chromosomal DNA between the regions of microhomology with the URA3 gene. DNA was prepared from UA+ transformants and deletion of the chromosomal copy of the gene confirmed by Southern analysis. PCR conditions were used 3 times at 95 °C, 45 °C, and 72 °C, and 30 times at 95 °C, 60 °C, and 72 °C (all steps 1 min). The primers used were: A-1, TATACAATAGTCC (forward: GTCGAATTC(A/T)CIGGIGCIGGIAA(A/T)CGAGTTG-GTGTTGCCCATC; ADP1-R, CAAGGGGAAATTGGTTTGCCAT- TGACGCTGTGATGTCAGCTGGTGTCGCGCAT; MDL1-F, GTTACATTCTCACCCTCCTTTTATGCCTCAGTGTGTGGAC- GCTTAC; MDL2-R, CGTCTTTTGGTCTGACCTCTTACATC; YD96-F, CAGTACGAGTTAGTAAAGAAAAGAGCTGCTGGTGTCGGCC- ATG; YD96-R, GTGAGTTCATATCGCAACACAAAGAGTATGTTTCTAGACGATTCGTTATC; YHD5-F, CTAGTGCGTCGATCAAAACCATTACATTGATGGTTGGTCGCG; YHD5-R, GTCTTGACGTCGCTGGTATGGCATGCGTATGCGTT; YHD5-F, CTAGTGCGTCGATCAAAACCATTACATTGATGGTTGGTCGCG; YHD5-R, GTCTTGACGTCGCTGGTATGGCATGCGTATGCGTT; YHD5-F, CTAGTGCGTCGATCAAAACCATTACATTGATGGTTGGTCGCG; YHD5-R, GTCTTGACGTCGCTGGTATGGCATGCGTATGCGTT.

**ATP-dependent transport of [3H]DNPSG and BA was measured as follows; radiolabeled substrate was added to 50 μl of 2 × reaction buffer (20 mM MES/Tris, pH 8.0, 8.0 μM ATP, 20 mM MgCl2, 10 mM creatine phosphate, 10 μM creatine kinase) and mixed with 50 μl of vacuolar vesicles (20–30 μg of protein) prewarmed to 30 °C. At the specified time points, 15–25–μl aliquots of the solution were diluted to 1 ml with cold wash buffer (10 mM MES/Tris, pH 8.0, 5 μM MgCl2, 20 mM KCl) and filtered under mild vacuum through glass fiber filters (25-mm GF/C, Whatman). The filter disks were washed with 5 ml of wash buffer, dried, and the retained counts determined by liquid scintillation.**
combined using a unique Sp6 site residing 2429 bp downstream of ATG to generate an 8-kb insert containing the 5-kb open reading frame flanked by 1.5 kb of upstream and downstream sequence, respectively. In pAA17 the Nra1-Sma1 fragment containing the URA3 gene in pSE936 was replaced with a HindIII fragment containing the LEU2 gene. pAA43 was generated by insertion of the 8-kb Bat1 genomic fragment in pAA17.

**Antibody Production and Immunoblot Analysis**—A 318-bp PCR fragment encoding aa 900–1006 of Bat1p was subcloned into the pQE70 expression vector (QiAGEN), which ligates it in frame with an ATG codon on the 5′ end and a six-histidine coding region on the 3′ terminus. Induction of E. coli containing this plasmid with isopropyl-1-thio-β-D-galactopyranoside led to overexpression of a 12.5-kDa peptide, which was purified by affinity chromatography on a nickel-agarose column (QiAGEN) and used to immunize two rabbits. Anti sera from both rabbits reacted strongly on immunoblots with the 12.5-kDa peptide. The rabbit sera were purified by affinity chromatography on a nickel-agarose column (Millipore) in 100 mM CAPS-piperidine, pH 9.5. The bound proteins were eluted with 300 mM imidazole, concentrated, and applied to 75% saturated polyethylene glycol. Protein A-Sepharose beads were bound to Protein A-Sepharose beads for 1 h (Sigma), washed three times with RIPA buffer, and dissolved in sample loading buffer by heating at 75 °C before running of SDS-polyacrylamide gels overlaid with a 4% stacking gel. Polyacrylamide gels were stained with Coomassie Blue, and the Bat1p-containing membranes were blotted unto PVDF-Immobilon (Millipore) in 100 mM CAPS-piperidine, pH 9.5. The bound proteins were stained with Coomassie Blue, and the Bat1p-containing membrane fraction was submitted for aa sequence determination using an Applied Biosystems Inc. model 477 protein sequencer.

**RESULTS**

**A BA Transporter in the Vacuolar Fraction**—Yeast secretory vesicles exhibited ATP-dependent transport of various BAs (see Table I and Ref. 23). Conjugated BAs were transported more efficiently than unconjugated BAs. A subcellular fraction enriched in vacuolar enzyme markers also exhibited ATP-dependent transport of [3H]taurocholate (Fig. 1A). The rate of BA transport in the vacuolar fraction was dependent on substrate concentration and was saturable. ATP-dependent transport as a function of taurocholate concentration can be fitted assuming a Michaelis-Menten kinetics with a predicted $K_m$ of 63 μM and $V_{max}$ of 14.9 nmol/mg/min (Fig. 1B). Because the specific activity and substrate affinity of ATP-dependent BA transport was higher in the vacuolar fraction than in purified secretory vesicles, and because preparation of the vacuolar fraction does not require temperature-sensitive strains, subsequent experiments were performed with the vacuolar fraction.

Transport of BAs into membrane-bound vesicles should be sensitive to changes in the intravesicular space. Increasing the osmolarity of the reaction mix inhibited ATP-dependent transport of taurocholate in vacuolar vesicles (Fig. 1C). When the rate of BA transport was plotted against the inverse of the sucrose concentration in the reaction mix, a straight line with an origin of approximately zero was obtained, indicating that taurocholate was translocated into the vesicle rather than merely binding to the membrane.

BA transport by the vacuolar fraction was virtually non-existent at 4 °C, in the absence of ATP, or when ATP was replaced by the non-hydrolyzable ATP analog, AMP-PNP. Vacuolar ATP-dependent BA transport was not inhibited by azide, an inhibitor of the mitochondrial proton ATPase, bafilomycin A1, an inhibitor of the vacuolar proton pump, or ionophores that dissipate membrane potential and ΔψH (Table II). NH₄Cl reduces uptake of taurocholate by 31%; however, the same degree of inhibition is observed when vacuoles lacking V-ATPase activity are used (see below), suggesting that the effect represents direct inhibition of the BA transporter rather than quenching of the ΔψH. ATP-dependent BA transport in the vacuolar fraction did not depend on activity of the V-ATPase. SF838–5AfP1-ΔS yeast lack the vma1 gene (31), which codes for the catalytic subunit of the V-ATPase, and consequently do not acidic the vacuolar space. Vacuoles purified from this strain exhibit no significant difference with regard to BA transport when compared with the vacuolar fraction prepared from an isogenic strain expressing VMA1 (Fig. 2). 100 μM sodium orthovanadate inhibits ATP-dependent BA transport by 56%. The plasma membrane proton ATPase Pma1p is also inhibited by vanadate with a $K_i$ of 1 μM (41). However, BA transport was not significantly affected by 10 μM vanadate, which inhibits Pma1p activity by 80%. This is consistent with previous findings, which show that secretory vesicles lacking Pma1p are still capable of vigorous taurocholate transport (23). Thus, vanadate appears to inhibit the vacuolar BA transporter directly, rather than by abrogation of Pma1p activity. These data indicate that BA transport in the yeast vacuolar fraction is energized directly by ATP.

**Identification of the Gene Encoding the BA Transporter**—Transport mediated by a number of ABC-type proteins is energized directly by ATP and, in some cases, is inhibited by vanadate, suggesting that BA transport may be mediated by an ABC-type protein. The YCF1 gene encodes an ABC-type protein that is sorted primarily to the yeast vacuole and transports glutathione conjugates, which are also amphipathic organic anions. However, vacuoles from the ycf1 deletion mutant JWS53 (28), although deficient in ATP-dependent glutathione conjugate transport, were normal with regard to ATP-dependent BA transport, indicating that Ycf1p is not responsible for this activity. Likewise, yeast strains deficient in expression of the ABC-type proteins Ste6p or Pdr5p (42), or which overexpress Snq2p (43) exhibited no alteration in ATP-dependent BA transport (not shown).

To identify other yeast ABC proteins, a homology search of the Saccharomyces Genome Database was performed using a consensus NBD amino acid (aa) sequence. At that time, the search identified seven putative yeast ABC-type proteins of

| Bile acid                      | Initial transport velocity (pmol/min/mg) |
|--------------------------------|------------------------------------------|
| Taurocholate                   | 51 ± 6.6                                 |
| Tauroursodeoxycholate          | 66.7 ± 1.6                               |
| Glycocholate                   | 4.5 ± 10.3                               |
| Chenoxycholate                 | 4.6 ± 4.0                                |
| Cholate                        | <1.0                                     |

**Table I**

Initial velocity of transport for radiolabeled BA was measured in secretory vesicles isolated from NY3 yeast. Reactions were carried out in the presence of 5 μM BA and 5 μM ATP as described (23). Values represent the mean ($n = 3$) ± standard error.
unknown function or intracellular location. Analysis of knock-out mutants can help identify the function of novel genes. Deletion of the chromosomal copy of a yeast gene of known sequence is facilitated by a PCR-based technique for generating disruption constructs that is based on the observation that 40 bp of homology are sufficient to elicit a homologous recombination event in yeast (15). Deletion mutants lacking the genes encoding the putative ABC-type proteins Adp1p (44), Mdl1p (45), Mdl2p (45), Yhd5p, Yk83p, Yib3p, and Yd96p (sequence accession numbers: 113449, 1346512, 170557, 731612, 549649, 558389, and 1077557, respectively) were generated by this technique. ATP-dependent transport of [3H]taurocholate was measured in vacuoles prepared from the disrupted strains; however, no significant differences in ATP-dependent BA transport were observed between samples derived from the disrupted strains and the RSY12 progenitor controls (not shown).

Because both the BA and glutathione conjugate transporters recognize small, negatively charged amphipathic molecules, are enriched in the vacuolar fraction, and exhibit similar substrate dose response (23), we hypothesized that the BA transporter was more closely related to Ycf1p than to other ABC-type proteins. Alignment of the Ycf1p aa sequence with Yhd5p, Yk83p, and mammalian MRP1, the proteins in the data base that most closely resembled Ycf1p, identified highly conserved aa sequence motifs in the carboxyl-terminal NBDs. Degenerate oligonucleotide primers, designed by reverse translation of these motifs, were used for PCR amplification of a genomic DNA template derived from DOY2J, a yeast double mutant deleted for the YCP1 and YHD5 genes. PCR products of the size expected for genes encoding ABC-type proteins (400–450 bp) were cloned and sequenced. Three novel DNA fragments were identified that contained open reading frames exhibiting similarity with the NBD of Ycf1p (Fig. 3).

BA transport activity was measured in vacuoles prepared from mutant yeast deleted for the DNA encompassed in the genome of SF838–5Afp1Δ8 strain that is deleted for the vma1 gene and the QL1 strain in which a copy of VMA1 has been integrated in the genome of SF838–5Afp1Δ8. Transport of 5 μM taurocholate was measured as in Fig. 1 for vacuoles from SF838–5Afp1Δ8 (●), and QL1 (○) in the presence (●) or absence (○) of 5 mM ATP. Values represent the mean (n = 3 ± standard error (except where smaller than symbol).

Table II

| Inhibitor          | Relative transport rate |
|--------------------|------------------------|
| ATP (5 mM)         | 100%                   |
| No ATP             | 6.4 ± 0.5              |
| AMP-PNP (5 mM)     | 5.7 ± 0.4              |
| Bafilomycin (0.5 μM) | 83 ± 9              |
| NaN3 (1 mM)        | 90 ± 2                |
| NH4Cl (1 mM)       | 69 ± 3                |
| CCCP (5 μM)        | 85 ± 4                |
| Gramicidin D (5 μM) | 103 ± 5              |
| NaN3O4 (1 μM)      | 95 ± 6                |
| NaN3O4 (10 μM)     | 86 ± 4                |
| NaN3O4 (100 μM)    | 44 ± 6                |
A Yeast Gene Encoding a Bile Acid Transporter

**FIG. 3.** Alignment of the Ycf1p NBD sequence with aa sequences deduced from DNA fragments generated by PCR amplification of yeast DNA. Fragment A is identical to EMBL accession number E283715 on chromosome XII, fragment B is identical to the YOR1 gene (65), and fragment C is identical to sequence accession number Z73153 on chromosome XII.

Alteration of the NBD abolishes transport activity of natural (46) and synthetic (47, 48) ABC-type protein mutants. Vacular BA transport in the DOY8 and DOY9 knockout strains, which are partially deleted for genes A and B, respectively, proved indistinguishable from the progenitor RSY12 strain. On the other hand, ATP-dependent transport of [3H]taurocholate was distinguishable from the progenitor RSY12 strain. On the other hand, ATP-dependent transport of [3H]taurocholate was completely abrogated in vacuoles derived from the DOY10 mutant that had been disrupted for gene C.

**Analysis of the BAT1 Sequence—DNA Clones Encapping PCR Fragment C and Flanking Sequences Were Isolated from a Bacteriophage λ Library of Genomic S. cerevisiae DNA by Hybridization to Radioactively Labeled Probes.** Analysis of the nucleotide sequence of the yeast DNA inserts identified a single long open reading frame encoding a putative 1661-aa protein with an estimated Mr of 189,147 (Fig. 4). The nucleotide sequence was derived from DNA clones of genomic origin. Posttranscriptional processing could therefore produce an mRNA that differs significantly from the DNA template. However, PCR fragments generated from genomic DNA and DNase-treated RNA are indistinguishable in size, suggesting that the BAT1 primary transcript does not contain large introns (Fig. 5).

The Bat1p (bile acid transporter) exhibits considerable aa sequence identity with a large number of ABC-type transporters in the protein sequence data bases. BLAST and FASTA homology searches of protein sequence data bases indicated that the aa sequences most closely resembling Bat1p include two putative proteins of unknown function encoded by the S. cerevisiae genes YHD5 (57% amino acid sequence identity with Bat1p) and YK83p (46%), the rat cMOAT/cMRP1 (32%) canalicular multispecific organic anion transporter, the human MRP1 (30%) glutathione conjugate transporter, and the yeast Ycf1p (27%) glutathione conjugate transporter. A cluster analysis showing putative phylogenetic relationships of Bat1p and several other ABC-type proteins is presented in Fig. 6.

The Bat1p deduced aa sequence contains two NBDs typical of ABC-type transporters. Both NBDs contain a reasonable match with the consensus Walker A and B motifs as well as the ABC signature sequence. Associated with each NBD is a hydrophobic domain capable of spanning a membrane multiple times (Fig. 5). It is difficult to ascertain the membrane topology of Bat1p from the primary sequence, as the predictions made by different algorithms do not match exactly. Thus TMAP (EMBL server) suggests an arrangement of 9 amino-proximal transmembrane helices + 5 membrane-spanning domains carboxyl-terminal to the first NBD, TMpred (49) proposes 11+, and PhdTopology (50) predicts 11+. It is unlikely that there are 5 transmembrane domains between the first and second NBD, as this would place the ATP binding domains on opposite sides of the membrane, suggesting that there are 4 or 6 transmembrane helices. Comparison of the hydropathy plots of closely related proteins reveals strong similarity in the arrangement and placement of the transmembrane domains with Yhd5p, Yk83p, MOAT, MRP1, and SUR (51). One notable difference is that Bat1p and the putative Yhd5p and Yk83p proteins appear to contain an additional amino-terminal transmembrane helix that is separated from the next transmembrane domain by a highly polar stretch of approximately 100 aa. This suggests the presence of a signal peptide. However, no good match to the von Heijne cleavage consensus sequence was detected in this region. Prosite analysis of the Bat1p sequence identified numerous potential glycosylation, myristylation, and phosphorylation sites, the relevance of which require experimental validation.

**BAT1 Deletion Mutants Are Deficient in BA Transport Activity—** ATP-dependent transport of BA was abolished in vacuoles prepared from DOY11 yeast (Fig. 7A) in which the BAT1 coding region has been deleted from the genome. It is conceivable that loss of Bat1p function affected ATP-dependent transport activities in the vacuolar fraction in a nonspecific manner; however, ATP-dependent transport of the glutathione conjugate DNPSG was unaffected in vacuoles derived from the DOY11 knockout strain (Fig. 7B), suggesting that this was not the case. Transformation of DOY11 with the centromeric plasmid pAA43, which carries an 8-kb genomic fragment containing the BAT1 coding region and 1.5 kb of upstream sequence, restored ATP-dependent BA transport to the level observed in the RSY12 control (Fig. 7A). Because overexpression of membrane proteins may result in mislocalization to the vacuole (52), a low copy number centromeric plasmid was used for the complementation experiments and samples from the progenitor RSY12 strain were included in all experiments.

A sec1–1 bat1a3 double mutant (DOY12) was generated by disruption of the BAT1 gene in the sec1–1 NY3 strain. Secretory vesicles prepared from DOY12 were essentially lacking ATP-dependent transport of taurocholate relative to the NY3 strain, indicating that Bat1p is also responsible for the BA transport activity detected in secretory vesicles.

Polyclonal antibodies were raised against a histidine-tagged
Bat1p partial peptide expressed in *E. coli*. The antibodies recognized a protein that was enriched 10–15-fold in the vacuolar fraction relative to total yeast membranes, and was absent in the DOY10 and DOY11 bat1 deletion mutants (Fig. 8). It was difficult to estimate the *M* <sub>r</sub> of Bat1p by SDS-PAGE as the electrophoretic mobility of Bat1p varied relative to common protein *M* <sub>r</sub> standards depending on the acrylamide percentage of the gel, *e.g.*, Bat1p has an apparent *M* <sub>r</sub> of 115,000 or 190,000 in immunoblots generated from protein extracts separated on 6% or 15% SDS-PAGE, respectively. Bat1p that had been de-lipidated by extraction with ether or ethanol-acetone behaved in an identical fashion, suggesting that it is not an effect of associated phospholipids. Migration of the major glycoprotein of human erythrocyte membranes is known to vary with the acrylamide concentration of the SDS-PAGE system (53). Other membrane proteins also migrate aberrantly on SDS-PAGE, including yeast uracil permease (54); mammalian sulfonylurea receptor SUR (51), which shows significant similarity with Bat1p at the level of aa sequence and transmembrane topology; and the bacterial ABC-type proteins, AbcA (55) and HlyB (56). At this time it is difficult to rule out post-translational modification or processing of Bat1p that would affect the size or mobility of the protein. The deduced aa sequence suggests the presence of a signal peptide that could be cleaved after membrane insertion. Bat1p was purified by immunoprecipitation and gel electrophoresis, and protein sequencing was attempted on the purified protein. However, the amino terminus was blocked. Further experiments will address this question.

**DISCUSSION**

The Bat1p ABC-type protein mediates ATP-dependent transport of bile acids and is overrepresented in a subcellular fraction enriched in vacuolar markers. Uptake by this fraction represents true translocation of the BA across the membrane, as transport is susceptible to reduction of the osmotically sensitive intravesicular space and permeabilization of the vesicles results in loss of the radiolabeled substrate. The yeast transporter is similar to the bile acid transport activity that resides in the canalicular membrane. Transport is completely dependent on the presence of Mg<sup>2+</sup>-ATP and is inhibited by non-hydrolyzable nucleotide analogs. Like canalicular transport, the yeast transporter is inhibited by the phosphate analog vanadate, which also inhibits the activity of a number of ABC-type proteins. Substrate competition studies with CMV indicate that the mammalian transporter has higher affinity for negatively charged, conjugated BA (57); likewise, yeast membrane fractions recognize glycine- and taurine-conjugated BA more efficiently than unconjugated BA. Transport kinetics are also similar, CMV manifest a *K*<sub>m</sub> for taurocholate of 2–47 μM and *V*<sub>max</sub> of 0.5–4 nmol/min/mg (4–6), whereas the vacuolar fraction of yeast has a *K*<sub>m</sub> of 63 μM and *V*<sub>max</sub> of 15 nmol/min/mg. While it is possible that the higher *V*<sub>max</sub> observed in the yeast vacuolar fraction represents a real difference between the two proteins, it may also reflect a higher yield of vacuoles in the right configuration for transport. Approximately 80% of CMV have a right-side-out orientation and do not manifest ATP-dep...
The deduced aa sequence indicates that Bat1p is similar to the subgroup of ABC-type proteins that includes mammalian MDR1 and related polypeptides, which are believed to have originated from duplication and fusion of an ancestral gene encoding a single NBD and polytopic transmembrane region (61). Many members of this subgroup exhibit an amino-terminal transmembrane region that is significantly longer than the carboxyl-terminal counterpart and spans the membrane more times. Although membrane topology of polytopic membrane proteins is hard to predict in the absence of experimental data, three different algorithms predicted that Bat1p contains 8–10 transmembrane helices in the amino terminus which are believed to have originated from duplication and fusion of an ancestral gene encoding a single NBD and polytopic transmembrane region (61). Many members of this subgroup exhibit an amino-terminal transmembrane region that is significantly longer than the carboxyl-terminal counterpart and spans the membrane more times. Although membrane topology of polytopic membrane proteins is hard to predict in the absence of experimental data, three different algorithms predicted that Bat1p contains 8–10 transmembrane helices in the amino terminus versus only 4 in the carboxyl region. This matches the predicted topologies for SUR, EHBH, cMOAT/cMRP1, and MRP1, the mammalian proteins most closely resembling Bat1p, and is similar to the topologies predicted for the yeast putative proteins, Yk83p and Yhd5p.

ATP-dependent transport of BA has also been observed in the vacuoles of plants (62) and the fission yeast, Schizosaccharomyces pombe (63). Conservation of this activity over such a broad evolutionary range suggests that BA transport may have a basic metabolic relevance. Yeast vacuoles are digestive compartments implicated in autophagy and degradation of cellular organelles (64). It is conceivable that Bat1p concentrates fun-
gal equivalents of BA in the vacuole where these detergents aid in breakdown of organelle membranes and lipids. Alternatively, transport of soluble sterol derivatives across the vacuolar membrane may fulfill a function in steroid or steroid metabolism. BAT1 is closely related to two genes implicated in detoxification of xenobiotics: YCF1 in Cd tolerance and YOR1/ YRS1, which is involved in oligomycin and multidrug resistance. It is therefore possible that BAT1 also plays a role in toxin resistance. Identification of BAT1 and the availability of a knockout strain should aid in elucidating the function of BA transport in plants and fungi.

ATP-dependent transport of BA in the liver directly affects cholesterol secretion and water flow into the canaliculus, and impaired BA secretion occurs in numerous inherited and acquired hepatobiliary diseases. Despite the importance of ATP-dependent BA transport, the protein responsible for this activity and the cognate gene have not been isolated, mainly due to low abundance in the liver. Hepatic BA transport activity resembles Bat1p-mediated BA transport with regard to energy requirements, substrate specificity, and response to inhibitors. Yeast Ycf1p, which exhibits similarities in substrate affinity, energy requirements, and inhibitor response with mammalian MRPI and cMOAT (24), also displays significant sequence identity with these proteins, suggesting that isolation of the yeast gene encoding an ATP-dependent BA transporter should facilitate identification of the mammalian counterpart.

REFERENCES

1. Meier, P. J., Meier-Abt, A. S., and Boyer, J. L. (1987) Biochem. J. 242, 465–469
2. Poupol, E., Poupol, M. L., Grodenouge, M., Dumont, M., Erlinger, S. (1976) Eur. J. Clin. Invest. 6, 279–284
3. Reichen, J., and Paumgartner, G. (1976) Am. J. Physiol. 231, 734–742
4. Nishida, T., Ganthai, Z., Chen, J. M., and Arias, I. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6590–6594
5. Adachi, Y., Kobayashi, H., Kurumi, Y., Shouji, M., Kitano, M., and Yamamoto, T. (1991) Hepatology 14, 655–659
6. Siess, E., O'Neill, B., and Meier, P. J. (1992) Biochem. J. 284, 67–74
7. Marin, J. J., Bravo, P., El-Mir, M. Y. A., and Serrano, M. A. (1995) Am. J. Physiol. 268, G685–G689
8. Sippel, C. J., Suchy, F. J., Ananthanarayanan, M., and Perlmuter, D. H. (1993) J. Biol. Chem. 268, 2083–2091
9. Lin, S. H. (1989) J. Biol. Chem. 264, 14403–14407
10. Sippel, C. J., McCollum, M. J., and Perlmuter, D. H. (1994) J. Biol. Chem. 269, 2820–2826
11. Kast, C., Siess, B., Winterhalter, K. H., and Meier, P. J. (1994) J. Biol. Chem. 269, 5179–5186
12. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
13. Kamimoto, Y., Ganthai, Z., Hsu, J., and Arias, I. M. (1989) J. Biol. Chem. 264, 11693–11698
14. Smit, J. J., Schinkel, A. H., Oude-Eferink, R. P. J., Groen, A. K., Wagenar, E., van Deemer, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) Cell 75, 451–463
15. Ruets, S., and Gros, P. (1994) Cell 77, 1071–1081
16. Nies, A. T., Ganthai, Z., and Arias, I. M. (1996) J. Lipid Res. 37, 1125–1136
17. Mayer, R., Kartenbeck, J., Buchler, M., Jedlitschky, G., Leier, I., and Keppler, D. (1995) J. Biol. Chem. 270, 137–150
18. Paulusma, C. C., Bosma, P. J., Zaman, J. R., Bakker, C. T. M., Otter, M., Scheffer, G. L., Schepers, R. J., Borst, P., and Oude-Eferink, R. P. J. (1996) Science 271, 1126–1128
19. Buchler, M., König, J., Bron, M., Kartenbeck, J., Spring, H., Horie, T., and Keppler, D. (1996) J. Biol. Chem. 271, 15991–15998
20. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, R. C., Stewart, A. J., Kurtz, E. U., Duncan, A. M. V., and Betsley, R. (G) (1992) Science 258, 1650–1654
21. Ruets, S., and Gros, P. (1994) J. Biol. Chem. 269, 12277–12284
22. Chumakov, K. M., Bat1p-mediated BA transport with regard to energy. Hepatic BA transport activity re- sults in an impairment of BA secretion occurs in numerous inherited and acquired hepatobiliary diseases. Despite the importance of ATP-dependent BA transport, the protein responsible for this activity and the cognate gene have not been isolated, mainly due to low abundance in the liver. Hepatic BA transport activity resembles Bat1p-mediated BA transport with regard to energy requirements, substrate specificity, and response to inhibitors. Yeast Ycf1p, which exhibits similarities in substrate affinity, energy requirements, and inhibitor response with mammalian MRPI and cMOAT (24), also displays significant sequence identity with these proteins, suggesting that isolation of the yeast gene encoding an ATP-dependent BA transporter should facilitate identification of the mammalian counterpart.