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Molecular Cloning and Functional Expression of the Human Golgi UDP-*N*-Acetylglucosamine Transporter[1]

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We have cloned the human UDP-*N*-acetylglucosamine (UDP-GlcNAc) transporter cDNA, which was recognized through a homology search in the expressed sequence tags database (dbEST) based on its similarity to the human UDP-galactose transporter. The chromosomal location of the UDP-GlcNAc transporter gene was assigned to chromosome 1p21 by fluorescence *in situ* hybridization (FISH). The transporter was expressed ubiquitously in every tissue so far examined. Expression of the transporter cDNA in CHO-K1 cells in its native and in a C-terminally HA-tagged form indicated that the human UDP-GlcNAc transporter was localized in the Golgi apparatus. The membrane vesicles prepared from yeast cells expressing the cDNA product exhibited UDP-GlcNAc-specific transporting activity. Comparison among UDP-galactose, CMP-sialic acid, and UDP-GlcNAc transporters from several organisms enabled us to identify residues highly conserved among the transporters and residues specific for each group of transporters.

Key words: CDG syndrome, CMP-sialic acid transporter, nucleotide-sugar transporter, UDP-galactose transporter, UDP-*N*-acetylglucosamine transporter.

Oligosaccharide chains on glycoconjugates play a wide variety of biological roles ranging from conformational and metabolic stabilization of proteins to involvement in the processes of cell-matrix and cell-cell interactions as ligands for specific recognition (*1*). Glycosylation of proteins and lipids is carried out in the lumenal regions of the endoplasmic reticulum and the Golgi apparatus. Nucleotide-sugar transporters are localized mainly in the membranes of the Golgi apparatus, where they provide various glycosyltransferases with proper nucleotide-sugar substrates (*2*). Therefore, the transporters are indispensable for glycoconjugate synthesis, and are strong candidates for risk factors of carbohydrate-deficient glycoprotein (CDG) syndrome (*3*). The CDG syndrome is a multisystem disorder characterized by mental retardation, lipocutaneous abnormalities, cerebellar hypoplasia, liver dysfunction, retinal degeneration, stroke-like episodes, and other features. Abnormalities of glycoprotein structure, including increased amounts of carbohydrate-deficient transferrin, are noted, but the exact biochemical and molecular defects have not yet been identified, and prenatal diagnosis is not yet available (*4*).

In recent years, several nucleotide-sugar transporter cDNAs have been molecularly cloned and their protein products have been characterized (*5*). Among these are the cDNAs of the UDP-Gal transporter and its isozyme, the CMP-Sia transporter and the UDP-GlcNAc transporter from mammalian species (*6-11*), the GDP-Man transporter from *Leishmania donovani* and *Saccharomyces cerevisiae* (*12, 13*), and the UDP-GlcNAc transporter from *Kluyveromyces lactis* (*14*). The gene locus of the UDP-Gal transporter from *Schizosaccharomyces pombe* was also identified (*15*). In addition, several related genes from humans, *S. cerevisiae*, and *Caenorhabditis elegans* have been reported based on similarity search of gene databases (*5, 6, 12, 14*). Only two human nucleotide-sugar transporters, namely, human UDP-Gal transporter and human CMP-Sia transporter, have been characterized so far in molecular and immunochemical detail (*6-8, 16*). Information concerning the molecular and immunochemical details of the individual human nucleotide-sugar transporters is indispensable if we are to define the significance of the nucleotide-sugar transporters in CDG syndrome and to have tools for its genetic diagnosis. We have therefore been continuing efforts to isolate and characterize yet unidentified human nucleotide-sugar transporter genes.

The structural basis of nucleotide-sugar transport has currently become a matter of intensive inquiry based on

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Abbreviations: CDG, carbohydrate-deficient glycoprotein; CHO, Chinese hamster ovary; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HA, hemagglutinin; mAb, monoclonal antibody; Man, mannose; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; Sia, sialic acid; TBS, Tris-buffered saline; UGT, UDP-galactose transporter.

successful molecular cloning of several nucleotide-sugar transporters (*5*). Comparison of the primary structures among nucleotide-sugar transporters would yield valuable information for elucidation of such critical issues as their domain structure, topology, and determinants of substrate specificity. Alignment of the amino acid sequences of nucleotide-sugar transporters for various substrates will reveal residues and submolecular regions important in common for the general structure and transport function of the nucleotide-sugar transporters. Alignment of nucleotide-sugar transporters for a particular nucleotide-sugar from various species will highlight substrate-specific residues. This is another reason why we have been attempting to characterize new members of the nucleotide-sugar transporter family.

In this report, we describe cloning of a novel human nucleotide-sugar transporter gene related to UDP-Gal transporter, and demonstrate that the transporter is responsible for UDP-GlcNAc transport in the Golgi apparatus.

MATERIALS AND METHODS

Materials—The radioactive substrates UDP-[6-^{3}H]-*N*-acetyl-D-glucosamine (34.8 Ci/mol), UDP-[4,5-^{3}H]-galactose (37.9 Ci/mol), and CMP-[9-^{3}H]-sialic acid (33.2 Ci/mol) were purchased from NEN Life Science Products and GDP-[2-^{3}H]-mannose (15 Ci/mol) was purchased from American Radiolabeled Chemicals.

Cell Culture—Chinese hamster ovary (CHO) K1 cells were maintained in α-minimum essential medium (MEM) (Life Technologies, Gaithersberg, MD) supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator. Transfection with expression vectors was carried out using LipofectAMINE reagent (Life Technologies), following the manufacturer's instructions.

Fluorescence In Situ Hybridization (FISH)—*In situ* chromosomal hybridization was performed by SeeDNA Biotech (York University, Ontario, Canada) as described (*17, 18*). The location of the hUGTrel2/human UDP-GlcNAc transporter gene was assigned using lymphocytes isolated from human blood and a 1.5-kbp cDNA fragment from IMAGE clone 138128 (Fig. 1) as a probe. The lymphocyte cultures were treated with 5-bromodeoxyuridine to synchronize the cells. The probe was biotinylated with dATP using a BioNick labeling kit (Life Technologies), and then hybridized to chromosomal spreads overnight in a hybridization mix consisting of 50% formamide and 10% dextran sulfate. FISH signals and the 4′,6-diamidino-2-phenylindole (DAPI) banding patterns were recorded separately by making photomicrographs, and the chromosomal location of the gene was determined by superimposing FISH signals with DAPI-banded chromosomes according to Heng and Tsui (*18*).

Northern Blot Analysis—Hybridization with human multiple tissue Northern blots (Clontech, Palo Alto, CA) was carried out as recommended by the manufacturer. Human β-actin cDNA was obtained from Clontech. Radioactivity was detected using Fuji BAS2000 (Fuji Photo Film, Tokyo).

Yeast Strain and Culture Conditions—The nucleotide-sugar transporting activity was examined in *S. cerevisiae* YPH500 cells (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*). Transformation was carried out according to the method described by Ito *et al.* (*19*). Transformants were selected on a selective medium containing 0.67% (w/v) bacto-yeast nitrogen base without amino acids, 2% glucose (YNBD), and auxotrophic supplements except uracil. For induction experiments, transformants were grown in liquid selective medium until they reached a density of 0.6 A_{660}. Cupric sulfate was added to the medium at a final concentration of 2.0 mM. The cells were further cultured for 2 h at 30°C with shaking at 400 rpm, and then harvested.

Preparation of Yeast Membrane Vesicles—Membrane vesicles were prepared essentially as previously described (*20*). Cells were harvested, washed once with ice-cold 10 mM NaN_3 and weighed. The cells were resuspended in five volumes of a spheroplast solution containing 1.4 M sorbitol, 50 mM potassium phosphate (pH 7.5), 10 mM NaN_3, 40 mM β-mercaptoethanol, and 2 mg of zymolyase-100T (Seikagaku, Tokyo) per g of cells, and incubated at 37°C for 20 min. The spheroplasts were collected, and resuspended in four volumes of lysis buffer containing 10 mM triethanolamine acetate (TEA) (pH 7.2), 0.8 M sorbitol, 1 mM EDTA, a protease inhibitor cocktail (Complete; Roche Diagnostics, Basel, Switzerland), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (*p*-APMSF), and homogenized with eight strokes of mechanical shear using a teflon homogenizer. The lysate was then centrifuged at 2,300 × *g* for 10 min to remove unlysed cells and debris. The supernatant was centrifuged at 10,000 × *g* for 10 min to remove larger membrane structures derived from vacuoles, plasma membrane, endoplasmic reticulum, mitochondria and nuclei. The Golgi-enriched membrane vesicles were collected by centrifugation at 100,000 × *g* for 60 min. The nucleotide-sugar transport assay was carried out as described previously (*20*).

Anti-Peptide Antibody—A peptide corresponding to the 17 amino acid residues from the hUGTrel2 C-terminus (Fig. 2) was synthesized, and rabbit antiserum against the peptide conjugated with keyhole limpet hemocyanin was prepared as described (*8*). The IgG fraction was prepared from the crude antiserum on an immobilized protein A column (Pierce, Rockford, IL) according to the manufacturer's instructions. Specific antibodies were enriched from the IgG fraction by affinity chromatography using a column of AF-Amino Toyopearl 650 (Tosoh, Tokyo) coupled with the antigen peptide as described (*16*).

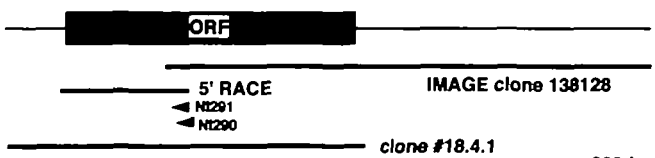

Fig. 1. **Cloning of the hUGTrel2/human UDP-GlcNAc transporter cDNA.** The shaded box and the thin lines denote the open reading frame and the 5′ and 3′ untranslated regions of hUGTrel2/human UDP-GlcNAc transporter, respectively. The thick bars indicate the regions covered by the IMAGE clone 138128 identified by BLAST search through dbEST, a cDNA fragment obtained by 5′ RACE, and the clone #18.4.1 isolated from a human liver cDNA library. The positions of gene-specific primers, NI290 and NI291, are also indicated.

A

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-197                                                    CCGCGGGAACCCGGAAA   -181
-180 CGCCTGGGCTGCACCGAGCTGGCGGTGGCTACGGCGACGGGAGCCGGCCGGCGCTGCGGGT   -121
-120 CAGCGGTCGCGTAGGACCCAGCGGACTCGGCAGCCTGGGGCGCCCGGCGGAGCTGAACCG    -61
 -60 CGGCCCCCGGTGGTGGGCTCAGCCGGTCGAGCTGCGCGGGAGGCAAATGAAGATAAAACA     -1

   1 ATGTTCGCCAACCTAAAATACGTTTCCCTGGGAATTTTGGTCTTTCAGACTACCAGTTTG     60
   1 MetPheAlaAsnLeuLysTyrValSerLeuGlyIleLeuValPheGlnThrThrSerLeu     20

  61 GTTCTAACAATGCGTTATTCCAGAACTTTAAAAGAAGAAGGACCTCGTTATCTATCTTCT    120
  21 ValLeuThrMetArgTyrSerArgThrLeuLysGluGluGlyProArgTyrLeuSerSer     40

 121 ACAGCAGTGGTTGTTGCTGAACTTTTGAAGATAATGGCCTGCATTTTATTGGTCTACAAA    180
  41 ThrAlaValValValAlaGluLeuLeuLysIleMetAlaCysIleLeuLeuValTyrLys     60

 181 GACAGCAAATGTAGTCTAAGAGCACTGAATCGAGTACTACATGATGAAATTCTTAATAAA    240
  61 AspSerLysCysSerLeuArgAlaLeuAsnArgValLeuHisAspGluIleLeuAsnLys     80

 241 CCTATGGAAACACTTAAACTTGCTATTCCATCAGGGATCTATACTCTTCAGAATAATTTA    300
  81 ProMetGluThrLeuLysLeuAlaIleProSerGlyIleTyrThrLeuGlnAsnAsnLeu    100

 301 CTGTATGTGGCACTATCAAATCTAGATGCAGCTACTTATCAGGTCACGTATCAGTTAAAA    360
 101 LeuTyrValAlaLeuSerAsnLeuAspAlaAlaThrTyrGlnValThrTyrGlnLeuLys    120

 361 ATTCTTACAACAGCATTATTTTCTGTGTCTATGCTTAGTAAAAAATTGGGTGTATACCAG    420
 121 IleLeuThrThrAlaLeuPheSerValSerMetLeuSerLysLysLeuGlyValTyrGln    140
                   C
 421 TGGCTGTCCCTAGTAATTTGATGACAGGAGTTGCTTTTGTACAGTGGCCCTCAGATTCT    480
 141 TrpLeuSerLeuValIleLeuMetThrGlyValAlaPheValGlnTrpProSerAspSer    160

 481 CAGCTTGATTCTAAGGAACTTCAGCTGGTTCTCAATTTGTAGGACTCATGGCAGTTCTC    540
 161 GlnLeuAspSerLysGluLeuSerAlaGlySerGlnPheValGlyLeuMetAlaValLeu    180

 541 ACAGCATGTTTTTCAAGTGGCTTTGCTGGGGTTTACTTTGAGAAAATCTTAAAAGAAACA    600
 181 ThrAlaCysPheSerSerGlyPheAlaGlyValTyrPheGluLysIleLeuLysGluThr    200

 601 AAACAATCAGTGTGGATAAGAAATATTCAGCTTGGTTTCTTTGGAAGTATATTTGGATTA    660
 201 LysGlnSerValTrpIleArgAsnIleGlnLeuGlyPhePheGlySerIlePheGlyLeu    220

 661 ATGGGTGTATACATTTATGATGGAGAACTGGTATCAAAGAATGGATTTTTTCAGGGATAT    720
 221 MetGlyValTyrIleTyrAspGlyGluLeuValSerLysAsnGlyPhePheGlnGlyTyr    240

 721 AACCGACTGACCTGGATAGTAGTTGTTCTTCAGGCACTTGGAGGCCTTGTAATAGCTGCT    780
 241 AsnArgLeuThrTrpIleValValValLeuGlnAlaLeuGlyGlyLeuValIleAlaAla    260

 781 GTTATTAAGTATGCAGATAATATTTTAAAAGGATTTGCAACCTCTTTATCGATAATATTA    840
 261 ValIleLysTyrAlaAspAsnIleLeuLysGlyPheAlaThrSerLeuSerIleIleLeu    280

 841 TCAACATTGATCTCCTATTTTTGGCTTCAAGATTTTGTGCCAACCAGTGTCTTTTTCCTT    900
 281 SerThrLeuIleSerTyrPheTrpLeuGlnAspPheValProThrSerValPhePheLeu    300

 901 GGAGCCATCCTTGTAATAACAGCTACTTTTTTGTATGGTTATGATCCCAAACCTGCAGGA    960
 301 GlyAlaIleLeuValIleThrAlaThrPheLeuTyrGlyTyrAspProLysProAlaGly    320

 961 AATCCCACTAAAGCATAGTTGTATACTATCTTTAACTGGTTTTTCACGATGGGGCACTAG   1020
 321 AsnProThrLysAla***

1021 GAATCTCGACATTAATCTTGCACAGAGGACTTCTACAGAGTCTGAGAAGATATCATCATG   1080
1081 CTGAATCTGATCATACTGTTTTTTAAAAGTTTAAGGATAAGACATGTGTATATGTAACAA   1140
1141 AACACATTGCATCTAGAAATCAAAACTTGAAAGTATTTCCAGGGATTAGGATTAGAAGGA   1200
1201 ATATTAGAGGAAACTTGAAATCTGAGTTTAAAAAGATTTTACCTTTTTGATTGCTGCAGA   1260
1261 AATGTCCTATGCACTCTTTGCAAGAGCACACAACAAATGTCAGATACCAATTTTTGCAAA   1320
1321 TTAGATTTAATCTTATTAAATGTTTTTATCTTACTCTTTCTGTACAGATATATCAAATCA   1380
1381 CATGAAATATTTAAAGTTTGAAAATTATAATTACCTATAAAGCTGTGAAAAATAGAAGTA   1440
1441 TAATTTGAAAAAACATTTCACTTATCAGAGATTTTTATATTTATACAAAAGATTACTAAA   1500
1501 TGAAGGATTGCTAAATGTTTTTGGTTCAATTACATAAAAATTAATATTCTGGGTCTGATC   1560
1561 TGTCAGAGAATAAATATCAAATCTAAATTTAATGTAGAGATACATACTATTTCTCCATAT   1620
1621 GAATTTTAAGATATTTTAGTGCTTCAAGACTGCTGAAAGCAATCCAGTTGCTCCTGTGCT   1680
1681 AGATGGTAGCCAGAGAATTTTATAGTAATGGAGGTTAGCCCTTAATCTCTTCATTGCATT   1740
1741 TCATTTCTGTAAATCAGATTAAGTCCTTAATATTATTTTAAATTAAAATTTGTGTGTAAT   1800
1801 TGCCATTAAATTTTCAAAATGTAATTTAAAAGGATTAAATACTCATTTAATAATTTAAAA   1860
1861 TAATTATTGTATAATATCTACATTTGGAGAATTTTGAACTATCAAGCATATACTGTATAC   1920
1921 AGTTAGAAAGTTATTAAATGAACATTTTACTCAAAAAAAAAAAAAAAAAAA            1971
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B

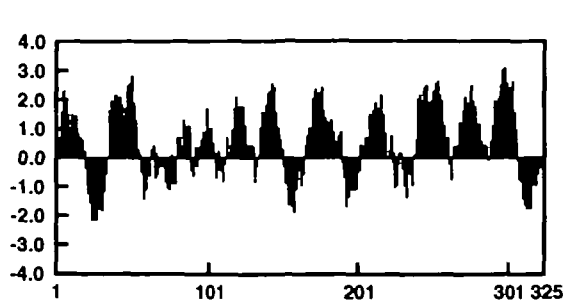

Fig. 2. **Sequence analysis of the hUGTrel2/human UDP-GlcNAc transporter.** (A) Nucleotide and deduced amino acid sequences of the hUGTrel2/human UDP-GlcNAc transporter. The GenBANK/EMBL/DDBJ accession number of the nucleotide sequence is AB021981. An allelic variation, T439C, found in IMAGE clone 138128 is also indicated above the replaced T residue. A putative poly(A) addition signal is underlined. (B) Hydrophobicity plot of the hUGTrel2/human UDP-GlcNAc transporter. The plot was calculated with a window size of nine amino acids using the hydrophobicity values of Kyte and Doolittle (*22*).

Preparation of Cells for Western Blot Analysis—CHO-K1 and vector-transfected cells were trypsinized and washed with PBS. The packed cell volume was estimated from the wet weight of the cell pellets. The cells were resuspended in two volumes of lysis buffer containing 10 mM HEPES-Tris (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 0.2% NP-40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The suspensions were vortexed six times for 10 s, and centrifuged for 1 min at 5,000 rpm at 4°C. The supernatants were recovered for analysis.

Western Blot Analysis—The samples were fractionated on a 12% SDS-PAGE gel and then electrotransferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk and 0.2% Tween 20 in Tris-buffered saline (TBS), consisting of 20 mM Tris-HCl (pH 7.6) and 0.14 M NaCl, the membrane was incubated with the hUGTrel2 antibodies or rat anti-HA monoclonal antibody (mAb) 3F10 (Roche Diagnostics) at room temperature for 1 h. The binding of the antibodies was detected by chemiluminescence using HRP-anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or HRP-anti-rat IgG antibodies (Santa Cruz Biotechnology) with Renaissance-plus reagents (NEN Life Science Products, Boston, MA). Biotinylated SDS-PAGE standards (low molecular weight range) (Bio-Rad, Hercules, CA) were used as molecular weight standards.

Immunofluorescence—Cells were transiently transfected with appropriate expression vectors using LipofectAMINE (Life Technologies) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were transferred onto a Lab-Tek 8-well chamber glass slide (Nalge Nunc International, Naperville, IL), and grown for another 24 h. Indirect immunofluorescence analysis was carried out as described previously (*8*). Fluorescence labeling was visualized under a Carl Zeiss laser scanning confocal microscope LSM510.

RESULTS

Molecular Cloning of the Human UDP-Gal Transporter Related Isozyme 2 (hUGTrel2)/Human UDP-GlcNAc Transporter—We previously found several UDP-Gal transporter-related genes, utilizing the expressed sequence tags data base (dbEST). The human CMP-Sia transporter and the human, murine, and rat UDP-Gal transporter related isozyme 1 (UGTrel1) cDNAs were thus found, and then isolated and characterized (*7, 8*). IMAGE clone 138128 (Fig. 1) was similarly found through a BLAST search of the dbEST by the procedure described previously (*7*). The partial amino acid sequence deduced from the clone's nucleotide sequence showed significant similarity with that of the human UDP-Gal transporter. We therefore tentatively named the gene "human UDP-Gal transporter related isozyme 2" (hUGTrel2), which turned out to be the human UDP-GlcNAc transporter, as shown later in this report. 5′ RACE was carried out using primers NI290 and NI291, which were specific for the hUGTrel2 gene, and partial cDNA fragments were obtained (Fig. 1). Clone #18.4.1 (Fig. 1) was isolated by screening a human liver

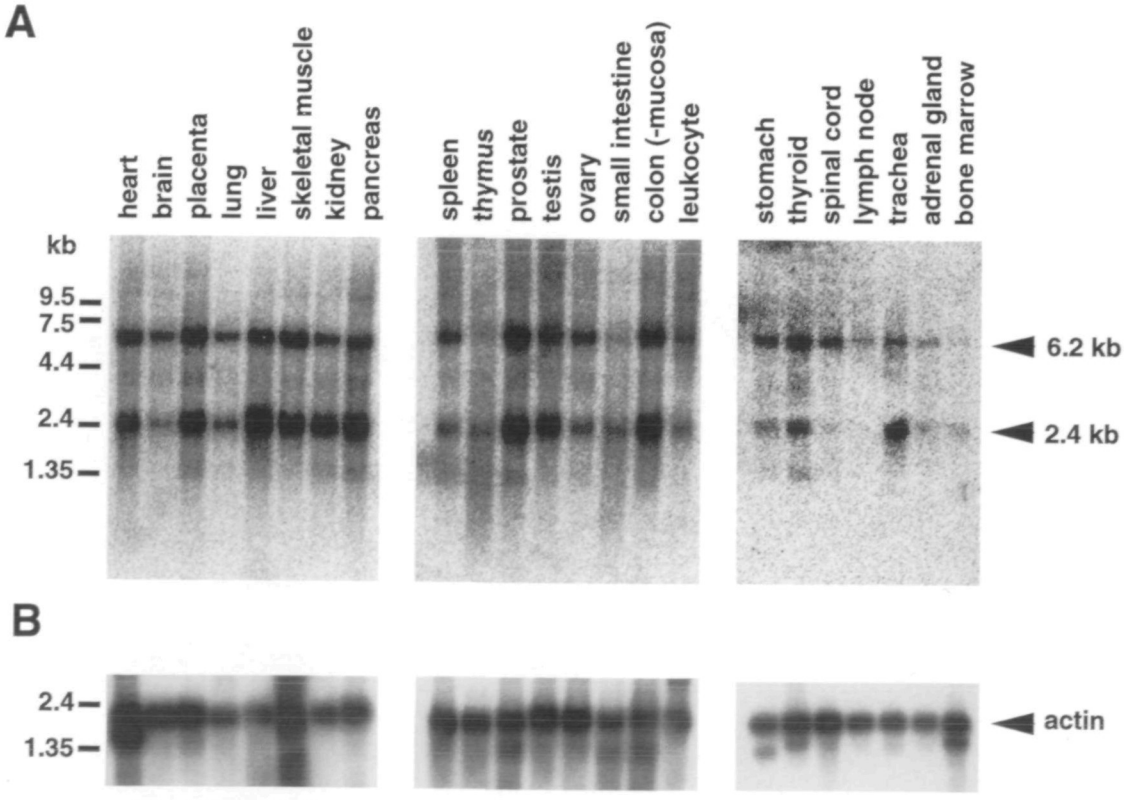

Fig. 3. **Northern blot analysis of the hUGTrel2/human UDP-GlcNAc transporter in various tissues.** (A) Each membrane was hybridized with a radiolabeled cDNA fragment encoding the entire ORF, which was amplified by PCR from clone #18.4.1. (B) After the remaining radioactivity was stripped off from the membranes, hybridization with human β-actin cDNA was carried out.

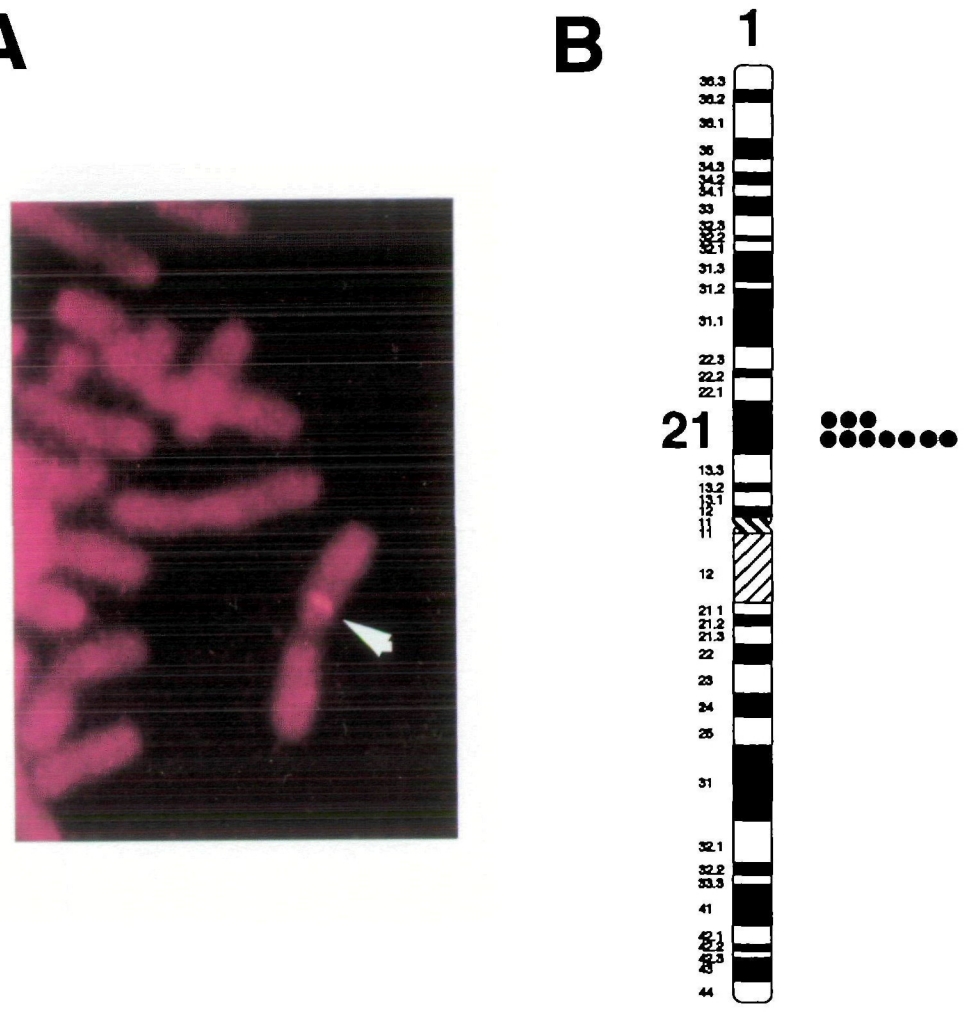

Fig. 4. **Determination of the chromosomal location of the hUGTrel2/human UDP-GlcNAc transporter gene.** (A) A representative result of FISH analysis. The arrowhead indicates the positive signal. (B) Summary of superimposition on DAPI banding images.

cDNA library with a 5′ RACE cDNA fragment as a probe. Clone #18.4.1 covered a complete open reading frame (ORF) that consisted of 325 amino acids (Fig. 2). The deduced amino acid sequence of hUGTrel2 was very similar to that of human UDP-Gal transporter (54% identity over the entire overlapping region). During the cloning process, we found an allelic variation, that is, TTG for Leu147 in clone #18.4.1 *versus* CTG in clone 138128. The mutation, T439C at the nucleotide level, was silent, as in the case of the human CMP-Sia transporter (*8*).

Tissue Distrubution of hUGTrel2/Human UDP-GlcNAc Transporter mRNA—Northern blot analysis revealed two hUGTrel2/human UDP-GlcNAc transporter mRNA species, 2.4 kb and 6.2 kb long, respectively (Fig. 3). This suggests that there exist at least two species of isozymes for the hUGTrel2/human UDP-GlcNAc transporter. Judging from the structure of the hUGTrel2 cDNA, it is likely that the cDNA cloned corresponds to the 2.4 kb-species. The expression of both mRNA species was ubiquitous in all the tissues examined (Fig. 3).

Chromosomal Assignment of the hUGTrel2/Human UDP-GlcNAc Transporter Gene—To determine the chromosomal location of the hUGTrel2 gene, we performed FISH analysis as described in "MATERIALS AND METHODS." Among 100 mitotic figures, 75 showed hybridization

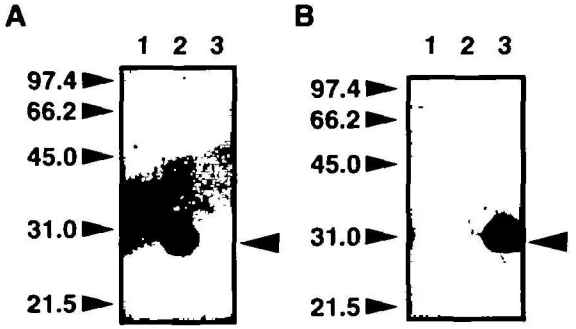

Fig. 5. **Expression of hUGTrel2/human UDP-GlcNAc transporter proteins.** CHO-K1 cells were transfected with pMKIT-neo control vector (lane 1), pMKIT-neo-hUGTrel2 (lane 2), or pMKIT-neo-hUGTrel2cHA (lane 3). Forty-eight hours after transfection, the cell lysates were prepared as described in "MATERIALS AND METHODS." Western blot analysis was carried out using 40 μg of protein of each lysate. The membranes were treated with anti-hUGTrel2 rabbit polyclonal antibody (1:400) (A) or anti-HA rat mAb 3F10 (1:50,000) (B), and then with HRP-conjugated anti-rabbit IgG antibody (1:10,000) or HRP-conjugated anti-rat IgG antibody (1:20,000), respectively.

signals on chromosome 1. A representative result is shown in Fig. 4A. Assignment in further detail was achieved by superimposing the FISH signals on the DAPI-banded chromosome, and the gene was thereby mapped to human chromosome 1p21 (Fig. 4B).

Expression of the hUGTrel2/Human UDP-GlcNAc Transporter in CHO Cells—The hUGTrel2 cDNA and a C-terminally HA-epitope-tagged hUGTrel2 cDNA were introduced into the mammalian expression vector pMKIT-neo. The HA-epitope tag was introduced by replacing the termination (*) codon with a coding sequence for "YPYDVPDYA*" by PCR. Addition of the C-terminal HA-tag and construction of these expression vectors were carried out as described previously (*6, 8*). The constructs were transiently expressed in CHO-K1 cells, and the cDNA products were analyzed by immunoblotting, using the rabbit anti-hUGTrel2 C-terminal peptide polyclonal antibody or anti-HA mAb 3F10. The results obtained are shown in Fig. 5. The anti-hUGTrel2 C-terminal peptide antibody was specific for the intact C-terminus (Fig. 5A, lane 2), and did not recognize the cognate peptide in the modified C-terminus with an attached HA-epitope tag (Fig. 5A, lane 3). The apparent molecular masses of the intact and the epitope-tagged hUGTrel2 proteins were 29.5 kDa (Fig. 5A, lane 2) and 30.5 kDa (Fig. 5B, lane 3), respectively.

Subcellular Localization of the hUGTrel2/Human UDP-GlcNAc Transporter—Transporting activities for a number of nucleotide-sugars are mainly associated with Golgi membranes. In fact, the human UDP-Gal transporter and the human CMP-Sia transporter cDNA products expressed in cultured cells are targeted to the Golgi apparatus (*8, 16*). To investigate whether the hUGTrel2 protein also associates with the Golgi apparatus, the cDNA was transiently expressed in CHO cells. Forty-eight hours after transfection, the localization of the hUGTrel2 protein was determined by indirect immunofluorescence using the anti-hUGTrel2 peptide antibody. The hUGTrel2 proteins were clearly localized in the hUGTrel2-expressing cells to perinuclear compartments which most likely represented the Golgi apparatus (Fig. 6). To conclusively identify the

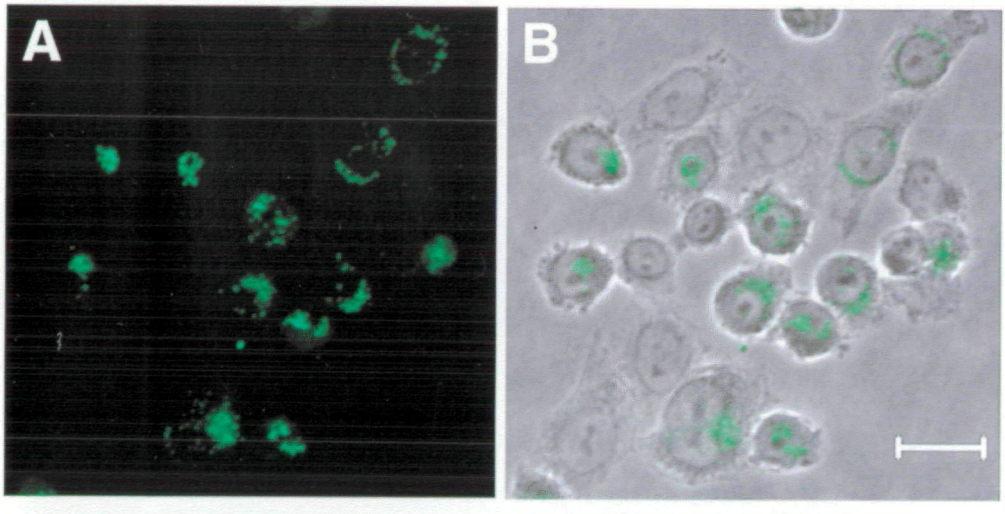

Fig. 6. **Subcellular localization of the hUGTrel2/human UDP-GlcNAc transporter.** CHO-K1 cells were transiently transfected with pMKIT-neo-hUGTrel2, encoding hUGTrel2/human UDP-GlcNAc transporter. Forty-eight hours after transfection, cells were fixed and permeabilized in cold methanol, and analyzed by indirect immunofluorescence using rabbit anti-hUGTrel2 polyclonal antibody. Bound primary antibodies were visualized with anti-rabbit IgG-fluorescein isothiocyanate conjugate (A), and then the image was merged with the phase-contrast view (B). Bar, 20 μm.

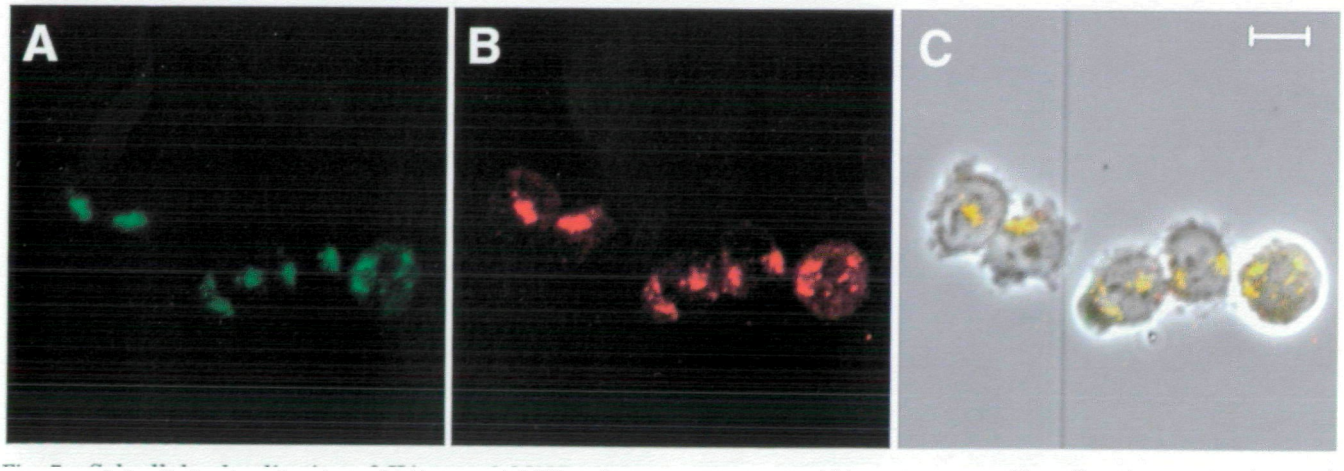

Fig. 7. **Subcellular localization of HA-tagged hUGTrel2/human UDP-GlcNAc transporter.** CHO-K1 cells were transfected with pMKIT-neo-hUGTrel2cHA. Forty-eight hours after transfection, the cells were fixed and permeabilized with cold methanol. Analysis by indirect immunofluorescence was performed using anti-HA rat mAb 3F10 and rabbit anti-α-mannosidase II antiserum simultaneously. Bound primary antibodies were visualized with anti-rat IgG-fluorescein isothiocyanate (A) and anti-rabbit IgG-Cy3 (B) conjugates. The images A and B were also merged with the phase-contrast view (C). Bar, 10 μm.

targeting sites of the hUGTrel2 product, the HA-tagged construct was transiently expressed in CHO cells and indirect immunofluorescence was carried out using anti-HA mAb 3F10 (Fig. 7A). The cells were simultaneously stained with an antiserum directed against α-mannosidase II, a marker protein for the Golgi apparatus (Fig. 7B). The HA-tagged hUGTrel2 products were distributed in the same way as the intact hUGTrel2 products (Figs. 6A and 7A), and were closely co-localized with α-mannosidase II (Fig. 7C). These results clearly indicate that the hUGTrel2 products are targeted to the Golgi apparatus.

Determination of the Transporter Substrate—In a recent report, we demonstrated that the human Golgi UDP-Gal transporters were heterologously expressed in *S. cerevisiae*, and that a significant portion of the transporter protein was targeted to the Golgi region in a functionally active form (*20*). This heterologous functional expression system was utilized to identify the nucleotide-sugar transported by the hUGTrel2 products. The hUGTrel2 cDNA was introduced into a yeast inducible expression vector, pYEX-BX (Clontech). The construct, pYEX-BX-hRel2-3HA, represents a copper-inducible expression vector for hUGTrel2 tagged by three repeated HA-epitopes attached to its C-terminus. The plasmid was transfected into yeast strain YPH500, and a transformant was obtained. After induction by copper, the microsomal fraction was prepared from the transformant. Western blot analysis using an anti-HA antibody revealed that a product of the expected size was produced from the introduced gene (Fig. 8, lane 2). The apparent molecular mass of 33.0 kDa agreed well with that expected for a molecule with three HA tag epitopes added to the native hUGTrel2 product (29.5 kDa). Membrane fractions enriched in Golgi-derived vesicles were prepared from *S. cerevisiae* YPH500 transformed with the vectors with and without hUGTrel2-3HA, and were investigated for their ability to transport various nucleotide-sugars. As expected, vesicles from both kinds of cells had similar endogenous GDP-mannose transport activity (Fig. 9, A and B, column 1), while they did not transport a significant amount of UDP-Gal or CMP-Sia (Fig. 9, A and B, columns 2 and 3). YPH500 transformed with pYEX-BX-

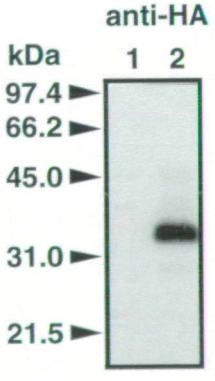

Fig. 8. **Expression of the HA-tagged hUGTrel2/human UDP-GlcNAc transporter in yeast.** The Golgi-enriched membrane vesicles were prepared from yeast carrying the expression vector pYEX-BX-3HA without/with hUGTrel2 cDNA (lanes 1 and 2, respectively). Western blot analysis was performed with 20 μg of protein of the membrane vesicles, as described in "MATERIALS AND METHODS." The blot was probed with anti-HA mAb 3F10 (1:50,000) as the primary antibody, and then treated with HRP-conjugated anti-rat IgG antibody (1:20,000).

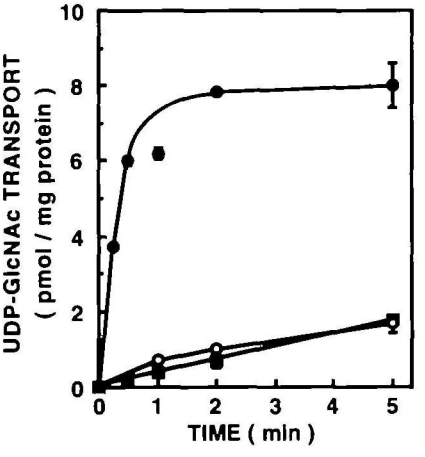

Fig. 10. **Time and temperature dependence of UDP-GlcNAc transport by the hUGTrel2 product.** Microsomal vesicles (50 μg protein/assay) were prepared and UDP-GlcNAc transport was assayed as described (*20*). Vesicles from YPH500/pYEX-BX-3HA (solid squares) and from YPH500/pYEX-BX-hRel2-3HA (solid circles) were incubated at 30°C. Incubation at 0°C was also carried out using vesicles from YPH500/pYEX-BX-hRel2-3HA (open circles). The amount of radioactivity retained on the filter at 0°C for 0 min of incubation (background) was subtracted from the corresponding experimental values.

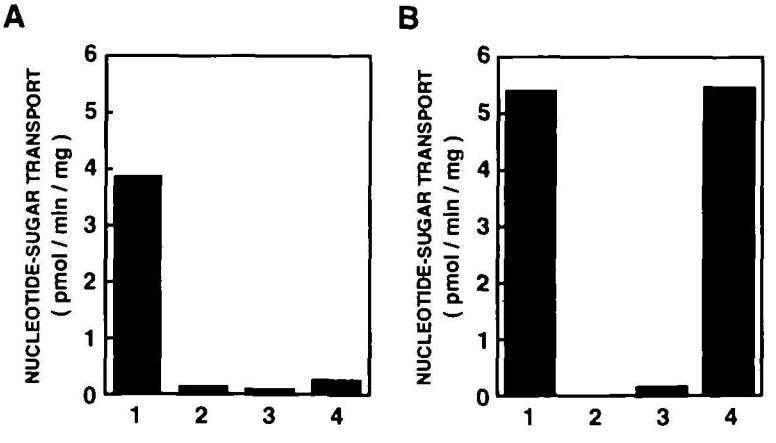

Fig. 9. **Nucleotide-sugar transport by the hUGTrel2/human UDP-GlcNAc transporter.** Microsomal vesicles (50 μg protein/assay) were prepared from YPH500/pYEX-BX-3HA (A) and YPH500/pYEX-BX-hRel2-3HA (B) (hRel2 denotes hUGTrel2). The amount of radioactivity retained on the filter at 0°C for 0 min incubation (background) was subtracted from the corresponding experimental values. Radioactive substrates used were: 1, GDP-mannose; 2, CMP-sialic acid; 3, UDP-galactose; 4, UDP-*N*-acetylglucosamine.

3HA without the hUGTrel2 insert similarly lacked UDP-GlcNAc transporting activity. In contrast, the membrane vesicles obtained from YPH500/pYEX-BX-hRel2-3HA cells expressing the hUGTrel2 product were highly active in transporting UDP-GlcNAc (Fig. 9, A and B, column 4). Based on these results, we concluded that the hUGTrel2 product is the human Golgi UDP-GlcNAc transporter. As shown in Fig. 10, the activity was time- and temperature-dependent, and was decreased to the background level at 0°C.

DISCUSSION

In this report, we have described the molecular cloning and characterization of human Golgi UDP-GlcNAc transporter. The hUGTrel2/human UDP-GlcNAc transporter cDNA encodes a highly hydrophobic, multiple-membrane-spanning protein with significant similarity to human UDP-Gal and human CMP-Sia transporters. The transporter was expressed ubiquitously in every tissue so far examined (Fig. 3). Expression of the human CMP-Sia and UDP-Gal transporters was similarly ubiquitous (*8*, and Ishida, N. *et al.*, unpublished observations). This is to be expected since these nucleotide-sugar transporters are involved in the delivery of fundamental constituents of glycoconjugates to the active sites of glycosyltransferases responsible for glycoconjugate synthesis. The UDP-GlcNAc transporter, as well as its derivative with a C-terminal HA-epitope tag, were correctly targeted to the Golgi apparatus in CHO cells transfected with the respective cDNAs. The UDP-GlcNAc-transporting activity was convincingly demonstrated by using a heterologous functional expression system in the budding yeast *S. cerevisiae*. The yeast provided us with a zero-background host to demonstrate clearly that it acquired the activity to selectively transport UDP-GlcNAc depending strictly upon the expression of the products of the hUGTrel2/human UDP-GlcNAc transporter cDNA. While our studies on hUGTrel2/human UDP-GlcNAc transporter were in progress, the cloning of the canine UDP-GlcNAc transporter cDNA, which is closely similar in structure to the human counterpart (Fig. 11), was reported by Guillen *et al.* (*11*). Our work on the human CMP-Sia transporter demonstrated previously that the orthologous mammalian transporters for a common nucleotide-sugar substrate show high degrees of identity with each other (*7, 8*). The close similarity between the human and canine UDP-GlcNAc transporters is consistent with this observation.

Figure 11 shows a comparison of the amino acid sequences of human (*6*) and fission yeast (*23*) UDP-Gal transporters, human (*7*) and murine (*9*) CMP-Sia transporters, and human and canine (*11*) UDP-GlcNAc transporters. In this alignment, there is inter-species conservation of 10 residues (enclosed by open boxes) among the transporters of a common nucleotide-sugar substrate, but are divergent among transporters with different substrate specificities. We tentatively call these 10 residues "substrate-specific" residues in the present discussion.

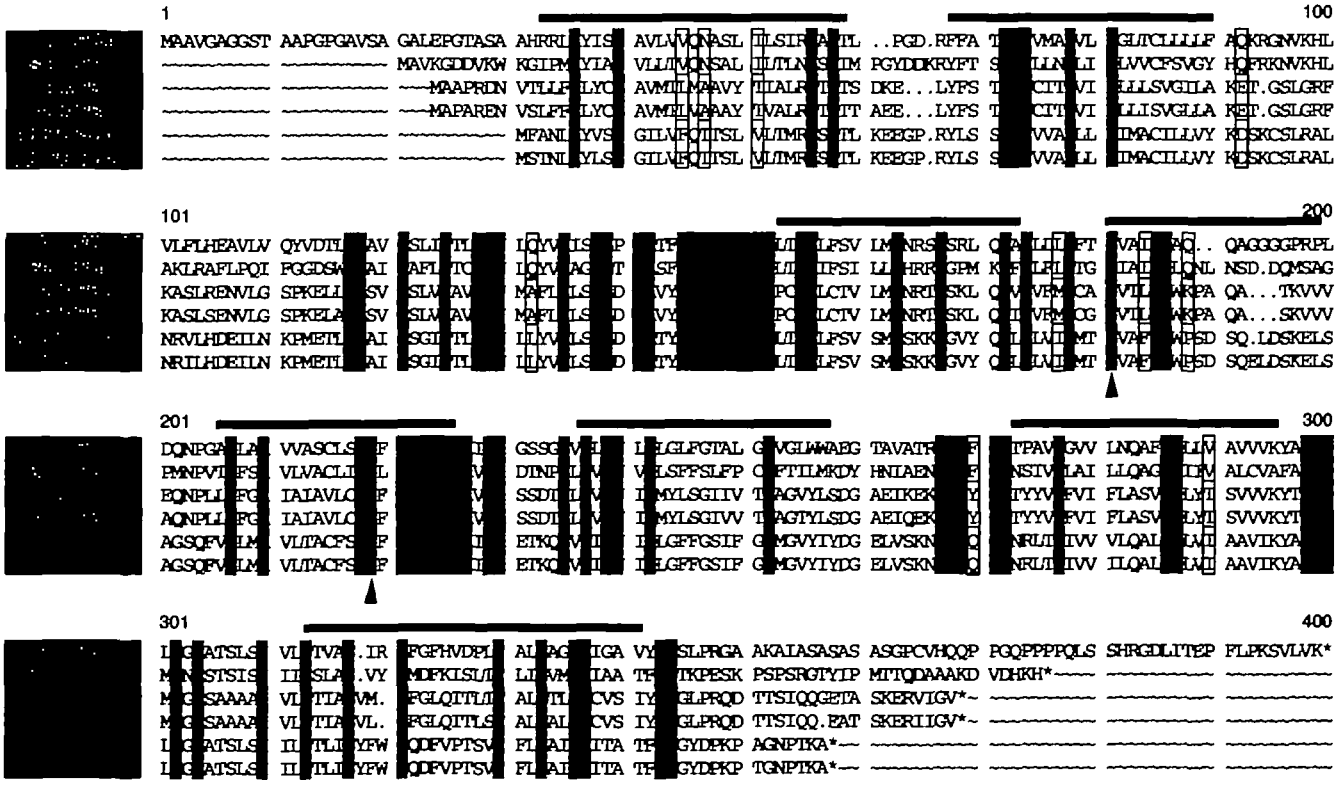

Fig. 11. **Alignment of UDP-Gal, CMP-Sia, and UDP-GlcNAc transporter sequences; conserved residues and substrate-specific residues.** For details, see the text. hUDPGalTr1, human UDP-Gal transporter 1 (GenBank Accession Number D84454); SpUDPGalTr, *Schizosaccharomyces pombe* UDP-Gal transporter (AB023425, see the text); hCMPSiaTr, human CMP-Sia transporter (D87969); mCMPSiaTr, murine CMP-Sia transporter (Z71268); hUDPGlcNAcTr, human UDP-GlcNAc transporter (this work, AB021981); cUDPGlcNAcTr, canine UDP-GlcNAc transporter (AF057365); thick bar, putative transmembrane helix.

A cluster of three substrate-specific residues is found in the most N-terminus-proximal putative transmembrane helix. This is interesting in view of the fact that the replacement of this segment of the human UDP-Gal transporter with the corresponding one of the human CMP-Sia transporter led to the loss of the UDP-Gal transporting activity (Aoki, K. *et al.*, manuscript in preparation). It should also be noted that we found very recently that the functional fission yeast UDP-Gal transporter is considerably larger than the one reported (*15*), and its newly identified extension in the N-terminal region shows a high degree of similarity to the N-terminal portion of the human UDP-Gal transporter (*23*). The sequence shown in Fig. 11 includes this N-terminal portion which was not previously recognized.

Of particular interest is the region from the 172nd to the 188th residues (on the alignment scale in Fig. 11) with scattered substrate-specific (enclosed in the open boxes) and conserved (shaded) residues. It is certain that the conserved Gly residue at the 181st position (arrowhead), which is located in the middle of this region, is functionally important, since its mutation to Asp in the Had-1m mutant leads to inactivation of the murine UDP-Gal transporter (Ishida, N. *et al.*, manuscript in preparation). The conserved as well as the substrate-specific residues neighboring the 181st Gly residue are also likely to be intimately involved in the transporter function, including the selection of a specific substrate.

A Gly residue whose mutation to Glu led to the inactivation of the Chinese hamster CMP-Sia transporter (*21*) is also conserved among these transporters (arrowhead at the 219th position). The stretch of 13 (or perhaps 25) amino acid residues which extends from Ser218 toward the C-terminus, including this Gly residue, is very well conserved among the transporters aligned in Fig. 11. This stretch, as well as another highly conserved stretch extending from Ala135 to Ala156, may contribute to the transport reaction as a part of the general transport device. It is of interest to test whether these segments are interchangeable among the transporters.

It seems highly likely that the substrate-specific residues are involved in the determination of the substrate specificity of the transporter. However, such substrate-specific residues are rather scattered on the linear sequence and may be interacting with each other. Disruption of such interactions as a result of swapping among these residues may often simply result in a loss of transporting activity rather than alteration of the substrate specificity. For this reason, the significance of these residues as possible determinants of the substrate specificity of the transporters will have to be addressed by systematic site-directed mutagenesis in future studies.

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