Point Mutations Identified in Lec8 Chinese Hamster Ovary Glycosylation Mutants That Inactivate Both the UDP-galactose and CMP-sialic Acid Transporters*

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Nucleotide-sugar transporters (NSTs) are critical components of glycosylation pathways in eukaryotes. The identification of structural elements that are involved in NST functions provides an important task. Chinese hamster ovary glycosylation mutants defective in nucleotide-sugar transport provide access to inactive transporters that can define such structure/function relationships. In this study, we have cloned the hamster UDP-galactose transporter (UGT) and identified defects in UGT gene transcripts from nine independent Chinese hamster ovary mutants that belong to the Lec8 complementation group. Reverse transcription polymerase chain reaction with primers that span the UGT open reading frame showed that three Lec8 mutants express a full-length open reading frame, while six Lec8 mutants predominantly express truncated UGT gene transcripts. Sequencing identified different single or triplet nucleotide changes in full-length UGT transcripts from three of the mutants. These mutations translate into three different amino acid changes at positions that are highly conserved in all the known mammalian NSTs. Transfection of a cDNA encoding either of the mutations Δserine 213 or G281D failed to correct the UDP-galactose transport defect in Lec8 transfecants. Most importantly, introducing these same mutations into the homologous region of the murine CMP-sialic acid transporter caused inactivation of this transporter. Thus, identifying point mutations that inactivate UGT in Lec8 mutants resulted in the discovery of amino acids that are critical to the activity of both UGT and CST, the two most divergent mammalian NSTs.

The maturation of glycoconjugates that are either secreted or become constituents of the plasma membrane occurs while passing through the Golgi compartments. Processing enzymes, glycosidases, and glycosyltransferases, are Golgi residents, and their spatial organization in the Golgi stack generally reflects the sequence of biosynthetic steps (1). The nucleotide-sugars, which are substrates for luminal glycosyltransferases, are produced in the cytoplasm or, in the case of CMP-sialic acid, in the cell nucleus (reviewed in Refs. 2 and 3) and are supplied to the Golgi lumen via specific nucleotide-sugar transporters (NSTs). The existence of NSTs, their functional properties, and subcellular locations have been described for many years (4). The isolation of NST genes, however, succeeded only recently by expression cloning using glycosylation-defective mutants (reviewed in Refs. 4 and 5).

Three genetic complementation groups that are affected in the UDP-galactose transporter (UGT) gene have been described: the CHO mutant Lec8 (6), murine Had-1 cells (7), and a vanadium-sensitive strain of Schizosaccharomyces pombe called gns1–1 (for galactomannan synthesis-negative) (8). Complementation cloning was carried out in Had-1 cells and in S. pombe and identified the human (9) and a truncated form of the yeast (10) UGT genes, respectively. Using PCR-based approaches, the murine UGT (11), a second isoform of the human UGT (12), and a full-length S. pombe UGT cDNA (13) were isolated. Sequence alignments reveal strong conservation of UGTs, since species as distant as human and S. pombe are 40% identical (see Fig. 1C).

Little information exists on the functional modi and architecture of the NSTs. Membrane topology has been determined for the murine CMP-sialic acid transporter (CST) and, in contrast to the theoretically predicted eight transmembrane domains, it was shown to contain 10 transmembrane domains (14). Evidence is accumulating that NSTs in the active state seem to be essential for the formation of the dimer, while the N-terminal cytosolic tail is required for export from the ER (15, 16). In contrast, the cytosolically located N- and C-terminal tails of murine UGT were found to be dispensable. Deletion of both ends affected neither the activity nor the subcellular destination of the UGT (11).

In order to define functional subdomains in NSTs, Aoki et al. (20) constructed chimeric molecules by exchanging segments of human UGT isofrom 1 with the corresponding domains of human CST. As expected from previous truncation experiments (11), the cytoplasmic tails were interchangeable. However, exchanges of amino acid stretches that represent potential transmembrane domains inactivated the UGT with a single exception. If the C terminus plus the C-terminal helix of the UGT

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF299335.

1 The abbreviations used are: NST, nucleotide-sugar transporter; UGT, UDP-galactose transporter; CST, CMP-sialic acid transporter; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PSA, polysialic acid; NCAM, neural cell adhesion molecule; mAb, monoclonal antibody; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; kb, kilobase pair(s); bp, base pairs.

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PSA detection has been used to identify the polysialyltransferase ST8SiaIV (24, 25), the CST from mouse (26) and hamster (27), and the CMP-Neu5Ac synthetase (3). In this study, PSA was a marker in analytical steps carried out to determine the functional consequences of lec8 mutations.

Nine independent Lec8 isolates were analyzed at the molecular level. Six were found to express truncated transcripts, while three mutants possess a UGT with a single amino acid change. Amino acids replaced or deleted in the missense mutants are highly conserved residues in all mammalian transporters. Two of these mutations inactivate both the UGT and CST transporters revealing their importance for the correct folding or functional assembly of NSTs.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal antibody (mAb) 12CA5, directed against the hemagglutinin (HA) epitope YPYDVPDYASL, was purchased from Roche Molecular Biochemicals, and mAb M5, directed against the FLAG sequence MDYKDDDDK, was from Sigma. mAb 735, directed against PSA, has been described (28). A rabbit antiserum against the catalytic domain of α-mannosidase II was a kind gift of Dr. K. Moremen (University of Georgia, Athens, GA). Secondary antibodies used in this study were anti-mouse Ig-alkaline phosphatase conjugate (Diannaov), anti-mouse Ig-Cy3 conjugate (Molecular Probes, Inc., Eugene, OR), and anti-rabbit Ig-Alexa488 conjugate (Molecular Probes).

**Cell Lines**—CHO K1 (ATCC CCL 61), COS-7 (ATCC CRL 1651), and Pro 5Leck cells (ATCC CRL 1737) were obtained from the American Type Culture Collection (Manassas, VA). Additional independent Lec8 mutants listed in Table I were selected for lectin resistance and shown by complementation in somatic cell hybrids to belong to the Lec8 complementation group. 6B2 cells belong to complementation group Lec2 and were characterized previously (21). CHO K1 cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, penicillin (50 units/ml), and streptomycin (50 µg/ml). Lec8 and 6B2 cells were cultured in α-minimal essential medium with Glutamax (Life Technologies, Inc.), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 µg/ml), and COS-7 cells in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). All cells were kept in a humidified atmosphere at 37 °C plus 5% CO₂.

**Isolation of the Hamster UGT**—A hamster UGT homologue was isolated by colony hybridization using a digoxigenin-labeled DNA probe generated by PCR from the 3’-end (nucleotides 640–1182) of human

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**Table I**

**Summary of Lec8 mutants independently selected for lectin resistance and shown to belong to complementation group Lec8**

| Summery name | Truncated name | Reference |
|--------------|----------------|-----------|
| Pro 5Lec8    | Lec8           | 6         |
| Pro 5Lec8.5H | Lec8.5H        | 47        |
| Gat 2Lec8.1C | Lec8.1C        | 47        |
| Gat 2Lec8.2B | Lec8.2B        | 47        |
| Pro 5Lec8.Lec8 | Lec8.2B | 47        |
| Pro 5Lec4.A.Lec8 | Lec8     | 48        |
| Pro 5Lec8.2.S | Lec8.2.S       | 48        |
| Pro 5Lec8.2.S.1 | Lec8.2.S.1  | 48        |
| Pro 5Lec10.Lec8 | Lec8.2.B  | 49        |

**Table II**

**Summary of the primer sequences used in this study**

| Primers | Sequences | Descriptions |
|---------|-----------|--------------|
| Flanking primers | | |
| SO18 (as) | GCGGATTCG*GCAGCGGTTGGGTTGCGG** | 5’ primer, hamster UGT, introduces BamHI site |
| SO19 (as) | GCGGATTC**GCGAACCTTACATCTGTGAGCC** | 3’ primer, hamster UGT, introduces BamHI site |
| SO23 (as) | GCGGATTCG*GCAGCGGTTGGGTTGCGG** | 5’ primer, hamster UGT, introduces EcoRI site |
| SO24 (as) | CGTCTAGA**TACGACAATCCTACACAGG** | 3’ primer, hamster UGT introduces XhoI site |
| ME41 (as) | GCGGATTCGATGGTCGCGCCAGAG** | 5’ primer, murine CST, introduces BamHI site |
| ME42 (as) | GCGGATTC**GACACCAATGTTCTCTCTTCTCT** | 3’ primer, murine CST introduces BamHI site |
| RT-PCR primers | | |
| SO11 (as) | 1225GCGGCCCCAGATGATGTCGTTCTC1229 | 5’ primer, hamster UGT, introduces BamHI site |
| SO45 (as) | 49GAGTACTCGTCTGTCGTTGCT128 | 3’ primer, hamster UGT, introduces BamHI site |
| SO46 (as) | 1266GTATAGTCAGCTGCGG1248 | 5’ primer, murine CST, introduces BamHI site |
| SO47 (as) | 1293AGCTACACTTCCTGTC1271 | 3’ primer, murine CST introduces BamHI site |
| SO48 (as) | 5’GACGAGGACTGCGGACTG5’ | |
| Mutagenesis primers | | |
| SO85 (as) | 369GTATTTTGCGCAAAGGTAGGATGAGAGACC** | 5’ primer, hamster UGT, introduces BamHI site |
| SO86 (as) | 357CTCTTCTGCAAATCAATTTTGAGGATAACC** | 3’ primer, hamster UGT, introduces BamHI site |
| SO97 (as) | 657CTCTGACAGGTGAGAGGCGG** | 5’ primer, hamster UGT, introduces EcoRI site |
| SO98 (as) | 622CTCTGCGACTCTTGTTGAGG** | 3’ primer, hamster UGT introduces XhoI site |
| SO105 (as) | 856CCTACGACTGCGCAATGACAGAGC** | 5’ primer, murine CST, introduces BamHI site |
| SO106 (as) | 833CAGGCGCTTTGAGCGCGTACCGTG1256 | 3’ primer, murine CST introduces BamHI site |
| SO114 (as) | 30GTTTCTGCGACAGTCGCTGAG1281 | 5’ primer, hamster UGT, introduces BamHI site |
| SO115 (as) | 28CATCAGATGCTGCGATTCAAGC** | 3’ primer, hamster UGT, introduces BamHI site |
| SO116 (as) | 57TCCTGCGAATCCACAAGAATACAGACACG** | 5’ primer, murine CST, introduces BamHI site |
| SO117 (as) | 54GCTATCGGCTGCTGATGTTGCGC** | 3’ primer, murine CST introduces BamHI site |
| SO118 (as) | 58GACGCTTGAGGCGGACACTACAGTAGG** | 5’ primer, murine CST, introduces BamHI site |
| SO119 (as) | 75CTCTGCACTTGTGGTTGAGG** | 3’ primer, murine CST introduces BamHI site |

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*Note: The asterisk (*) indicates the position of the restriction enzyme site.*
UGT isoform 1 (UGT1; Ref. 9; accession no. D84454). A CHO-K1 cDNA library was constructed as described earlier (24). In a first round, 3 x 10^3 colony forming units were plated per 140-mm dish and after overnight incubation were transferred to nitrocellulose filters. 25 positive colonies were selected and plated on 94-mm dishes. Preparation of the probe, hybridization, and detection of positive colonies were performed as described in the DIG System user's guide for filter hybridization (Roche Molecular Biochemicals). In the second hybridization round, 10 colonies gave positive signals on duplicate filters. Positive colonies were picked and amplified in Luria broth (LB) medium supplemented with 100 μg/ml ampicillin, and plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Inserts were released by XhoI digestion, and after separation on a 2% agarose gel and transfer onto nitrocellulose membranes were verified by a second hybridization step with the digoxigenin-labeled probe described above. Positive clones of 1 k b were sequenced by the dideoxy chain termination method using a DNA sequencing kit (AbiPrism). Only one clone, pABE-SO1, contained the full-length hamster UGT sequence in inverse orientation.

Northern Blot Analysis—Total RNA was isolated from CHO and Lec8 cells using the RNeasy Midi Kit (Qiagen). Polyadenylated RNA (Poly(A)^+ RNA) was selected from total RNA using the QuickPrep Micro...
mRNA Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Poly(A)+ RNA (2 μg/sample) was electroforesed in a 1% agarose gel in 20 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and transferred to nylon membranes (Qiagen). Blots were hybridized overnight at 68 °C in high nucleotide SO47 annealing to nucleotides 1273–1291 in the hamster UGT subcloned into pBluescript SK (Stratagene). The membrane was washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 0.1× SSC, 0.1% SDS at 68 °C for 20 min. Bound probe was detected by incubation with anti-digoxigenin IgG–alkaline phosphatase conjugate (Roche Molecular Biochemicals) and chemiluminescence detection using disodium-3-(4-methoxyxipirrol,1,2-dioxetane-3,2,-(5'-chloro)tricyclo-[3,3,1,1^3,7]decane-4-yl)phenylphosphate (Roche Molecular Biochemicals) as a substrate.

Construction of Epitope-tagged Transporters—to generate N-terminally FLAG-tagged hamster UGT, the insert contained in the vector pBE-501 was amplified with oligonucleotide primers SO90 and SO74, which include EcoRI and XbaI restriction sites, respectively (Table II). PCR products were treated with EcoRI and XbaI, purified, and ligated into the vector pME8.1, which is a derivative of the eukaryotic expression vector pcDNA3 containing the FLAG-sequence (MDYKDDDDK) (6). 9

Site-directed Mutagenesis—Missense mutations identified in Lec8-derived UGT cDNAs as well as other point mutations described in this study were introduced into the wild type sequences of both epitope-tagged transporter cDNAs and the murine CST. Site-directed mutations were introduced by PCR using Pfu polymerase (Stratagene) followed by fusion PCR. Oligonucleotide primers used to introduce point mutations and to generate full-length constructs by fusion PCR are listed in Table II. In order to generate HA-tagged UGT variants in the vector pEVRF0-HA, the primer combinations SO18/SO55 and SO19/SO86, SO18/SO97 and SO19/SO98, and SO18/SO105 and SO19/SO106 were used to introduce the mutations Y122H, ΔS213, and C281D, respectively. DNA fragments resulting from these PCRs were purified on 2% agarose gels using the GFX-PCR Kit (Amersham Pharmacia Biotech), corresponding fragments were combined, and fusion PCRs were carried out with the primers SO18/SO19, which add BamHI restriction sites to the 3′- and 5′-ends of the DNA sequence (see Table II). Two wild type N-terminally FLAG-tagged UGT variants in the vector pcDNA3, the reactions described above were repeated, but SO18 and SO19 were replaced by SO23 and SO24, which add EcoRI and XbaI restriction sites, respectively, to the DNA sequences. Mutations in the murine CST were introduced with the primer combinations SO114/ SO115 for Y98H, SO116/SO117 for Δs188, and SO118/SO119 for G256D. Flanking primers used in the mutagenesis step were ME41 (used together with SO114, SO116, and SO118) and ME42 (used together with SO115, SO117, and SO119). ME41 and ME42, which add BamHI restriction sites to the 3′- and 5′-ends of the DNA sequences (see Table II) were also used in the fusion reaction. All constructs were confirmed by sequencing.

Transfections—Recipient cells for transient transfections were the Lec8 subclone Lec4.8.7A and the Lec2 subclone 6B2 (21). Expression was monitored by Western analysis, and UGT function was measured by the ability to produce sialylated NCAM detected by anti-PSA antibody (22). While Lec2 cells do not produce spontaneous PSA-positive revertants at a detectable level (even after several months of culture), spontaneous Lec revertants arose at a significant frequency. Thus, it was necessary to deplete revertants by panning on anti-PSA antibody every 3–5 days (for details, see Ref. 3). Of the nine Lec8 subclones used in this study, Lec4.8.7A cell populations produced the fewest revertants, and therefore this cell line was routinely used as the recipient in transfection experiments.

Transfections were carried out with 5 × 10^5 cells seeded in 6-cm Petri dishes (or on glass coverslips placed into 6-cm Petri dishes) 24 h prior to transfection. Cells were washed twice with Opti-MEM and transfected with 1 μg of cDNA mixed with 6 μl of LipofectAMINE in 1 ml of Opti-MEM (Life Technologies). Cells were cultured for 6–8 h in the presence of the transfection mixture. Transfections were stopped with 2 volumes of the normal culture medium supplemented with 10% fetal calf serum. After an additional 24–72 h of culturing, analytical steps were performed.

Western Blot Analysis—SDS-polycrylamide gel electrophoresis was performed according to Laemmli (30) on 15 and 7% polyacrylamide gels. Lysate of transfected COS-7 cells was carried out in 20 μl Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 200 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2% Nonidet P-40. CHO cells were lysed in 50 mM Tris-HCl (pH 8.0), 1 mM MgCl2, 2 mM EDTA, 200 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. After electrophoretic separation, proteins were transferred to nitrocellulose membranes by semidy blotting (31). Membranes were blocked in 2% nonfat dry milk in phosphate-buffered saline (PBS) for at least 30 min at room temperature. Immunostaining with mAb 735 was performed as described (32). Transport proteins were detected with either the anti-α-HA mAb 12CA5 (Roche Molecular Biochemicals) or the anti-FLAG mAb M5 (Sigma). Bound primary antibodies were detected with anti-α-HA- or anti-FLAG-alkaline phosphatase conjugate (Diaclone), and blots were developed with nitro blue tetrazolium (Rotth) and 5-bromo-4-chloro-3-iodyl phosphate (Rotth) as substrates.

Indirect Immunofluorescence—Cells grown overnight on glass coverslips were washed with PBS and fixed in 4% parformaldehyde in PBS for 20 min. After three washing steps with PBS, the cells were permeabilized for 30 min with 0.2% saponin in PBS containing 0.1% bovine

Fig. 2. Epitope-tagged forms of hamster UGT restore PSA expression in Lec8 cells. PSA expression was determined by Western blot with mAb 735 to analyze the complementing ability of untagged or N-terminally FLAG-tagged or C-terminally HA-tagged hamster UGT. 35 μg of protein were loaded on each lane. PSA is visible as a heterogeneous band that disappears after EndoNE digestion (+). Wild-type (CHO K1) cells express PSA, while Lec8 (mutant Lec4.8.7A) and Lec2 (mutant 6B2) cells are PSA-negative. Transient expression of wild type UGT (UGT wt), FLAG-UGT, or UGT-HA in Lec4.8.7A restores PSA expression. Transfer of FLAG-UGT into 6B2 cells did not result in PSA expression. The bottom panels show Western blot analyses with anti-FLAG or anti-HA antibodies and demonstrate equivalent expression of tagged UGTs.
serum albumin (BSA). After permeabilization, samples were incubated with the primary antibodies diluted in 0.1% BSA in PBS (anti-FLAG mAb M5, 5.4 μg/ml; anti-HA mAb 12CA5, 2.5 μg/ml). The α-mannosidase II antiserum (33) was diluted 1:2000. Antibodies were incubated for 2 h at room temperature. After three washing steps in 0.1% BSA in PBS, bound antibodies were detected with secondary antibodies: anti-mouse Ig-Cy3 (Molecular Probes; diluted 1:500) or anti-rabbit Ig-Al-dase II antiserum (33) was diluted 1:2000. Antibodies were incubated for 2 h at room temperature. After three washing steps in 0.1% BSA in PBS, bound antibodies were detected with secondary antibodies: anti-mouse Ig-Cy3 (Molecular Probes; diluted 1:500) or anti-rabbit Ig-Al-
lso expressed UGT-122H is inactive, but overexpressed UGT-122H in transfectants is active (see “Results”).

RESULTS

Molecular Cloning and Expression of the Hamster UGT—To isolate a hamster UGT cDNA, a cDNA library from CHO K1 cells was screened using colony hybridization with a digoxigenin-labeled probe derived from the 3’ part (nucleotides 640–1182) of the coding region of isoform 1 of the human UGT (UGT1; Ref. 9; accession number D84454). One of 10 colonies that gave positive signals on duplicate filters pABE-SO1 harbored an insert of sufficient size (~1.4 kb) to encode a full-length UGT. Sequencing revealed an open reading frame (ORF) of 1197 bp, predicting a protein of 398 amino acids with a calculated molecular mass of 41.5 kDa. Since only nine nucleotides upstream of the translation initiation codon were present (see Fig. 1A), 5’-rapid amplification of cDNA ends was used to search for additional start sites. 295 nucleotides were analyzed, but no in frame ATG was found. The 3’-noncoding region contained a polyadenylation signal AATAAA starting 23 bp upstream of the poly(A) tail.

In Fig. 1B, the amino acid sequence deduced from the newly cloned gene is aligned to UGT sequences from human (9, 12), mouse (11), and yeast (13). The high conservation between the mammalian genes (overall identity >93%; see Fig. 1, B and C) strongly suggests that the new cDNA represents the hamster homologue of UGT. In humans, two UGT cDNAs that result from alternative splicing have been isolated. The two predicted UGT proteins differ only at their C termini (see Fig. 8) (12). Interestingly, the hamster UGT is similar to human UGT2, while the recently cloned murine UGT resembles human UGT1 (see Fig. 1B). RT-PCR was used in this study to search for alternative UGT splice variants in CHO cells, but none were isolated.

Northern blot analysis was used to examine UGT gene transcripts in the Lec8 mutant (Fig. 1D). A digoxigenin-labeled cRNA transcribed from the 5’-coding region (nucleotides 32–503) of the hamster UGT was used as a probe. Bands of about 2.4 and 1.4 kb represent UGT gene transcripts in CHO K1 cells. Only the higher molecular weight band could be detected in the Lec8 mutant, and this was present at drastically reduced intensity, as revealed by the glyceraldehyde-3-phosphate dehydrogenase control. These data suggest reduced production or stability of UGT gene transcripts in Lec8.

The ORF Contained in pABE-SO1 Encodes a Functional UGT—To test functional activity, the ORF contained in pABE-SO1 was amplified by PCR and subcloned into pcDNA3-FLAG and pEVRF0-HA, that direct the expression of N-terminally

| Clone       | Nucleotide change | Amino acid change | Protein expressed | Transport activity | Localization |
|-------------|-------------------|-------------------|-------------------|-------------------|-------------|
| Lec8        | Δ 275–374         | E92stop           | +                 | –                 | ER          |
| Lec8.5H     | Δ 636–638         | ΔS213             | +                 | –                 | Golgi       |
| Lec8.1C     | G844A             | 281D              | +                 | +                 | Golgi       |
| LEC10.Lec8  | T364C             | Y122H             | +                 | +                 | Golgi       |

* Endogenously expressed UGT-122H is inactive, but overexpressed UGT-122H in transfectants is active (see “Results”).
UDP-Galactose Transporter Mutations

Fig. 5. Lec8 mutations affect primary sequence elements that are invariant in mammalian NSTs. Primary sequence stretches that carry a lec8 mutation in hamster UGT are shown together with the corresponding domains of other NSTs. Point mutations identified in the hamster UGT are given in the top lane. Conserved amino acid residues are shown on a black background. Numbers in parentheses indicate the amino acid residue with which the conserved domains start. Accession numbers are given at the end of each lane. The alignment demonstrates that amino acids changed in Lec8 mutants reside in highly conserved domains present in all mammalian NSTs. The three positions are also preserved in the S. pombe UGT but not in yeast transporters of different substrate specificity (UDP-GlcNAc transporter from S. cerevisiae; UDP-GlcpA transporter from L. donovani).

(PSA-NCAM), which is present in CHO wild type cells (Fig. 2) (34). The defect in UDP-galactose-transport in Lec8 mutants precludes the addition of galactose to glycoconjugates and consequently also prevents the addition of sialic acid, resulting in a PSA-negative phenotype in Lec8 cells (see Fig. 2). Both the epitope-tagged constructs and the UGT without an epitope tag rescued the lec8 mutation, leading to reappearance of PSA, which was specifically detectable with mAb 735 (28). To demonstrate specificity, the FLAG-UGT construct was also expressed in Lec2 cells (6B2; see Fig. 2). Due to a defect in CST that severely reduces Golgi import of CMP-sialic acid (35), Lec2 cells are PSA-negative. In accordance with the high substrate specificity of NSTs, 6B2 cells could not be rescued with the hamster UGT cDNA (see Fig. 2). In all samples, the specificity of the PSA signal was shown by EndoNE digestion, which degrades PSA and abolishes binding of mAb 735 (36). Expression levels of the recombinant epitope-tagged proteins were monitored by Western blot analysis using the anti-FLAG mAb M5 and the anti-HA mAb 12CA5. The data presented in Fig. 2 (bottom panel) demonstrate that, while epitope-tagged transporters were expressed in both the Lec8 clone Lec4.8.7A and the Lec2 clone 6B2, rescue of PSA expression by transfected UGT occurred only for the Lec8 phenotype, while the Lec2 phenotype was not corrected.

Molecular Analysis of UGT in Independent Lec8 Mutants—To investigate the molecular basis of UGT defects in nine independent Lec8 CHO mutants, UGT gene expression was examined by RT-PCR in wild type and mutant cells. Reverse transcription was performed with the gene-specific primer SO47 that annealed to the 3'-untranslated region (see Fig. 1A). Nested PCR was performed with primer pairs SO45/ SO46 in the first and SO48/SO11 in the second round to obtain the −1.2-kb UGT ORF. A strong band of −1.2 kb was present in CHO K1 wild type cells and in the mutants Lec8.5H, Lec8.1C, and LEC10.Lec8 interfere with the expression of stable proteins, site-directed mutagenesis was used to introduce each change into the wild type UGT sequence. Both FLAG- and HA-tagged hamster UGT constructs were subjected to site-directed mutagenesis and transiently expressed in COS-7 cells. UGT expression was analyzed by Western blotting 48 h post-transfection using the anti-HA mAb 12CA5 (Fig. 4B) and the anti-FLAG mAb M5 (Fig. 4A). Wild type and mutant UGTs (∆S213, G281D, Y122H) migrated with an apparent molecular mass of about 42 kDa. Other bands detected by mAb 12CA5 were not related to the HA tag, since they were present in all lanes including control. Interestingly, the truncated protein FLAG-UGT-E92stop isolated from clone Lec8 was also stable and migrated with an apparent molecular mass of 22 kDa.

Lec8 Mutations Occur at Highly Conserved Amino Acid Residues—In Fig. 5, the three segments of the hamster UGT sequence that harbor a lec8 missense mutation are aligned with the corresponding homologous region of other NSTs. It can be seen that the amino acid residues mutated in Lec8 mutants are invariant in all mammalian transporters, irrespective of their substrate specificity. Moreover, the three positions appear to be conserved in the UGT from S. pombe, and two are present in the GDP-mannose transporter isolated from Saccharomyces cerevisiae, but different residues occur in the UDP-GlcNAc transporter from Kluyveromyces lactis or the GDP-mannose transporter from Leishmania donovani.

The high conservation among mammalian transporters of different substrate specificities strongly suggests that lec8 mutations affect structural elements that fulfill essential functions in this protein family. To test this hypothesis, the Lec8 UGT mutations were introduced into a transporter of different
substrate specificity, the HA-tagged murine CST. The functional activity of CST mutants was tested in the Lec2 mutant 6B2 (21) by monitoring PSA reexpression after transfection (Fig. 6). Both Lec4.8.7A and 6B2 mutants are PSA-negative due to their respective NST defect, but each expresses PSA after transfection with the appropriate epitope-tagged NST cDNA (wild type). Deletion of the invariant serine-residue (D\textsubscript{S213} in UGT; D\textsubscript{S188} in CST) and exchange of the invariant glycine to aspartic acid (G\textsubscript{281}D in UGT; G\textsubscript{256}D in CST) abolished the ability of these cDNAs to rescue PSA expression, and thus transport of both UDP-galactose and CMP-sialic acid was inhibited by these mutations. By contrast, the amino acid exchange Y\textsubscript{122}H identified in the LEC10.Lec8 mutant did not lead to an inactivated UGT; nor did the corresponding Y\textsubscript{98}H change lead to inactivation of CST (Fig. 6A).

The T364C mutation was the only change in sequence in LEC10.Lec8 UGT cDNAs, and the same mutation was shown to be present in genomic DNA of LEC10.Lec8 cells. When 100 base pairs surrounding this site were amplified by PCR of genomic DNA from LEC10.Lec8 and control cells, LEC10.Lec8 DNA gave two products; about 50% carried the wild type sequence with T at nucleotide position 364, which was also present in genomic DNA of CHO K1 and the LEC10 parent of LEC10.Lec8, and about 50% carried the point mutation T\textsubscript{364}C, which causes the exchange Y\textsubscript{122}H. Therefore, the mutation T\textsubscript{364}C is present in the genomic DNA of LEC10.Lec8 cells and must represent the active UGT allele, since no cDNAs with T at position 364 were found by RT-PCR of LEC10.Lec8 mRNA.

Because UGT-122H in the LEC10.Lec8 genome is present on the LEC10 mutant background, it seemed possible that the dominant mutation LEC10, which causes the \textit{de novo} expression of \beta-4-N-acetylglucosaminyltransferase III and introduces a bisecting GlcNAc into the core of complex N-glycans (37),
might somehow cause the UGT-122H mutation not to be phenotypically expressed. However, when LEC10.Lec8 cells were used as hosts to express the mutant UGT-122H cDNA, correction of the PSA-negative phenotype was again observed, demonstrating that complementation by UGT-122H also occurred in this background (Fig. 6B). The lower PSA signal obtained after transient transfection of LEC10.Lec8 cells is caused by LEC10.Lec8 mutant populations generating PSA-positive revertants with considerable frequency and requiring daily panning to avoid background staining, which interferes with optimal transfection conditions. Nevertheless, Western analysis showed readily detectable expression of each transfected construct in all cells (see bottom panels). It is therefore apparent that expression of EndoNE-sensitive PSA did not depend on differential levels of expression of transfected plasmids. Thus, it seems likely that the ability of UGT-122H to function is due to overexpression in transfectants. Whereas overexpression of the two NST mutants (∆S213 in UGT; ∆S188 in CST and G281D in UGT; G256D in CST) did not obscure their inability to transport nucleotide-sugar, overexpression of the Y122H mutation allowed this mutant UGT-122H (and the corresponding CST-98H) to function. By contrast, endogenous levels of the UGT-122H expressed in LEC10.Lec8 cells are clearly inactive (Fig. 6B). A similar expression-dependent activity has most recently been described for variants of the yeast GDP-mannose transporter that exhibit single amino acid exchanges in a highly conserved primary sequence motif (38).

**Subcellular Localization of Wild Type and Mutant NSTs—** Biochemical studies carried out on isolated organelles have demonstrated that UDP-galactose and CMP-sialic acid transport activity are strictly associated with Golgi vesicles (39). Later studies confirmed these results by demonstrating Golgi localization of epitope-tagged versions of both UGT and CST (40). To find out whether UGT and CST carrying a lec8 mutation are correctly targeted inside the cell, indirect immunofluorescence was used to compare the subcellular localization of HA-tagged wild type and mutant proteins. Constructs were transiently expressed in CHO K1, and 30 h later, cells were fixed in paraformaldehyde and permeabilized with saponin, and the localization of epitopes was determined by indirect immunofluorescence with the anti-HA mAb 12CA5. Simultaneously, the cells were stained with an antiserum directed against α-mannosidase II, a known marker for the Golgi apparatus (33). In accordance with the earlier data (26, 40) Golgi localization was found for the wild type transporters (Fig. 7), and the same strict co-localization with α-mannosidase II was observed for UGT and CST proteins with a missense mutation. Lec8 missense mutations do not interfere with the signals that are responsible for Golgi destination and do not appear to significantly affect the folding of UGT. In contrast, the FLAG-tagged mutant UGT-E92stop isolated from subclone Lec8 showed an ER-like distribution.

**DISCUSSION**

In order to define structure/function relationships in the NST family of proteins, we have identified mutations that weaken or inactivate mammalian CST (21) and UGT (this study). The hamster UGT gene was cloned from a CHO cDNA library, and epitope-tagged versions of the encoded protein were shown to complement the Lec8 CHO glycosylation mutant (see Fig. 2). The conservation between UGT sequences of different animal species is high (>93% amino acid identity; see Fig. 1, B and C). However, while two UGT isoforms exist in humans (12) only one form was found in CHO cells (this study) and in mice (11). Moreover, the mouse UGT resembles human UGT1, while the CHO gene encodes a protein most homologous to UGT2. Differences in human UGT isoforms are confined to the extreme C terminus (see Fig. 1B) and result from alternative splicing as schematically shown in Fig. 8. The human UGT gene contains five exons, and the ORF contains stop codons in exons 4 and 5. Translation of exons 1–4 results in isoform 1. If a splice consensus motif contained in exon 4 is used, the 3′ part of exon 4 is removed, and exon 5 may be translated to generate UGT2.
CST-98H were active following transfection and were able to complement the defect in Lec8 and Lec2 cells, respectively. The point mutation T364C, which gives rise to UGT-122H, is present in the genomic DNA of LEC10.Lec8 cells, and the rest of the UGT sequence is identical to the UGT from three other CHO lines. Since LEC10.Lec8 cells are PSA-negative (see Fig. 6F) and express normal levels of UGT-122H mRNA (not shown), the loss of PSA in Pro5LEC10.Lec8 cells must be caused by the point mutation Y122H. Defective UGT transport activity is, however, manifest only if the mutant gene is expressed at endogenous levels. The gain of function in transfected cells is most likely due to overexpression, which may improve folding processes, resulting in stabilized transport units. Support for this interpretation comes from a recent study by Gao et al. (38). The authors identified variants of the yeast GDP-mannose transporter (VRG4), whose GDP-mannose transport activity was severely reduced. However, if the mutant proteins were expressed at higher levels by using strong promoter sequences, transport activity could be restored, and yeast cells grew normally. In the in vitro situation, the transport defect could also be compensated for by increased GDP-mannose concentration, indicating a reduced affinity for the nucleotide-sugar. Mutants described by Gao et al. (38) map to a region conserved in both confirmed and putative GDP-mannose transporter ORFs but not in NSTs of a different specificity. The analysis of these mutants allowed the authors to identify a sequence motif that is involved in the binding of GDP-mannose.

In mutant Lec8.5H, the in frame excision of the triplet 636–638 removes the highly conserved serine at position 213 in hamster UGT. This serine is the starting point of a homology box consisting of 13 amino acids. 11 positions are invariant in all mammalian transporters cloned so far (see Fig. 1B). The importance of this region was previously identified in lec2 CST mutations, since the mutant CST-G189E (G189 in CST is identical to G214 in the hamster UGT) was shown to inactivate the mutants allowed the authors to identify a sequence motif that is involved in the binding of GDP-mannose.

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