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Indispensability of Transmembrane Domains of Golgi UDP-Galactose Transporter as Revealed by Analysis of Genetic Defects in UDP-Galactose Transporter-Deficient Murine *Had-1* Mutant Cell Lines and Construction of Deletion Mutants[1]

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UDP-galactose transporter is a membrane protein localized in the Golgi apparatus. It translocates UDP-galactose from the cytosol into the Golgi lumen, thus providing galactosyltransferases with their substrate. We characterized murine UDP-galactose transporter through molecular cloning for the following purposes: (i) to elucidate the molecular bases underlying the genetic defects of murine *Had-1* mutants, which are deficient in UDP-galactose transporting activity, and (ii) to obtain information that would help us in planning rational approaches to identify functionally essential regions, based on comparison of primary structures between human and murine UDP-galactose transporters. We identified five nonsense mutations, one missense Gly178Asp mutation, and two aberrant splicing mutations. Although glycine178 is highly conserved among nucleotide-sugar transporters, a Gly178Ala variant was functional. The species-differences between human and murine UDP-galactose transporters were largely confined to the N- and C-terminal regions of the transporters. Substantial deletions in the N- and C-terminal regions did not lead to loss of UDP-galactose transporting activity, indicating that these cytosolic regions are dispensable for the transporting activity. The transporter was fused with green-fluorescent protein at the C-terminal cytosolic tail without impairing the functions of either protein. Our results demonstrate the importance of the transmembrane core region of the UDP-galactose transporter protein.

Key words: FISH, green fluorescent protein, nucleotide-sugar transporter, structure-function relationships, UDP-galactose transporter.

Nucleotide-sugar transporters are localized in the membranes of the Golgi apparatus, and translocate nucleotide-sugars from the cytosol into the Golgi lumen, thus providing glycosyltransferases with their substrates. In the nucleotide-sugar transport reaction in the Golgi membranes, the whole substrate molecule is delivered to the lumen. The reaction comprises electroneutral one-to-one exchange of a nucleotide-sugar molecule for a corresponding nucleoside monophosphate molecule in its dianionic form. Nucleoside monophosphate, which serves as the counter-substrate in this antiport process, is usually supplied continuously in the Golgi lumen through the hydrolysis of nucleoside diphosphate, which is produced from nucleotide-sugars as a result of ongoing glycosyltransfer reactions (*1, 2*).

In the past few years, some nucleotide-sugar transporter genes have been cloned [reviewed by Kawakita *et al.* (*1*)]. These include the human and *Schizosaccharomyces pombe* UDP-galactose (Gal) transporters (*3-5*), the human, murine and hamster CMP-sialic acid (Sia) transporters (*4, 6-8*), the human, canine, and *Kluyveromyces lactis* UDP-*N*-acetylglucosamine (GlcNAc) transporters (*9-11*), the *Leishmania donovani* and *Saccharomyces cerevisiae* GDP-mannose transporters (*12-15*), and human, murine, and rat putative nucleotide-sugar transporters for a yet unidentified substrate (*4*).

Recently, progress has been made in investigating the membrane-topology of nucleotide-sugar transporter mole-

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Abbreviations: CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GFP, green fluorescent protein; HA, influenza virus hemagglutinin; HRP, horseradish peroxidase; ORF, open reading frame; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; Sia, sialic acid; TBS, Tris-buffered saline.

cules. An eight transmembrane helices-model was proposed at early stages of molecular cloning based on computer-aided analysis (*3, 8*). Immunofluorescence microscopy after plasma membrane-selective permeabilization was first applied to epitope-tagged CMP-Sia transporter to demonstrate that the C-terminus is exposed to the cytosol on the outer surface of the Golgi membrane (*6*). In the same way, its N-terminal region was shown to face the cytosol (*16*). Using this technique, Eckhardt *et al.* recently carried out internal HA-epitope tagging studies, and proposed a ten transmembrane helices-model. This model suggests the occurrence of two additional helices that had escaped our notice when we proposed the eight transmembrane helices-models (*16*). Meanwhile, there is only limited information available concerning the determinants for substrate specificity and fundamental structural motifs common among nucleotide-sugar transporters and essential for the transporting activity. We have recently compared the structures of mammalian UDP-Gal, UDP-GlcNAc, and CMP-Sia transporters and *S. pombe* UDP-Gal transporter, and proposed "substrate-specific" residues as candidates for determinants of substrate specificity and highly conserved residues as candidates for components of functionally important common structural motifs (*9*), although these should be tested experimentally.

In this report, we describe the molecular cloning of murine UDP-Gal transporter and characterization of mutant transporters in UDP-Gal transporter-deficient *Had-1* cells. Comparison of the human and murine UDP-Gal transporters revealed variations between the species. Molecular characterization of *Had-1* mutants led to identification of several mutations that abolish the UDP-Gal transporting activity. Based on these findings, we carried out experimental modification of the murine UDP-Gal transporter protein in order to obtain insights into the structure-function relationships of the transporter.

EXPERIMENTAL PROCEDURES

Materials—The radioactive substrate UDP-[4,5-^{3}H]galactose (48.3 Ci/mmol) was purchased from NEN Life Science Products, Boston, MA.

Cell Culture—CHO-K1 wild-type cells and Lec8 mutant cells (ATCC CRL1737) (*17*) were maintained in minimum essential medium α (Life Technologies, Gaithersberg, MD) supplemented with 10% fetal calf serum (FCS). FM3A wild-type cells (*18*) and the *Had-1* mutant clones (*19*) were maintained in ES medium (Nissui, Tokyo) supplemented with 2% FCS. Both *Lec8* and *Had-1* mutants are defective in UDP-Gal transport (*20, 21*). Transfection with expression vectors was carried out using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) or LipofectAMINE (Life Technologies) reagents, following the manufacturer's instructions. Stable transformants were selected in media containing 0.8 mg/ml G418.

DNA Sequencing—Nucleotide sequences of both strands of polymerase chain reaction (PCR) products were determined by the dideoxy chain termination method (*22*) using a Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech) with an ABI Prism A377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Polymerase Chain Reaction (PCR)—5′-RACE and 3′-RACE were carried out utilizing mouse liver Marathon-Ready cDNA from BALB/c mice (Clontech, Palo Alto, CA) according to the manufacturer's instructions. RT-PCR was carried out using a Titan RT-PCR kit (Roche Diagnostics). Template poly(A)$^+$ RNAs were isolated from appropriate cells with a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). Addition of FLAG-epitope tag (DYKDDDDK) to the N-terminus of mUGT1 was achieved with PCR using the primer NI417 (5′-CGGAATTCACCATGGATTACAAGGACGACGACGATAAGGCAGCGGTTGGGGTTGGT-3′) for introducing the coding sequence of the FLAG tag (underlined).

Northern Blot Analysis—Hybridization of murine multiple tissue Northern blots (Clontech) was carried out as recommended by the manufacturer. Human β-actin cDNA was obtained from Clontech, and used to detect murine actin mRNAs. Radioactivity was visualized using Fuji BAS2000 (Fuji Photo Film, Tokyo).

Lectin-Sensitivity Assay—Equal number of cells of each clone (10^3 cells in 0.1 ml of culture medium) were initially inoculated into ES medium supplemented with 2% FCS containing various concentrations of *Griffonia simplicifolia* lectin II (GS-II) or wheat germ agglutinin (WGA) (EY Laboratories, San Mateo, CA), and then grown for 3 days in 96-well culture plates. The number of cells was determined using a Cell Counting Kit-8 (Wako, Osaka) and measuring the absorbance at 450 nm according to the manufacturer's manual.

Preparation of Microsomal Membrane Vesicles—The microsomal fraction was prepared as described previously with slight modifications (*4*). Cells were harvested, washed with an ice-cold 10 mM HEPES-Tris buffer (pH 7.4) containing 0.25 M sucrose, and then suspended in four volumes of a solution containing 0.25 M sucrose, 10 mM HEPES-Tris buffer (pH 7.4), 1 mM EDTA, and 1 μg/ml each of leupeptin, aprotinin and pepstatin A. The cells were treated twice with a homogenizer with a rotor/stator shaft (Physcotron Micro Homogenizer NS-310E; Niti-On, Chiba) at the speed setting of 10 for 30 s. The post-mitochondrial supernatant obtained through successive centrifugation of the homogenate at $1,000\times g$ for 5 min and $7,700\times g$ for 5 min was further centrifuged at $100,000\times g$ for 70 min to recover microsomal membrane vesicles in the pellet. The microsomal membranes were suspended in 0.25 M sucrose, 10 mM HEPES-Tris (pH 7.4), and stored frozen at −80°C. The protein concentrations of the vesicle preparations were determined by using a BCA kit (Pierce, Rockford, IL).

Nucleotide-Sugar Transport Assay—The uptake of UDP-Gal was measured as described (*23*). The transport reaction was started by mixing 50 μl of microsomal vesicles with an equal volume of a 2-fold concentrated reaction medium to obtain a 100-μl reaction mixture containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 0.5 mM dimercaptopropanol, and 1 μM UDP-[^{3}H]Gal (6,400 Ci/mol). The samples were incubated at 30°C for various times, as indicated, and then the reaction was stopped by 10-fold dilution with an ice-cold 0.25 M sucrose, 10 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, and 1 μM nonradioactive UDP-Gal ("stop buffer"). The entire reaction mixture was then poured onto an Advantec Toyo A020-A025A nitrocellulose filter (Advantec Toyo, Tokyo). The filter was washed three times with 1 ml of the ice-cold stop buffer, and dried, and the radioactivity remaining on the filter was determined. As a control for nonspecific adsorp-

tion, the reaction kept at 0°C was stopped immediately after it was started.

Anti-Peptide Antibody—A peptide corresponding to the 17 amino acids of the murine UDP-Gal transporter C-terminus was synthesized and a rabbit anti-serum against the peptide conjugated with keyhole limpet hemocyanin was prepared at a commercial firm (Hokudo, Abuta). The IgG fraction was prepared from the crude anti-serum using an immobilized protein A column (Pierce) according to the manufacturer's instructions. Specific antibodies (anti-mUGT1 antibodies) were enriched from the IgG fraction using affinity columns, AF-NH_2 Toyopearl 650 (Tosoh, Tokyo) coupled with the antigen peptide, as described (*23*).

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 0.5% 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 appropriate primary antibodies (anti-mUGT1 antibodies) at room temperature for 1 h. Binding of antibody was detected through chemiluminescence using HRP-conjugated anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) with Renaissance-plus reagents (NEN Life Science Products).

Immunofluorescence—Cells cultured on a Lab-Tek II 8-well chamber glass slide (Nalge Nunc International, Naperville, IL) were fixed with cold methanol for 6 min, and then incubated in PBS containing 0.2% gelatin (PBSG) for 30 min. Cells were incubated with primary antibodies in PBSG for 1 h, washed three times with PBSG, and then incubated with FITC-conjugated anti-rabbit IgG antibody (Vector Laboratories, Barlingame, CA) and Cy3-conjugated anti-mouse IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA). Fluorescence labeling was visualized by using a laser scanning confocal microscope, model LMS510 (Carl Zeiss, Germany).

RESULTS

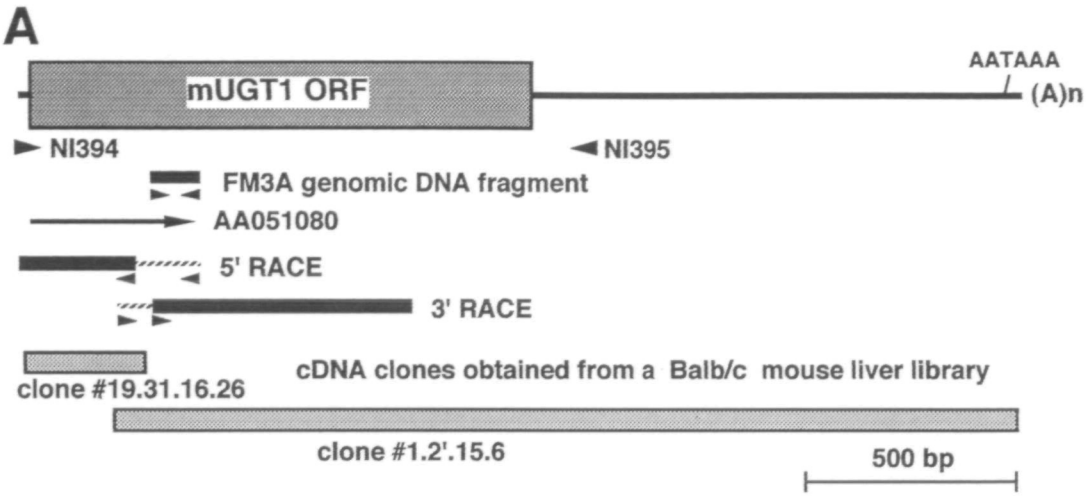
Molecular Cloning of Murine UDP-Gal Transporter—During the course of cloning the human UDP-Gal transporter gene, we found that a pair of primers in the human 2.7k exon (exon 3) (*3*) was able to amplify the corresponding portion of the murine UDP-Gal transporter gene from the genomic DNA isolated from FM3A cells (Fig. 1A). We then found a nucleotide sequence, AA051080, in the expressed sequence tags data base (dbEST; http://www.ncbi.nlm.nih.gov/dbEST/) which shares a 395-base-long overlapping stretch with the nucleotide sequence of the genomic fragment. Based on these sequences, 5′-RACE and 3′-RACE were carried out. Complementary DNA fragments obtained by the 5′-RACE contained the initiating methionine, while those obtained by the 3′-RACE coded for only a part of the open reading frame (ORF) probably due to technical difficulties in the RACE reaction. Clones #19.31.16.26 and #1.2′.15.6 were isolated by screening a BALB/c mouse liver cDNA library (Clontech) with the RACE fragments. An ORF consisting of 390 amino acids was identified. The predicted protein sequence was similar to that of human UDP-Gal transporter 1, and hence designated murine UDP-Gal transporter 1. Its calculated molecular mass was 40.8 kDa.

The expression profile of murine UDP-Gal transporter was examined using Northern blot analysis. When the ORF portion of the cDNA was used as a probe, we observed ubiquitous expression of two species of transcripts, 2.6- and 1.7-kb long, in every tissue examined, as shown in Fig. 2A. They may correspond to the two isozymes observed in humans (*4*). Judging from the length of the transcripts, the 2.6-kb species must be the one coding for murine UDP-Gal transporter 1. While an additional 1.1-kb-long transcript was observed in skeletal muscle, it seems too short to code for an entire functional transporter protein. Both the 2.6- and 1.7-kb species were expressed from an early stage of development, in 7- to 17-day embryos. Expression of another nucleotide-sugar transporter, the CMP-Sia transporter, was also examined for comparison. Using the ORF portion of human CMP-Sia transporter cDNA (*4*) as a probe, a 2.3-kb-long transcript of murine CMP-Sia transporter was observed in all tissues and at all the stages of development examined (Fig. 2B) as observed for murine UDP-Gal transporter. A minor 1.4-kb-long band reported by Eckhardt *et al.* (*7*) was also detected in all tissues, especially distinctly in liver and kidney, but only in trace amounts in embryos. The ubiquitous expression of the UDP-Gal and CMP-Sia transporters is consistent with their housekeeping role in glycoconjugate biosynthesis.

The chromosomal location of the murine UDP-Gal transporter gene was also determined by fluorescence *in situ* hybridization (FISH). As shown in Fig. 3, the murine UDP-Gal transporter gene was assigned to chromosome X, region A2. This location is in accordance with the syntenic relationship with the human UDP-Gal transporter gene on human chromosome Xp11.23-p11.22 (*24*).

Functional Expression of Murine UDP-Gal Transporter—The entire ORF of murine UDP-Gal transporter 1 was isolated from FM3A cells by RT-PCR using primers NI394 and NI395 (Fig. 1A), and an expression vector, pMKIT-neo-mUGT1, was constructed as described before (*3*). The sequence of the isolated cDNA was identical to that obtained from BALB/c mice (Fig. 1B). A stable transformant, Had-1a/mUGT1, was isolated. An anti-peptide antibody was prepared against 17 amino acid residues of the C-terminus (see Fig. 6C) to detect expression of the introduced murine UDP-Gal transporter 1 (Fig. 4A, lane 1). The apparent molecular mass of the transporter was 36 kDa, which is the same as that of human UDP-Gal transporter 1 (*23*). Using the antibody, endogenous murine UDP-Gal transporter 1 was detected in FM3A cells (Fig. 4A, lanes 4 and 5), while no detectable transporter protein was observed in Had-1a cells (Fig. 4A, lanes 3 and 6). The antibody was cross-reactive with human UDP-Gal transporter 1, and efficiently detected a 36-kDa band in Had-1a/hUGT1 cells (*3*) (Fig. 4A, lane 2). We also detected a weak but significant band of 36 kDa (or slightly larger) in CHO-K1 wild-type cells (Fig. 4A, lane 7), but not in Lec8 cells (Fig. 4A, lane 8), a UDP-Gal transporter-deficient mutant line of CHO cells established by Stanley and Siminovitch (*25*).

As shown in Fig. 4B, microsomal vesicles prepared from Had-1a/mUGT1 cells incorporated UDP-Gal in a time-dependent manner comparably to vesicles from FM3A cells. The incorporation was temperature-dependent. No significant incorporation was observed with microsomal vesicles from Had-1a/mUGT1 cells incubated on ice.



B

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-18                                                                GGACGGGCAGATGCCAAC    -1
  1 ATGGCAGCGGTTGGGGTTGGTGGATCTACCGCTGCGGCCGGGGCTGGGGCTGTGTCCTCGGGCGCGTTGGAACCT     75
  1  M  A  A  V  G  V  G  G  S  T  A  A  A  G  A  G  A  V  S  S  G  A  L  E  P     25
 76 GGGTCCACTACAGCGGCTCACCGGCGCCTCAAGTATATATCCTTAGCTGTGCTGGTGGTCCAGAATGCCTCCCTC    150
 26  G  S  T  T  A  A  H  R  R  L  K  Y  I  S  L  A  V  L  V  V  Q  N  A  S  L     50
151 ATCCTCAGCATCCGCTACGCTCGCACACTGCCTGGGGACCGCTTCTTTGCCACCACTGCTGTGGTCATGGCTGAA    225
 51  I  L  S  I  R  Y  A  R  T  L  P  G  D  R  F  F  A  T  T  A  V  V  M  A  E     75
226 GTGCTCAAAGGTCTCACCTGTCTCCTGCTGCTCTTCGCACAAAAGAGGGGTAATGTGAAGCACCTGGTCCTCTTC    300
 76  V  L  K  G  L  T  C  L  L  L  L  F  A  Q  K  R  G  N  V  K  H  L  V  L  F    100
301 CTCCATGAGGCTGTCCTGGTCCAATATGTGGACACACTCAAGCTTGCGGTGCCCTCTCTCATCTATACCTTGCAG    375
101  L  H  E  A  V  L  V  Q  Y  V  D  T  L  K  L  A  V  P  S  L  I  Y  T  L  Q    125
376 AATAACCTCCAGTATGTTGCCATCAGCAACCTGCCAGCTGCCACTTTCCAGGTGACATATCAGCTGAAGATCCTG    450
126  N  N  L  Q  Y  V  A  I  S  N  L  P  A  A  T  F  Q  V  T  Y  Q  L  K  I  L    150
451 ACTACAGCGCTCTTCTCTGTGCTCATGTTGAATCGCAGCCTCTCACGCCTGCAGTGGGCCTCTCTGCTGCTGCTC    525
151  T  T  A  L  F  S  V  L  M  L  N  R  S  L  S  R  L  Q  W  A  S  L  L  L  L    175
526 TTCACTGGTGTCGCCATTGTCCAGGCACAGCAAGCTGGTGGGAGTGGCCCACGGCCACTGGATCAGAACCCGGGG    600
176  F  T  G  V  A  I  V  Q  A  Q  Q  A  G  G  S  G  P  R  P  L  D  Q  N  P  G    200
601 GCGGGCTTAGCGGCAGTTGTGGCCTCCTGTCTCTCCTCAGGCTTCGCTGGGGTCTACTTTGAGAAGATCCTCAAA    675
201  A  G  L  A  A  V  V  A  S  C  L  S  S  G  F  A  G  V  Y  F  E  K  I  L  K    225
676 GGCAGCTCAGGTTCTGTGTGGCTTCGTAACCTACAGCTCGGCCTCTTTGGCACAGCGCTGGGCCTGGTGGGGCTC    750
226  G  S  S  G  S  V  W  L  R  N  L  Q  L  G  L  F  G  T  A  L  G  L  V  G  L    250
751 TGGTGGGCTGAGGGCACCGCCGTGGCCAGTCAAGGCTTCTTCTTTGGGTACACACCTGCTGTCTGGGGTGTAGTA    825
251  W  W  A  E  G  T  A  V  A  S  Q  G  F  F  F  G  Y  T  P  A  V  W  G  V  V    275
826 CTAAACCAAGCCTTTGGTGGGCTTCTGGTGGCTGTTGTTGTCAAGTATGCTGACAACATCCTCAAGGGCTTTGCC    900
276  L  N  Q  A  F  G  G  L  L  V  A  V  V  V  K  Y  A  D  N  I  L  K  G  F  A    300
901 ACCTCCCTGTCTATTGTGCTGTCCACTGTTGCCTCCATTCGCCTCTTTGGCTTCCACCTGGACCCATTATTTGCC    975
301  T  S  L  S  I  V  L  S  T  V  A  S  I  R  L  F  G  F  H  L  D  P  L  F  A    325
976 CTGGGCGCTGGGCTCGTCATTGGTGCTGTCTACCTCTACAGCCTTCCCCGAGGTGCAGTCAAAGCCATAGCCTCG   1050
326  L  G  A  G  L  V  I  G  A  V  Y  L  Y  S  L  P  R  G  A  V  K  A  I  A  S    350
1051 GCCTCGGCCTCTGGGCCCTGCATTCACCAGCAGCCTCCTGGGCAGCCACCACCACCGCAGCTGTCTTCTCGAGGA   1125
351  A  S  A  S  G  P  C  I  H  Q  Q  P  P  G  Q  P  P  P  P  Q  L  S  S  R  G    375
1126 GACCTCACCACGGAGCCCTTTCTGCCAAAGTCAGTGCTGGTCAAGTGAGAGCTGGTGGTGGTTGGGGGAACAGGG   1200
376  D  L  T  T  E  P  F  L  P  K  S  V  L  V  K  *                              390
1201 CAGGGGGTTGGGTTGGAGGGGGTTGGGCTTCTGCAGGTCCAAAAAGTTGTTGCCAGGGCCTGGCTTTTGTGGGTT   1275
1276 GGAGGTTTATTTTCTCCCATAATTCTAGAGGGATATGGAACTAGGGCTGAATGTCACATGAACGCTTCCTGATAG   1350
1351 ATGGACTCTCCTCTCCTGGAGGAGCTTTTTAGAGCTGCTTCCTCTGCCTTGGGCTAACCTCTTTGGGAACAGGGT   1425
1426 TGGGGTACTGCTATTCCAGGCCTTTCCCCCATGACCCTGTGCTGGAGATGTCCTGTCTCGCATGCCTGGGACAGT   1500
1501 CCCTCCCAGCCATCCTGCAGACTGGATAAAAGCCCTGCAGCTCTCCAATAACGACTAATGACTCCTCGTGGGGTT   1575
1576 CATTTCCTATTGTATGAGGTCTTCTCTCCTGCACCATCACCCTGGATCATGACAACAGCGTGGTCTCTGCTGTGG   1650
1651 CTTTGGGGCAGTTTCCCCGGTACAGAGACCCTTGAAGAGCAATCAGCCTGTTGTAAGTGCCCGGAACGAGGAGAG   1725
1726 AGGCACAAGCTGAGGATTTCGCATCAGCTCATCTTGGGGAGGGTGCAATAAGTGCCATTTGCTTTCCAATAGTTT   1800
1801 GGGATGGAGATTTGTTTGTGCTATCATTAGATCAATGATCCAGCGTGTTGGGAAAATGGGGAGGGGAGGTCCTTG   1875
1876 AAGTGGCTGAATCTCATGTAATTGACGAGACAGAGGCGAAGGATTCTCTTCAGAAACATCCAGACAAAAACTAAA   1950
1951 GCTGCCGATCCGCCTGCAGCTCCATTGGCGTTTAAAACATCCTTTCAGGATGGGTGTCAGAATTGATGAAACATG   2025
2026 GAACCTGCGCCCCTGCACTCCCCAAAGCTTATTAACTCCTTAACTGTATCCCAGATGTGTGTGTGTGTGTGTT   2100
2101 CGCGCGCGCGTGGGGATGTGTGTGGATGTGTGCACGCACACTTAAGTGCATGCATGGAAGGTGCCTGGGCTGTCT   2175
2176 TTGCTATATGTAAATAGGGCCACTGGATCTTTATTTTTGATTAATTTGTTCTGATTTTTTTTTGGTTTGTTTTTT   2250
2251 AAGGAACTGTAATGAACAAATGTCAGGATACCCAATGCCAAATAAAGATGATGTATTTATTTAGAAAAAAAA      2322
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Fig. 1. **Cloning strategy and the nucleotide sequence of murine UDP-Gal transporter 1.** (A) Murine UDP-Gal transporter cDNA is shown in a schematic model where the shaded box and thin lines represent the ORF and untranslated regions, respectively. Arrowheads indicate the positions and directions of primers used in this study. Regions amplified by PCR are shown by thick bars together with the respective PCR primers. Shaded bars represent clones obtained from a BALB/c mouse liver cDNA library. (B) Nucleotide and deduced amino acid sequences of murine UDP-Gal transporter 1 are shown. Amino acid residues where nonsense or missense mutations were found in *Had-1* mutants are indicated in outlined letters. A putative poly(A) addition signal is underlined.

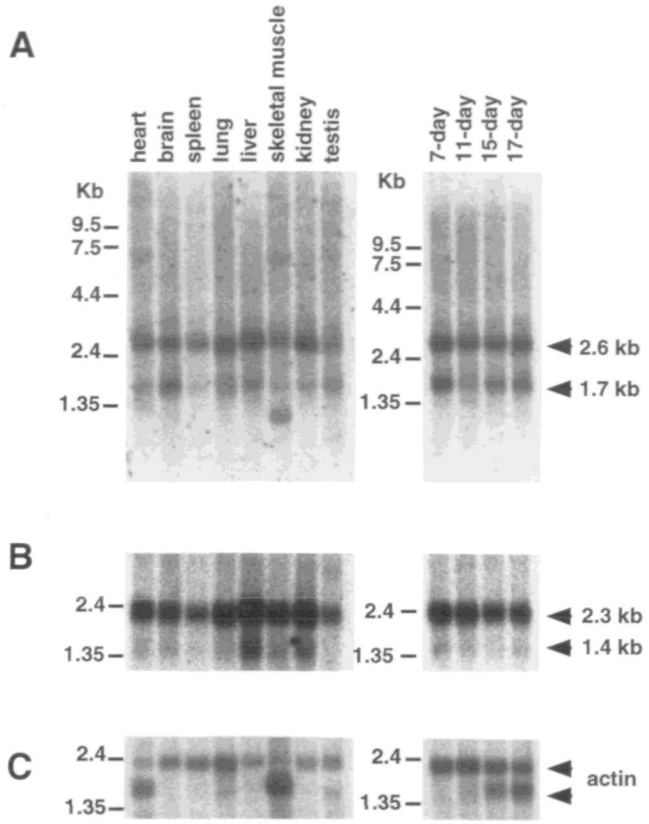

Fig. 2. **Northern blot analysis of expression of murine UDP-Gal transporter.** Each membrane was hybridized first with a radiolabeled cDNA fragment encoding the entire ORF of the human CMP-Sia transporter (B). The radioactivity retained on the filter was stripped off from the membranes, and hybridization with a radiolabeled cDNA fragment encoding the entire ORF of the murine UDP-Gal transporter was performed (A). The radioactivity retained on the filter was removed again, and then hybridization with human β-actin cDNA was carried out (C).

Molecular Basis of Murine Had-1 Mutations—We previously characterized several mutant cell lines belonging to the murine *Had-1* mutant complementation group (*19*). Murine UDP-Gal transporter 1 cDNA was amplified from several mutant cell lines by RT-PCR using the primers NI394 and NI395 (Fig. 1A), and then sequenced. Table I summarizes the mutations revealed by such molecular cloning.

We found five nonsense mutations. Had-1a exhibited a nucleotide change, G752A, resulting in the alteration of Trp251 to a stop codon. This is consistent with the results obtained through Western blot analysis using anti-mUGT1 C-terminal peptide antibody (Fig. 4A, lanes 3 and 6), since C-terminally truncated proteins, which are the products of this and other nonsense mutants, would not be recognized by the antibody against the C-terminal portion of murine UDP-Gal transporter 1. Other nonsense mutations included C547T in Had-1c (Gln183 to a stop codon), G756A in Had-1e and g (Trp252 to a stop codon), and C556T in Had-1h (Gln186 to a stop codon). Had-1l, with a point mutation, G816A, changing Trp272 to a stop codon, encoded the longest of the nonsense mutants examined so far. We also found one missense mutation, an amino acid change of Gly178 to Asp due to the nucleotide change G533A. This mutation was observed in Had-1i, m and n. As discussed below, Gly178 is highly conserved among the nucleotide-sugar transporters (*9*).

In Had-1b cells, we observed a 5- or 38-base insertion at the starting point of the human E10k exon (exon 2) (*3*). UDP-Gal transporter cDNAs from Had-1f cells were characterized by various deletions with/without an insertion in putative exon 2 leaving portions corresponding to the human E2.7k exon (exon 3) (*3*) and its down-stream region intact. These include complete loss of putative exon 2, a 39-base deletion, and a 162-base deletion followed by a 95-base insertion. It is highly likely that these represent typical products generated by aberrant splicing.

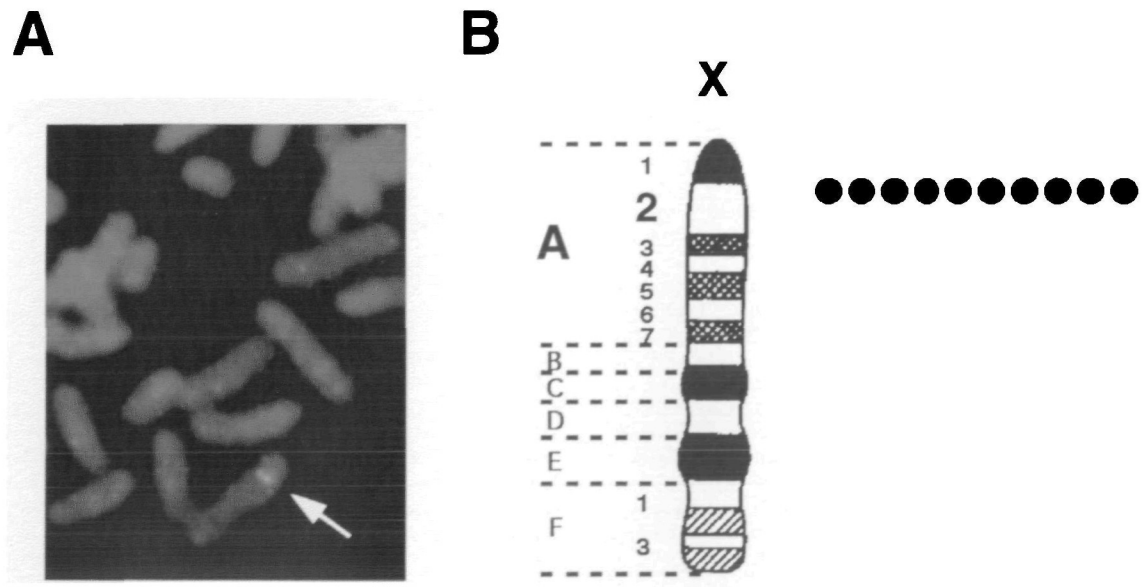

Fig. 3. **FISH analysis for murine UDP-Gal transporter gene.** (A) A representative result of FISH analysis. The arrow indicates the positive signal. (B) Summary of superimposition on DAPI banding images indicating that murine UDP-Gal transporter gene is located to chromosome X, region A2. The FISH analysis and chromosomal assignment were performed as described previously (*9*).

The UDP-Gal transporter-deficiency of these *Had-1* mutants was confirmed with a lectin sensitivity assay (data not shown), since the mutants had previously been characterized only by their resistance to infection with Newcastle disease virus (NDV) (*19*).

As described above, the Gly178Asp mutation occurred at conserved glycine178 (*9*). To examine the effect of this single amino acid change on the transporting activity, we established a stable transformant of Lec8 cells expressing Gly178Asp murine UDP-Gal transporter 1, mUGT1-(G178D), protein. Expression of the mutant protein was verified by Western blot analysis, as shown in Fig. 5. The activity of the mutant transporter was assessed by the lectin sensitivity. The growth of the transformant was arrested completely at 5 μg/ml of GS-II as was the growth of Lec8 cells, while that of CHO-K1 cells was not (Fig. 5B). Conversely, Lec8/mUGT1(G178D) cells and Lec8 cells were resistant against lectin WGA, while CHO-K1 were growth-arrested at concentrations of 3–5 μg/ml (data not shown). These results indicate that alteration of a single amino acid at Gly178 to aspartic acid is sufficient to abolish the UDP-Gal transporting activity.

Molecular Alterations of Murine UDP-Gal Transporters and Their Functional Consequences—Since glycine178 is conserved among nucleotide-sugar transporters (Fig. 6B) and a single amino acid change from Gly to Asp had a drastic effect on the transporting activity, we were interested in whether substitution by other amino acids would also diminish the activity of the transporter. Accordingly, we constructed an expression vector, pMKIT-neo-mUGT1-(G178A), to express murine UDP-Gal transporter 1 of which the 178th glycine residue has been changed to alanine (Fig. 6B).

To assess the expression of modified UDP-Gal transporter proteins and their transporting activity, we devised a simple assay involving transient expression in the CHO mutant cell line Lec8 and binding of FITC-conjugated lectin GS-II to the cells. Lec8 cells are defective in UDP-Gal transport (*20*) and have truncated cell-surface glycans with

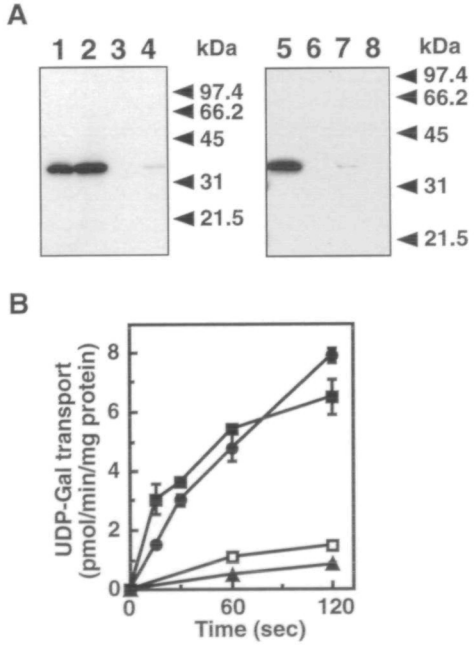

Fig. 4. **Functional expression of murine UDP-Gal transporter.** (A) Identification of UDP-Gal transporter protein. Microsomal vesicles (lanes 1–8) were prepared from Had-1a/mUGT1 (lane 1), Had-1a/hUGT1 (lane 2), Had-1a (lanes 3 and 6), FM3A (lanes 4 and 5), CHO-K1 (lane 7), and Lec8 (lane 8) cells as described previously (*4*). Forty micrograms (lanes 1–4) or 60 μg (lanes 5–8) of microsomal vesicles were fractionated on a 12% SDS-PAGE gel, transferred to a PVDF membrane, and developed with the anti-mUGT1 peptide polyclonal antibody (1:2,000). The bound primary antibodies were labeled using HRP-conjugated anti-rabbit IgG antibody (1:20,000) (Santa Cruz Biotechnology). Binding of the antibodies was detected through chemiluminescence using Renaissance-plus reagents (NEN Life Science Products). For lanes 5–8, longer exposure was carried out in order to detect binding of anti-mUGT1 antibodies to cross-reactive hamster UDP-Gal transporter endogenously expressed in CHO-K1 cells (lane 7). (B) UDP-Gal transporting activity of murine UDP-Gal transporter. Microsomal vesicles (50 μg/assay) were prepared from FM3A (●), Had-1a/mUGT1 (■, □) and Had-1a (▲) cells as described previously (*4*). Assays were performed as described in "EXPERIMENTAL PROCEDURES." The reaction mixture was incubated at 30°C for the periods indicated. The preparation from Had-1a/mUGT1 cells was also incubated at 0°C (□). The amount of radioactivity retained on the filter at 0°C, 0 min of incubation (background) was subtracted from the corresponding experimental values.

TABLE I. **Summary of the mutations found in the UDP-Gal transporter.**

Had-1 mutant	Mutation (s)	Amino acid changes
a	Point mutation G752A	Trp251Stop
b	Various insertions between exon 1 and 2	Frame-shift after Ala30
c	Point mutation C547T	Gln183Stop
e, g	Point mutation G756A	Trp252Stop
f	Various deletions with/without insertion(s) in exon 2	Frame-shift after Ala30
h	Point mutation C556T	Gln186Stop
i, m, n	Point mutation G533A	Gly178Asp
l	Point mutation G816A	Trp272Stop

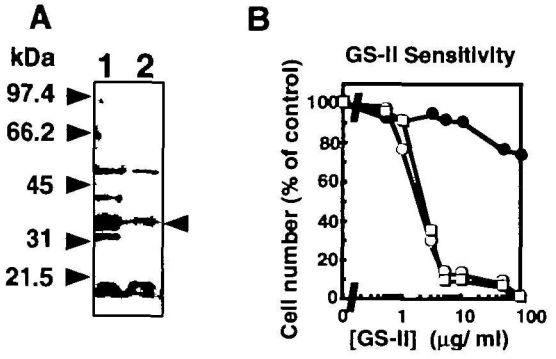

Fig. 5. **G178D UDP-Gal transporter is inactive in transporting UDP-Gal.** The expression vector pMKIT-neo-mUGT1(G178D) was stably transfected into Lec8 cells, and clone Lec8/mUGT1(G178D) was established. (A) Western blot analysis was performed with cell lysates from FM3A (lane 1) and Lec8/mUGT1(G178D) (lane 2) cells. (B) GS-II lectin-sensitivity was assessed. Equal numbers of cells (10^5 cells) of each cell line were initially inoculated into growth medium containing various concentrations of GS-II as indicated. After incubation for 3 days, the relative numbers of viable cells were compared by measuring the color intensity produced by a formazan derivative generated by the dye-reduction process in living cells, and expressed as the percentage of the value of control cultures grown in the absence of the lectin. ●: CHO-K1, ○: Lec8, □: Lec8/mUGT1(G178D).

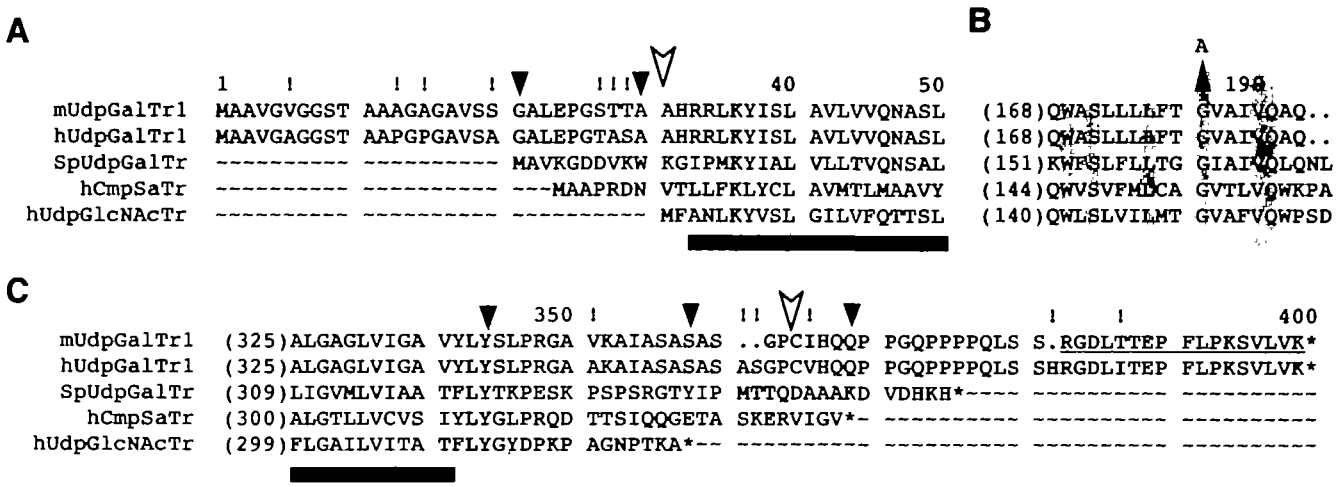

Fig. 6. **Modifications introduced to UDP-Gal transporter.** Residues of aligned amino acid sequences are numbered in the same way as in a previous report (*9*). The numbers indicated above the alignment are based on this numbering. Amino acid residues within shaded boxes are highly conserved among the nucleotide-sugar transporters whose sequences have been described (*9*). Specific differences between human and mouse UDP-Gal transporters are indicated by exclamation marks above the murine UDP-Gal transporter amino acid sequence. mUdpGalTr1: murine UDP-Gal transporter 1 (this work); hUdpGalTr1: human UDP-Gal transporter 1 (*3*); SpUdpGalTr: *Schizosaccharomyces pombe* UDP-Gal transporter (*5*); hCmpSaTr: human CMP-Sia transporter (*4*); hUdpGlcNAcTr: human UDP-*N*-acetylglucosamine transporter (*9*). (A) Mutants lacking the N-terminus of murine UDP-Gal transporter were created by replacing the residues indicated by solid arrowheads with initiating methionines. An open arrowhead indicates the position of the junction site between the putative first exon and the E10k exon of human UDP-Gal transporter (*3*) at which insertions likely due to aberrant splicing in Had-1b cells are noted. The thick bar indicates a part of the N-terminus-proximal putative transmembrane helix (TM1) (*3*). (B) Conserved glycine178 was changed to alanine as indicated. (C) The portions of the C-terminal tail downstream of the solid arrowheads were deleted by changing appropriate codons into termination codons. Murine and human UDP-Gal transporters were fused with GFP at the position indicated by the open arrowhead. The C-terminal peptide utilized in preparing an anti-peptide antibody is underlined. The thick bar indicates a part of the C-terminus-proximal transmembrane region (*3*).

GlcNAc at their termini, to which FITC-conjugated GS-II is bound (*6*). When the UDP-Gal transporting activity is restored upon introduction of modified but still functional UDP-Gal transporter, mature sugar chains appear on the transformed cells, resulting in the loss of binding of FITC-conjugated GS-II, which may be easily assessed by fluorescence microscopy. Expression of the transporter proteins may be examined in the same transient expression system, using specific antibodies (*26*).

Expression of mUGT1(G178A) proteins was clearly detected, and the proteins showed typical subcellular localization to the Golgi apparatus in cells transformed with pMKIT-neo-mUGT1(G178A) (as indicated by open arrowheads, Fig. 7, panel a). FITC-conjugated GS-II did not bind to the transformants expressing mUGT1(G178A) proteins. These results indicated that mUGT1(G178A) proteins were active in transporting UDP-Gal.

Comparison of the primary structures of human and murine UDP-Gal transporters revealed that UDP-Gal transporters were highly conserved between the two species, especially in putative transmembrane regions. The differences in amino acid sequence were found to be somewhat clustered, and largely confined to the N- and C-terminal regions. We presumed that these species-diverged regions would be non-essential for the transporting activities, and might tolerate modifications. To test this, we constructed several deletion mutants lacking the N- and C-terminal regions, as shown in Fig. 6.

Deletion mutants mUGT1(NΔ20) and mUGT1(NΔ29) lacked 20 and 29 amino acid residues of the N-terminus, respectively. Murine UGT1(NΔ20) was constructed so that it has an N-terminal region of the same length as that of *Schizosaccharomyces pombe* UDP-Gal transporter (*5*), while mUGT1(NΔ29) lacked the entire translated region of putative exon 1 (*3*). As shown in Fig. 7, both mUGT1-(NΔ20) and mUGT1(NΔ29)-expressing cells showed the wild-type phenotype (no GS-II-binding) and the expressed proteins were evidently localized in the Golgi regions (panels b and c).

Deletion mutants mUGT(CΔ30), mUGT(CΔ39), and mUGT(CΔ53) lacked 30, 39, and 53 amino acid residues of the C-terminal tail, respectively. A FLAG-epitope-tag was added to their N-termini for detection of these mutant proteins. Murine UGT(CΔ30) and mUGT(CΔ39) were terminated at the sites corresponding to the termination sites of human CMP-Sia transporter (*4*) and human UDP-GlcNAc transporter (*9*), respectively. Murine UGT(CΔ53) was terminated at the conserved tyrosine residue located most proximally to the C-terminus among the conserved residues (Fig. 6C). As shown in Fig. 7, cells expressing mUGT(CΔ30) and mUGT(CΔ39) did not bind FITC-conjugated GS-II, and the expressed proteins were detected in the Golgi region (panels d and e). We could not find any cells expressing mUGT(CΔ53) or any cells which had reverted to the wild-type phenotype in panel f.

UDP-Gal transporter was fused at its C-terminus with the green fluorescent protein (GFP), which replaced the terminal 34 amino acid residues of murine or human UDP-Gal transporter, to see whether another functional protein can be added to the C-terminus without impairing the enzyme's ability to transport UDP-Gal or to localize in the Golgi apparatus. As shown in Fig. 8, expression of UDP-Gal transporter-GFP fusion proteins was clearly detected under a fluorescent microscope (Fig. 8A, panels a and d).

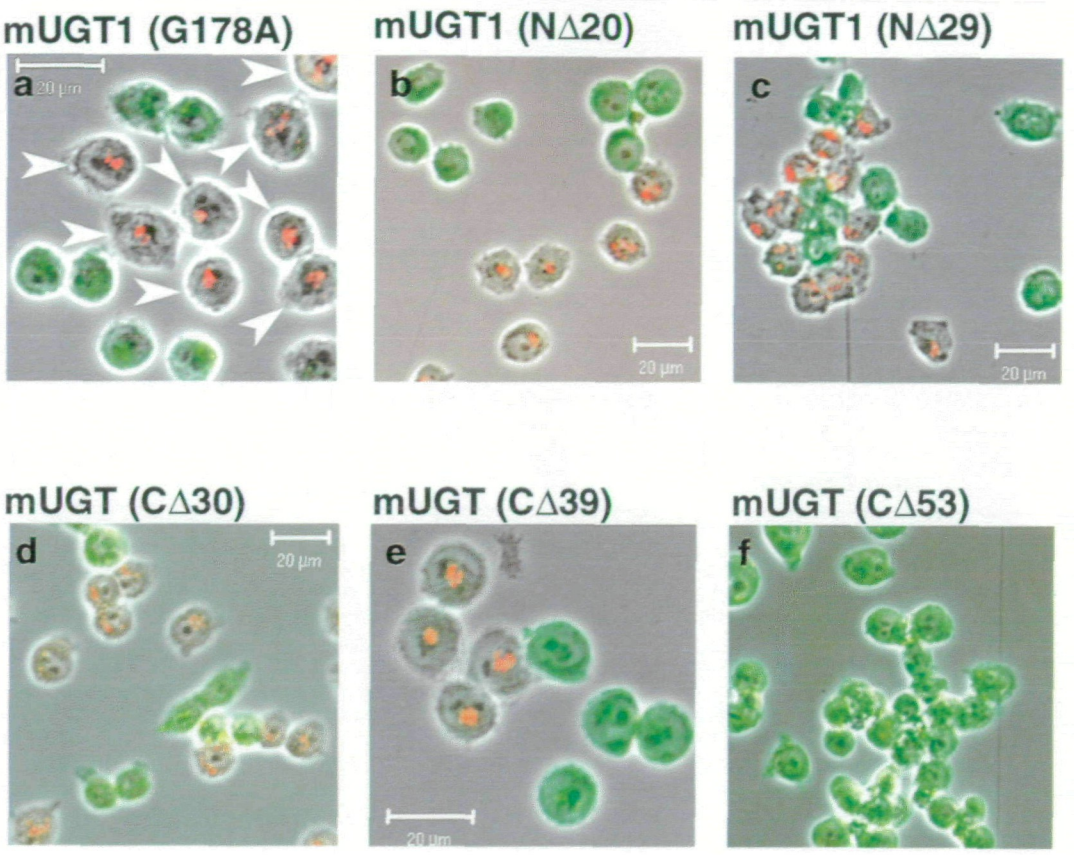

Fig. 7. **Expression and activity of modified murine UDP-Gal transporter.** Lec8 cells were transfected with pMKIT-neo expression vectors encoding various modified murine UDP-Gal transporters. The cells were grown for 2 days, and transfected cells were examined for expression of murine UDP-Gal transporter protein and binding of FITC-conjugated GS-II. The transporter protein was detected by incubation with rabbit anti-mUGT1 peptide antibody (1:100) or anti-FLAG monoclonal antibody 5M followed by anti-rabbit IgG antibody-Cy3 (1:80) or anti-mouse IgG antibody-Cy3 (1:100), respectively. Fluorescence labeling was visualized by using a laser scanning confocal microscope model LMS510, and the merged images with the phase contrast images are shown. a: pMKIT-neo-mUGT1(G178A), b: pMKIT-neo-mUGT1(NΔ20), c: pMKIT-neo-mUGT1(NΔ29), d: pMKIT-neo-mUGT(CΔ30), e: pMKIT-neo-mUGT(CΔ39), f: pMKIT-neo-mUGT(CΔ53). Bar: 20 μm.

The subcellular localization in the Golgi apparatus was demonstrated by showing colocalization of the murine UDP-Gal transporter-GFP fusion protein with α-mannosidase II, as revealed by immunostaining of the cells with anti-α-mannosidase II antibodies (Fig. 8A, panels b and c). As shown in Fig. 8B, stable transformants expressing UDP-Gal transporter-GFP fusion proteins manifested resistance against GS-II comparable to that of wild-type CHO-K1 cells. These results indicate that the fusion proteins are functional both in green fluorescence emission and in UDP-Gal transport.

DISCUSSION

In this report, we have described the molecular characterization of a murine UDP-Gal transporter 1 which is the murine counterpart of human UDP-Gal transporter 1 (*3*). This enabled us, in the first place, to identify the molecular basis of murine UDP-Gal transporter-deficient mutations (*Had-1* mutations). Secondly, comparison between murine and human UDP-Gal transporters yielded valuable information that led us to investigate the functional importance of the transmembrane core region of UDP-Gal transporter protein. As the result, we were able to demonstrate that the N- and C-terminal regions are dispensable for UDP-Gal transporting activity.

The murine UDP-Gal transporter 1 protein consisted of 390 amino acids. Compared with the human counterpart, murine UDP-Gal transporter 1 showed 14 amino acid differences and three deletions. The differences in amino acid sequence were found to be clustered, and largely confined to the N- and C-terminal regions. Namely, the N-terminal region has seven amino acid differences and the C-terminal tail has three amino acid differences and all of the three deletions (Fig. 6).

It has been demonstrated that the N- and C-terminal regions of UDP-Gal transporter are exposed to the cytosol (*26*). We showed previously that the cytosolic C-terminal portion of UDP-Gal transporter could tolerate minor modifications such as addition of an HA-epitope tag (*5, 26*). This was also the case with CMP-Sia transporter and GDP-mannose transporter (*6, 7, 13, 15*). In the present study, we were able to further demonstrate that a nucleotide-sugar transporter can be fused even with a functional protein at its C-terminal. That is, UDP-Gal transporter and GFP in the fusion product retained their respective functions unimpaired (Fig. 8).

Deletion of these cytosolic regions did not destroy the

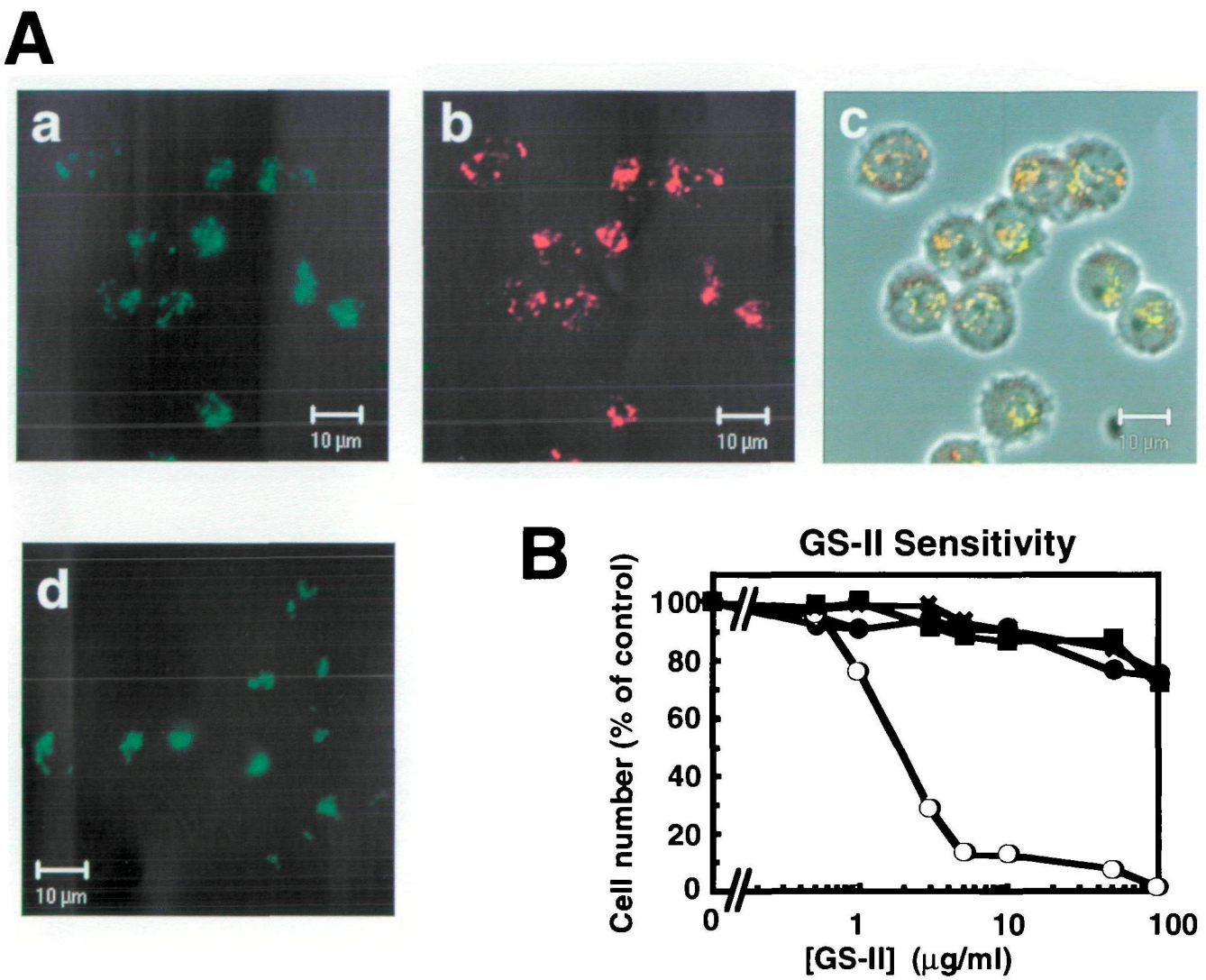

Fig. 8. **Subcellular localization and UDP-Gal transporting activity of murine and human UDP-Gal transporter-GFP fusion proteins.** Expression vectors pEGFP-mUGT and pEGFP-hUGT were constructed by introducing an appropriate restriction fragment of cDNA into pEGFP-N2 vector (Clontech) utilizing an *Apa*I recognition site in the C-terminal region of UDP-Gal transporters to generate an in-frame fusion protein joined at the point indicated in Fig. 7C. (A) Lec8 cells were stably transfected with pEGFP-mUGT (a-c) or pEGFP-hUGT (d) encoding murine or human UDP-Gal transporter-GFP fusion proteins, respectively. Immunolabeling of α-mannosidase II protein (b) was performed with a rabbit anti-α-mannosidase II antiserum followed by anti-rabbit IgG antibody-Cy3 (1:100). Bound antibodies (b) and fluorescence of fused GFP (a, d) were visualized using a Carl Zeiss laser scanning confocal microscope LSM510. Panel c represents a phase contrast image merged with the images in panels a and b. Bar: 10 μm. (B) GS-II sensitivity of stable transformants shown in (A) was assessed as in Fig. 5. ●: CHO-K1, ○: Lec8, ×: Lec8/hUGT-GFP, ■: Lec8/mUGT-GFP.

transporting activity or alter the substrate specificity (Fig. 7), suggesting that UDP-Gal transporter has portions dispensable for these basic functions. Some properties of the Had-1b mutant may be pertinent here. The virus infection assay indicated that the defect of Had-1b cells was leaky (*19*), but the lectin-sensitivity assay showed that the residual UDP-Gal transport activity must be very slight. This is consistent with the previous observation that glycosylation of VSV G protein was not detected in these cells (*19*). A trace of UDP-Gal transport activity, which would not alter the binding of GS-II to cytotoxic sites, might be left in Had-1b cells and be able to generate a limited amount of sialo-glycoconjugates to serve as the receptor for NDV. The Had-1b mutation resulted in insertions between putative exons 1 and 2 (Table I). Translation of the Had-1b UDP-Gal transporter mRNAs, which have longer inserts, would be terminated five residues from the beginning of the inserts due to an in-frame UGA termination codon. If an AUG codon distal to this termination codon and in-frame with the exon 2 sequence occurs in the insert, then it is possible that a minute amount of active UDP-Gal transporter with an altered N-terminal region would be produced, since the translated region of putative exon 1 is not required for function of the UDP-Gal transporter (Fig. 7, panel c).

The dispensability of the N- and C-terminal portions raises the question of the identity of the minimum essential core structure required for the UDP-Gal-specific transporting activity and for the correct targeting to the Golgi apparatus. Segawa *et al.* demonstrated recently that the

N-terminus-proximal transmembrane helix (TM1) is indispensable for both the transporting activity and targeting to the Golgi apparatus (*5*). This implies that TM1 is a part of such an essential core.

The Had-1m mutation occurred at the Gly178 residue and changed it to Asp178. Since Gly178 is highly conserved among the nucleotide-sugar transporters (*9*), we presumed that any amino acid changes at this residue might result in inactivation of the transporter. Unexpectedly, however, the Gly178Ala mutant was active in transporting UDP-Gal (Fig. 7, panel a). One possible explanation is that this conserved residue may contribute to maintaining a conformation fundamental to the transporting activity rather than constituting a sugar- or nucleotide-binding motif in forming transporter-substrate intermediates. Since both glycine and alanine are small, non-polar amino acids, the Gly to Ala substitution likely resulted in minimal alteration of the conformation. Similar amino acid substitutions may be tolerated at this highly conserved residue, but the phenotype of the Had-1m mutant underscores the importance of the integrity of the transmembrane region.

Deletion of conserved Tyr338, the most proximal residue to the C-terminus among the conserved residues, rendered the UDP-Gal transporter extremely unstable, probably because the deletion of the conserved residue disrupted some intramolecular interaction essential for the maintenance of the fundamental conformation of the transporter. Such interactions may involve the transmembrane helices, since the conserved Tyr residue is located at the border between a putative transmembrane helix and the C-terminal cytosolic tail.

We have demonstrated that the cytosolic N- and C-terminal regions are dispensable for the transporting activity and Golgi-targeting of UDP-Gal transporter. Currently available evidence indicates that the transmembrane domains are important for the activity and proper targeting of the enzyme. The structure-function relationships of nucleotide-sugar transporters currently constitute our major interest (*1*). Investigations on such issues, including intensive analyses of deletion mutants and chimeric transporter molecules, and systematic alteration of amino acid residues, are in progress in our laboratory.

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