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Functional Expression of the Human UDP-Galactose Transporters in the Yeast *Saccharomyces cerevisiae*[1]

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We describe the functional expression of the putative human Golgi UDP-galactose transporters (hUGT1 and hUGT2) in the yeast *Saccharomyces cerevisiae*. Both hUGT1 and hUGT2 were expressed under the control of the yeast constitutive GAPDH promoter. The expression level of hUGT1 seemed to be considerably lower than that of hUGT2, although hUGT1 has an amino acid sequence identical to that of hUGT2 except for 5 amino acid residues at the C-terminus. The hUGT product was expressed in the membranes of Golgi and other organellar compartments. The membrane vesicles prepared from the hUGT1- or the hUGT2-expressing yeast cells exhibited UDP-galactose specific transport activity. The apparent K_m values of the yeast-expressed hUGT1 and hUGT2 for UDP-galactose were 1.2 and 2 μM, respectively, which were comparable with the K_m obtained with mammalian Golgi vesicles. Transport was dependent on temperature and integrity of vesicles, and was inhibited by UMP, as observed with mammalian Golgi vesicles. Our results demonstrate that the previously described hUGT1 and hUGT2 encode the UDP-galactose transporters, rather than regulatory proteins. The development of a convenient yeast expression system should facilitate analysis of the structure-function relationships of the UDP-galactose transporters.

Key words: heterologous expression, human UDP-galactose transporter, nucleotide-sugar transporter, *Saccharomyces cerevisiae*.

Glycosylation of secretory and membrane-bound proteins and of lipids is carried out in the Golgi compartment. Translocation of nucleotide sugars, the substrates of glycosylation reactions, from the cytosol into the lumen of the Golgi apparatus is accomplished by a group of specific transporters (*1, 2*). Recently, cDNAs encoding putative nucleotide sugar transporters have been identified from human (*3, 4*), rodent (*4, 5*), protozoa (*6*), and yeast (*7, 8*) on the basis of their ability to complement the phenotypes of mutants defective in the transport activities. The products of these cDNA clones share 40-60% similarity to each other, and are predicted to be intrinsic membrane proteins with multiple membrane-spanning domains (*2-4*). We reported previously the cloning of two human cDNAs (hUGT1 and hUGT2) which correct the phenotype of Had-1 mutant cell (*9*) defective in UDP-galactose transport activity (*3, 4*). The Golgi vesicles from the hUGT-transfected Had-1 mutant cells were shown to have recovered the transport activity (*3, 4*). Immunocytochemical and subcellular fractionation studies confirmed that hUGT products are localized in the membranes of the Golgi compartment (*10*).

To demonstrate that hUGT1 and hUGT2 (*3, 4*) encode the UDP-galactose transporters rather than proteins that regulate the transporter or supplementary factors of the transporter, we tried to express the UDP-galactose transport activity by introducing the hUGTs into the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* cells do not naturally add galactose to proteins (*11*), and there is no gene exhibiting high homology to the mammalian UGT genes in the whole yeast genome. The absence of endogenous UDP-galactose transport into the yeast Golgi lumen was also confirmed by another group (*12*). Therefore, this unicellular organism provides a suitable system for addressing the question of whether hUGTs encode the transporters or associated factors. The other advantages of the yeast expression system are its simple manipulation and the potential to produce massive quantities of eukaryotic proteins efficiently. Large quantities of purified functional transporter protein would facilitate detailed analysis of the structure and function of the UDP-galactose transporters.

MATERIALS AND METHODS

Materials—The radioactive substrates UDP-[4,5-^{3}H]-galactose (48.3 Ci/mmol) and CMP-[9-^{3}H]sialic acid (33.2 Ci/mmol) were purchased from NEN Life Science Products. The microsome fractions from the mouse Had-1/hUGT1 cell line were prepared as described in detail elsewhere (*10*).

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Strains and Transformations—*Escherichia coli* strain DH5α (GIBCO) was used for transformation and propagation of the recombinant plasmids. *S. cerevisiae* strain YPH501 (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-*Δ*63/trp1-*Δ*63 his3-*Δ*200/his3-*Δ*200 leu2-*Δ*1/leu2-*Δ*1)* (*13*) was used in the expression study. Yeast transformations were performed using the lithium acetate method as described previously (*14*).

Construction of the Expression Plasmids—The 1.1 kb and the 1.2 kb *Eco*RI fragments from pMKIT-neo-hUGT1 and pMKIT-neo-hUGT2 (*3, 4*), which contain the coding region of hUGT1 and hUGT2, respectively, were inserted into the *Eco*RI site of vector pKTΔATG under the control of the GAPDH promoter. The resulting plasmids were designated as pKTΔATG/hUGT1 and pKTΔATG/hUGT2, respectively. pKTΔATG, a modified version of pKT10 (*15*), was obtained by digesting pKT10 with *Eco*RI and *Kpn*I, blunting the ends with T4 polymerase, and self-ligation. Thus the "ATG" site under the GAPDH promoter and the *Kpn*I site were removed and the *Eco*RI site was regenerated in this modification (*16*).

Yeast Cultures—Yeast strains were grown in a synthetic medium containing 0.67% (w/v) Bacto-yeast nitrogen base without amino acids, 2% (w/v) glucose (YNBD) or 1% (w/v) glucose and 1% (w/v) galactose (YNBDG), supplemented with L-leucine, L-histidine, L-tryptophan, L-lysine, and adenine. Uracil was omitted for selection and growth of transformants. For preparation of membrane vesicles, the transformants were grown to early log phase ($OD_{600} = 1.0$) at 30°C by inoculation with fresh overnight cultures.

Immunoblotting Analysis—To determine the expression of hUGT products, the crude membranes were prepared as described (*17*) with some modifications. Briefly, cells were harvested, washed once with the ice-cold lysis buffer [0.25 M mannitol, 1 mM HEPES-Tris (pH 7.4), 1 mM EDTA, and 20 mM KCl] and resuspended in the lysis buffer containing a protease inhibitor cocktail (Complete, EDTA-free, Boehringer Mannheim) and 1 mM *p*-APMSF. An equal volume of glass beads was added into the suspension, and cells were broken by vigorous vortexing at 4°C for 2 min with cooling of the suspension on ice every 30 s. Intact cells and debris were separated from the membrane suspension by centrifugation at $1,500 \times g$ for 5 min. The resultant supernatant was used as cell lysate. The cell lysate was then centrifuged at $10,000 \times g$ for 10 min, followed by $100,000 \times g$ for 60 min. The resultant membrane pellet after $100,000 \times g$ centrifugation (P100) was resuspended in the resuspension buffer [0.25 M mannitol, 1 mM HEPES-Tris (pH 7.4), 1 mM $MgSO_4$, and 20 mM KCl] plus the protease inhibitor cocktail (Boehringer Mannheim) and 1 mM *p*-APMSF, and used in immunoblotting analysis. The protein concentrations of the membrane preparation were determined by using a BCA kit (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE (*18*) with 12% polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membrane. Immunoblotting analysis was performed as previously described (*10*).

Subcellular Fractionation and Nucleotide-Sugar Transport Assay—The subcellular fractionation was performed as described by Walworth and Novick (*20*). Cells were grown in 1 liter of synthetic medium to the early exponential phase at 30°C. The cells were pelleted, washed once with ice-cold 10 mM NaN_3 and converted into spheroplasts in a spheroplast solution containing 1.4 M sorbitol, 50 mM potassium phosphate (pH 7.5), 10 mM NaN_3, and 40 mM β-mercaptoethanol. The spheroplasts were pelleted and broken by resuspending the pellet in 30 ml of the TEA-solution containing 10 mM triethanolamine acetate (TEA) (pH 7.2), 0.8 M sorbitol, 1 mM EDTA, the protease inhibitor cocktail (Boehringer Mannheim), and 1 mM *p*-APMSF. The suspension was passed twice through a thin pipette tip, and then centrifuged at $2,300 \times g$ for 10 min to remove unlysed cells. The supernatant was then centrifuged successively at $10,000 \times g$ for 10 min and $100,000 \times g$ for 45 min to yield P10 and P100 membrane fractions, respectively. The pellet was resuspended in the TEA-solution and used in immunoblotting analysis as described previously (*10, 19*).

The membrane fractions used in the transport assay were the combined P10 and P100 fractions. The transport assay was performed as previously described (*10, 21*). Briefly, the reaction mixture (100 μl) used in assays contained 10 mM TEA (pH 7.2), 0.6 M sorbitol, 100 mM KCl, 1 mM $MgCl_2$, and 0.5 mM dimercatopropanol (TSKM buffer) (*22*) plus 1 μM UDP-[^{3}H]galactose (6,400 Ci/mol) or 1 μM CMP-[^{3}H]sialic acid (6,400 Ci/mol). The reaction mixture was incubated at 30°C. Reactions were initiated by addition of membranes, and terminated by 10-fold dilution with an ice-cold stop buffer containing 10 mM TEA (pH 7.2), 0.6 M sorbitol, 100 mM KCl, and 1 mM $MgCl_2$, and 1 μM non-radioactive UDP-galactose or CMP-sialic acid. The entire reaction mixture was poured onto an Advantec Toyo A045A025A nitrocellulose filter (Advantec Toyo, Tokyo). The filter was washed 3 times with 1 ml of the ice-cold stop buffer, and then dried. The radioactivity trapped on the filter was determined.

RESULTS

Expression of Human UDP-Galactose Transporter in the Yeast Saccharomyces cerevisiae—The putative human UDP-galactose transporters (hUGT1 and hUGT2) were expressed in *S. cerevisiae* from the pKTΔATG vector, a multicopy expression vector constructed for constitutive expression from the strong promoter of glyceraldehyde-3-phosphate dehydrogenase, GAPDH promoter (*15*). The coding regions of hUGT1 and hUGT2 cDNAs including parts of their 3′-terminal untranslated regions (*3, 4*) were cloned into pKTΔATG without any modification.

To analyze the expression of the transporters, the membrane fractions were prepared from the hUGT transformant cells grown in YNBD medium and examined by immunoblotting (Fig. 1). In all the hUGT1 and hUGT2 transformants analyzed, a 36 kDa protein, which showed exactly the same mobility as the hUGT1 product expressed in the Had-1 cells, was recognized by the anti-hUGT1 (Fig. 1, lane 3) and anti-hUGT2 antibodies (Fig. 1, lane 6), respectively. No specific band was detected in the vector-transformed cells (Fig. 1, lanes 2 and 5). A band of approximately 33 kDa was specifically detected in the membrane fractions from the hUGT2 transformant cells. This band may represent a major degraded form of the transporter protein. A band of approximately 46 kDa was also specifically detected from the membrane fractions of the hUGT1 transformant. The 46 kDa band possibly repre-

sents a glycosylated form of the hUGT1 product, although the glycosylation state of the hUGT protein in yeast cells still needs further investigation.

Immunoblotting analyses using antibodies to the C-terminal showed that the signals of the hUGT1 and the hUGT2 products in the same amount of membrane protein were considerably different (Fig. 1). In one of our previous reports, we noted that the anti-hUGT1 and anti-hUGT2 C-terminal antibodies exhibit similar avidities to their respective peptide antigens (*10*). Assuming that reactivity of antibodies to the antigen peptide and to the respective C-terminal of cognate hUGT products bound on PVDF blot is proportional, if not the same, this result suggests that the expression levels of hUGT1 and hUGT2 were different, the former being much lower than the latter. The reason for this is unclear at present, since the amino acid sequences of the two transporters are identical except for several amino acid residues at the C-termini (*3*).

Subcellular fractionation studies using the hUGT2 transformant were carried out to localize the hUGT product in yeast cells. The hUGT2-expressing cells were lysed from spheroplast mildly, and the lysates were subjected to differential centrifugation (see "MATERIALS AND METHODS"). The hUGT product was recovered in both the P10 (approximately 60%) and P100 (40%) fractions (Fig. 2, panel A), but the 33 kDa degraded form was almost exclusively found in the P10 fraction. Under the same conditions, the vacuolar membrane marker protein, alkaline phosphatase (ALP), was found predominantly in the P10 fraction (Fig. 2, panel B) (*23*). Other large membrane structures, such as the plasma membrane and endoplasmic reticulum, in addition to the mitochondria and nuclei, are also expected to be part of this pelletable material (*20*). Most of the late Golgi marker protein, Kex2p, was detected in the P100 fraction (Fig. 2, panel C). No hUGT2 product was detected in the S100 fraction where the soluble protein marker, alcohol dehydrogenase (ADH), was present (Fig. 2, panels A and D). These results suggest that the mammalian Golgi UGT proteins were expressed in membranes of the Golgi and other cellular compartments in yeast cells.

Enzymatic Characteristics of the Yeast-Expressed hUGT—To determine whether the hUGT products expressed in *S. cerevisiae* were functional, the membrane fractions were prepared from the transformant cells (*20*), and the transport of UDP-[^{3}H]galactose into the membrane vesicles was measured by membrane filtration assay (see "MATERIALS AND METHODS"). We used the combined P10 and P100 fractions for the transport measurements below, since both fractions were active in UDP-galactose transport and showed similar transport characteristics

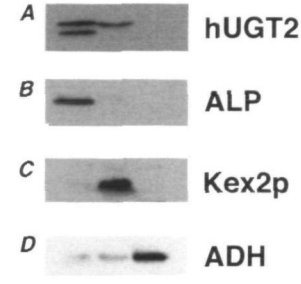

Fig. 2. **Distribution of the hUGT2 product among subcellular compartments in yeast cells.** Yeast cells carrying the hUGT2-expressing plasmid were converted into spheroplasts, lysed, and subjected to sequential differential centrifugation (see "MATERIALS AND METHODS"). Equivalent amounts of subcellular extracts were subjected to SDS-PAGE and analyzed by immunoblotting using anti-hUGT2 (panel A), anti-ALP (panel B), anti-Kex2p (panel C), and anti-ADH antibodies (panel D).

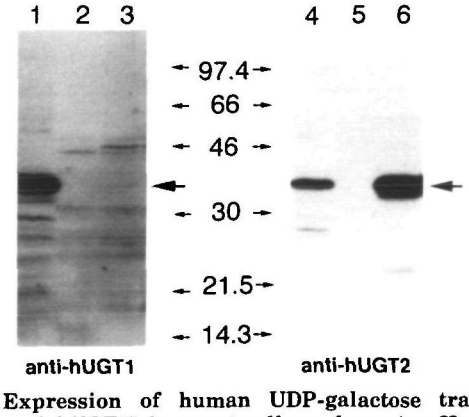

Fig. 1. **Expression of human UDP-galactose transporters (hUGT1 and hUGT2) in yeast cells and murine Had-1 cells.** Golgi-enriched P100 fractions prepared using the glass bead lysis method from pKTΔATG/hUGT1, pKTΔATG/hUGT2 and vector pKTΔATG transformant yeast cells were analyzed by immunoblotting using anti-hUGT1 and anti-hUGT2 affinity-purified polyclonal antibodies (see "MATERIALS AND METHODS"). Microsome fraction prepared from Had-1/hUGT1 was used as a positive control (lanes 1 and 4). Lanes 2 and 5: P100 fractions from the vector transformant. Lane 3: P100 fractions from the hUGT1 transformant; lane 6: P100 fractions from the hUGT2 transformant. Note that the hUGT1 product was recognized by both the anti-hUGT1 and the anti-hUGT2 antibodies (*10*). The amount of membrane fractions used was 40 μg per lane.

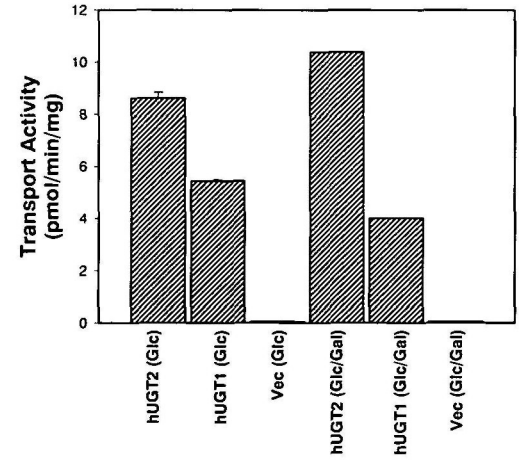

Fig. 3. **UDP-galactose transport activity of vesicles prepared from hUGT1- or hUGT2-expressing yeast cells.** Uptake of UDP-[^{3}H]galactose into vesicles prepared from hUGT-expressing cells and vector-transformed cells as described in "MATERIALS AND METHODS" was measured by means of filtration assay. Vesicles (50 μg/assay) were incubated in 100 μl of TSKM buffer (pH 7.2) containing 1 μM UDP-[^{3}H]galactose (6,400 Ci/mol) at 30°C for 30 s. The amount of radioactivity trapped on the filter at 0°C, 0 min of incubation (background) was subtracted from the corresponding experimental values. "Glc" and "Glc/Gal" indicate membrane vesicles obtained from cells grown in YNBD and YNBDG, respectively. Points represent mean values ± SE from duplicate experiments.

(data not shown). The integrity and correct topographical orientation of the membrane vesicles prepared by this procedure were examined and confirmed previously (*22, 24*). The membrane fraction prepared from vector-transformed cells was used as a reference. Figure 3 shows that UDP-[^{3}H]galactose was transported into the vesicles prepared from either the hUGT1 or the hUGT2 transformant cells. In contrast, no specific uptake of UDP-[^{3}H]-galactose was observed into the vesicles from cells harboring the vector only. The rate of transport observed with the hUGT2-expressing vesicles was approximately 2-fold higher than that obtained with the hUGT1-expressing vesicles. Provided that the above consideration on the level of hUGT1 and hUGT2 is valid, the observed transport activity of hUGT2-expressing vesicles seems to be relatively low.

Addition of galactose into the culture medium induces the *GAL1-GAL7-GAL10* promoters and raises the concentration of intracellular UDP-galactose in yeast cells (*25, 26*). To examine the effect of intracellular UDP-galactose on the activity of hUGT products, we have measured the uptake of UDP-[^{3}H]galactose with vesicles prepared from cells grown in medium where the *GAL* promoters are induced. Since the growth of hUGT transformants in medium containing galactose as the sole carbon source was retarded, we chose YNBDG medium (1% glucose and 1%

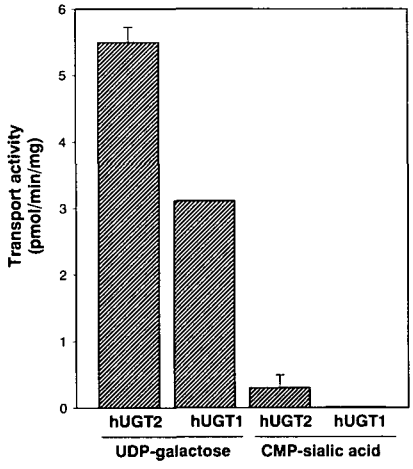

Fig. 4. **Substrate specificity of yeast hUGT-expressing yeast membrane vesicles.** The hUGT2-expressing yeast membrane vesicles (50 μg/assay) were incubated in 100 μl of TSKM buffer at pH 7.2 containing 1 μM UDP-[^{3}H]galactose (6,400 Ci/mol) or 1 μM CMP-[^{3}H]sialic acid (6,400 Ci/mol) at 30°C for 30 s. Assays were performed as described in "MATERIALS AND METHODS." The amount of radioactivity trapped on the filter at 0°C, 0 min of incubation (background) was subtracted from the corresponding experimental values. Values are means±SE from duplicate experiments.

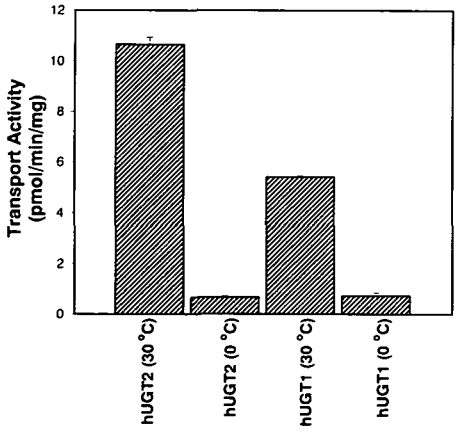

Fig. 5. **Temperature-dependence of UDP-[^{3}H]galactose transport into yeast hUGT-expressing vesicles.** The hUGT-expressing yeast membrane vesicles (50 μg/assay) were incubated in 100 μl of TSKM buffer (pH 7.2) containing 1 μM UDP-[^{3}H]galactose (6,400 Ci/mol) at 30 or 0°C for 30 s. The amount of radioactivity trapped on the filter at 0°C, 0 min of incubation (background) was subtracted from the corresponding experimental values. Values are means±SE from duplicate experiments.

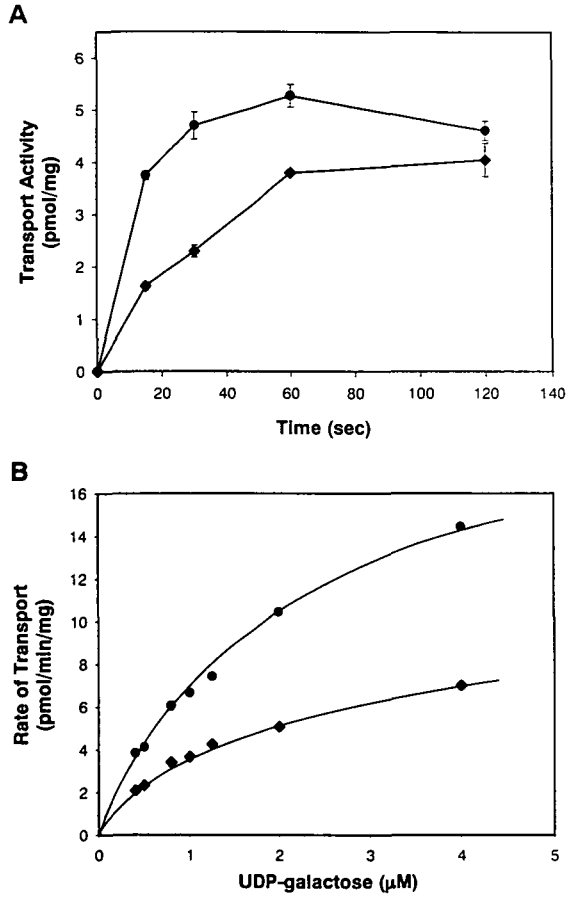

Fig. 6. **Time and substrate concentration dependence of UDP-[^{3}H]galactose transport into hUGT-expressing yeast membrane vesicles.** (A) hUGT1- (solid circles) and hUGT2- (solid diamonds) expressing vesicles (50 μg/assay) were incubated in 100 μl of TSKM buffer (pH 7.2) containing 1 μM UDP-[^{3}H]galactose (6,400 Ci/mol) at 30°C for the indicated time and transport of UDP-[^{3}H]galactose into vesicles was measured by means of filtration assay as described in "MATERIALS AND METHODS." (B) UDP-galactose transport was assayed as described above, except that the concentrations of UDP-[^{3}H]galactose were varied as indicated in the figure, and incubations were conducted for 30 s. The amount of radioactivity trapped on the filter at 0°C, 0 min of incubation (background) was subtracted from the corresponding experimental values. K_m values were calculated from a double-reciprocal plot fitted by linear regression analysis. Points represent mean values±SE from duplicate experiments.

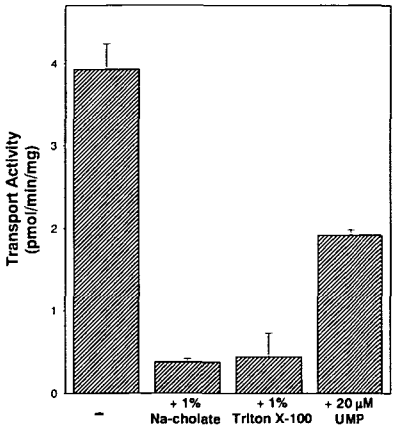

Fig. 7. **Effects of Triton X-100, Na-cholate, and UMP on the UDP-galactose transport.** Vesicles prepared from hUGT2-expressing yeast cells were incubated in 100 μl of TSKM buffer, containing detergent or UMP at the indicated concentration in the figure, at 30°C for 1 min. Values are means±SE from duplicate experiments.

galactose) as the induction medium. The *GAL* promoters were induced in the medium containing equal concentrations of galactose and glucose (*27*), and the induced expression level was sufficient to raise the intracellular concentration of UDP-galactose. No significant changes were observed in the transport activity (Fig. 3), suggesting that the increase in the intracellular concentration of UDP-galactose did not have any effect on the function of the yeast-expressed hUGT products.

The characteristics of UDP-[^{3}H]galactose uptake and substrate specificity of the yeast-expressed hUGT products were also examined. Since the yeast cells do not transport CMP-sialic acid into the Golgi lumen (*12*), uptake of CMP-[^{3}H]sialic acid into the same membrane vesicles as described above was measured. As shown in Fig. 4, uptake of CMP-[^{3}H]sialic acid was insignificant, being less than 20 cpm above the background (approximately 50 to 100 cpm) under experimental conditions where the uptake of UDP-[^{3}H]galactose was over 600 cpm. The uptake of UDP-*N*-acetylglucosamine was also not detected under a similar condition (data not shown). These results confirmed that the uptake of UDP-galactose into the yeast membrane vesicles was due to the expression of hUGT, and the hUGT products did not transport CMP-sialic acid or UDP-*N*-acetylglucosamine.

In accordance with the previous observation with mammalian Golgi vesicles, the vesicles prepared from the hUGT transformant cells transported UDP-galactose in a temperature-dependent manner. The rate of transport at 30°C was approximately 7 to 17 times that at 0°C (Fig. 5). The transport was a saturable reaction (Fig. 6). The apparent K_m values for UDP-galactose were 1.2 and 2.0 μM with hUGT1 and hUGT2, respectively.

The transport of UDP-galactose was also dependent on vesicle integrity. Addition of 1% Triton X-100 or 1% Na-cholate eliminated the UDP-galactose uptake (Fig. 7). UMP is reported to inhibit the UDP-galactose transport competitively (*1*). The UDP-galactose transport of the yeast-expressed hUGT products was also inhibited by UMP (Fig. 7).

DISCUSSION

The mouse galactosylation-defective mutant cell line Had-1 was originally isolated as a Newcastle disease virus receptor-deficient mutant cell line (*9, 28, 29*). Biochemical and genetic lines of evidence suggested that the gene defective in Had-1 cells encoded the Golgi UDP-galactose transporter (*28*). Recently we have shown that the products of human cDNA clones hUGT1 and hUGT2 (*3, 4*) complementing the defect of Had-1 cells are exclusively expressed in the membranes of the Golgi apparatus in Had-1 cells (*10*). In this report, we have shown that heterologous expression of hUGT1 and hUGT2 on the zero background of the yeast *S. cerevisiae* resulted in active UDP-galactose transport. This result clearly demonstrates that the hUGT1 and hUGT2 cDNAs do encode the transporter proteins *per se* rather than regulatory factors.

The yeast cells carrying the expression plasmid of hUGT1 or hUGT2 cDNA produced a protein with an apparent molecular mass of 36 kDa, which was not detected in the vector-transformed cells. The yeast-expressed hUGT products showed exactly the same mobility as the murine Had-1 cell-expressed hUGT protein and the endogenous UGT detected in HeLa cells (*10*), suggesting that no specific processing or modification reactions occur in the yeast cells. The constitutive expression of hUGT1 and hUGT2 did not show any effect on the growth of yeast cells (data not shown).

Our previous studies showed that the levels of expression of hUGT1 and hUGT2 in mammalian cells were comparable (*10*). However, for an unknown reason the expression level of hUGT1 product in yeast cells seemed to be significantly lower than that of hUGT2, although the two cDNA products differ only in several amino acid residues at the C-termini and the 3′-terminal untranslated regions.

Both hUGT1 and hUGT2 products were expressed in mammalian cells exclusively in the membranes of Golgi compartment (*10* and Yoshioka *et al.*, unpublished data). Subcellular fractionation analyses showed that a significant portion of the hUGT products in yeast cells was found in the P100, a fraction enriched with late Golgi vesicles. So far, it has been shown that the membrane anchor region of mammalian glycosyltransferase is able to target a reporter protein to the yeast Golgi, and a fusion of the membrane anchor region of yeast Golgi type II membrane protein to the catalytic domain of mammalian glycosyltransferase can be targeted to the Golgi in yeast cells (*30, 31*). However, the Golgi-targeting mechanism for membrane proteins with multiple membrane-spanning regions is still an unsolved problem. Our result suggests that essential features affecting the localization and function of Golgi membrane transporter proteins are conserved between higher eukaryotes and yeast. The hUGT2 product was also found in the P10, a fraction containing the plasma membrane, vacuolar membrane, endoplasmic reticulum, mitochondria and nuclei. It is possible that overexpression from the strong GAPDH promoter may affect the localization of the hUGT product in yeast cells. The hUGT products expressed in the P10 fraction seemed to be functionally competent, since both the P10 and P100 fractions were active in UDP-galactose transport (data not shown).

Detailed enzymatic analysis of hUGT as a UDP-galactose

transporter must await purification and reconstitution of hUGT products. Availability of the yeast expression system allows the large-scale production of the hUGT protein and will facilitate further elucidation of structure-function relationships of UDP-galactose transporters.

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