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Expression and Activity of Chimeric Molecules between Human UDP-Galactose Transporter and CMP-Sialic Acid Transporter[1]

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Human UDP-galactose transporter (hUGT1) and CMP-sialic acid transporter (hCST) are related Golgi proteins with eight putative transmembrane helices predicted by computer analysis. We constructed chimeric molecules in which segments of various lengths from the C- or N-terminus of hUGT1 were replaced by corresponding portions of hCST. The chimeras were transiently expressed in UGT-deficient mutant Lec8 cells, and their UGT activity was assessed by the binding of GS-II lectin to the transfected cells. The replacement of either the N- or C-terminal cytoplasmic segment by that of hCST did not affect the expression or activity of hUGT1. A chimera in which the eighth helix and the C-terminal tail were replaced also retained the UGT activity, indicating that this helix is not involved in the determination of substrate specificity. In contrast, three types of chimeras, in which the first helix, the first and the second helices, and a segment from the seventh helix to the C-terminus were replaced, respectively, were expressed very infrequently in the transfected cells, and had no UGT activity. They are likely folded incorrectly and degraded by a quality-control system, since the amounts of their mRNAs were normal and the proteins were mainly localized in the ER. The first and the seventh helices are important for the stability of the transporter protein.

Key words: chimeric transporter, CMP-sialic acid transporter, Golgi apparatus, nucleotide-sugar transporter, UDP-galactose transporter.

Glycoproteins and glycolipids are variously modified in the Golgi apparatus by sequential addition of sugar residues to their oligosaccharide chains. They are then transported to their proper destinations, and fulfill a variety of functions including those as specific ligands in cellular recognition processes. The nucleotide-sugar transporters are indispensable components of this glycosylation system, in which they deliver the substrates for glycosylation reactions into the lumen of the Golgi apparatus (*1, 2*). Thus, for instance, mouse Had-1 mutant cells defective in UDP-galactose (UDP-Gal) transporter (UGT) show a pleiotropic aberrance in glycoproteins and glycolipids characterized by a severe reduction in the amount of sialylated glycoconjugates with a concomitant increase in that of *N*-acetylglucosamine (GlcNAc)-terminated glycoconjugates (*3, 4*). Interestingly, the Galβ1-4GlcNAc linkage in *N*-linked oligosaccharides was reduced but persisted in Had-1 cells, while the Galα1-3Gal linkage was totally absent (*4*). This raises the possibility that the spectrum of glycoconjugates synthesized by a given cell may be altered by regulating the delivery of nucleotide-sugars into the Golgi lumen, or in other words, by regulating the activity of nucleotide-sugar transporters (*1, 2*). In order to gain insight into this challenging aspect of glycoconjugate regulation, however, we first have to learn much about the structure and mode of action of nucleotide-sugar transporters and the regulation of their expression and function.

We have recently cloned the cDNA for human UGT by phenotypic correction of UGT-deficient Had-1 mutant cells (*5, 6*). Through this and subsequent studies, we obtained three major human nucleotide-sugar transporter cDNAs, namely human UDP-Gal transporter (hUGT1 and 2 isoforms) (*6, 7*), human CMP-sialic acid transporter (hCST) (*7, 8*), and human UDP-GlcNAc transporter (*9*). Transporters for these nucleotide-sugars from other mammalian species (*10-12*) or from yeast (*13, 14*), and GDP-mannose transporter from yeast (*15*) and *Leishmania* (*16*) have also been cloned in several laboratories, including ours. These transporters constitute a family of related membrane proteins. Each member of the family is highly specific for a

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Abbreviations: CHO, Chinese hamster ovary; CST, CMP-sialic acid transporter; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GlcNAc, *N*-acetylglucosamine; GS-II, *Griffonia simplicifolia* lectin II; HA, hemagglutinin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; SLO, streptolysin O; UDP-Gal, UDP-galactose; UGT, UDP-galactose transporter.

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particular nucleotide-sugar, but the structural bases for the transport function and substrate specificity remain to be elucidated.

Human UGT1 and hCST are closely related multiple-membrane-spanning proteins with 43% identity in amino acid sequence and eight putative transmembrane helices predicted by computer analysis (*6, 11*). Human UGT1 and hCST proteins are strictly localized in the Golgi membranes (*8, 17*), and we showed previously that the C-terminal portion of hCST is exposed to the cytoplasmic surface of the Golgi membrane (*8*). In a recent report on murine CST, the authors confirmed this intramembrane orientation and further proposed a model for its structure in which the membrane is spanned 10 times (*18*). These lines of evidence which suggest structural similarity between hUGT1 and hCST prompted us to study the expression and properties of chimeric proteins between hUGT1 and hCST that were designed for obtaining information concerning the structure-function relationship of hUGT1. Studies on hUGT1/hCST chimeras indicated that the first and the seventh transmembrane helices are important for the transport function and targeting to the Golgi membranes.

MATERIALS AND METHODS

Construction of Chimeric cDNAs between hUGT1 and hCST—Chimeric cDNAs between hUGT1 and hCST were constructed by a sequential PCR procedure (*19*) using the primers listed in Table I, and were inserted into the mammalian expression vector pMKIT-neo as described before (*6, 7*). For example, to construct cDNA coding for chimera UC-8, which codes for the N-terminal 341 amino acids of hUGT1 joined to the C-terminal 21 amino acids of hCST, each part was first amplified separately with hUGT1 cDNA and hCST cDNA as templates, and UGT(F)/UC-8(R) and UC-8(F)/CST(R) as primer sets, respectively. Since UC-8(R) and UC-8(F) are complementary to each other, the amplified products are overlapping at one of the two ends, so that the second PCR using the mixture of the first PCR products as templates and UGT(F) and CST(R) as a primer set resulted in the desired chimeric cDNA. Chimeras UC-7 and UC-6 were constructed in the same way except that primers UC-7(F or R) and UC-6(F or R), respectively, were used instead of UC-8(F or R). An influenza virus hemagglutinin (HA) epitope-tag coding for the sequence YPYDVPDYA was then attached to the C-termini of UC-6, UC-7, and UC-8 chimeras by PCR using UGT(F) and UC-HA(R) as primers. Chimeras CU-1 and CU-2, in which the N-terminal portions of hUGT1 are replaced by those of hCST, were constructed in a similar manner using CST(F)/CU-1(R) or -2(R) and CU-1(F) or -2(F)/UGT(R) as primer sets in the first PCR, and CST(F) and UGT(R) as primers in the second PCR. Chimera CU-0 was obtained by one-step PCR using hUGT1 cDNA and CU-0(F)/UGT(R) as a template and primers, respectively.

Addition of the HA-tag to either the N- or C-terminus of hUGT1 was carried out by PCR using either UGT-HA(F)/UGT(R) or UGT(F)/UGT-HA(R) as the primer set.

Nucleotide sequences of all the constructs were confirmed before use in transfection experiments.

Cell Culture and Transfection—Chinese hamster ovary (CHO)-K1 and its UGT-defective mutant, Lec8, were maintained in minimum essential medium alpha supplemented with 10% fetal calf serum (growth medium). To obtain Lec8/hUGT1, a stable hUGT1 transformant of Lec8, Lec8 cells were transfected with pMKIT-neo-hUGT1 (*6, 7*) using LipofectAMINE reagent (Life Technologies, Rockville, MD) following the manufacturer's instructions. A transformant was selected in growth medium containing 1.2 mg/ml of G418 and cloned by limiting dilution.

Staining with Lectin and Antibody—Transfection was performed with LipofectAMINE reagent following the manufacturer's instructions. Twenty-four hours after transfection, cells were transferred onto a chamber slide (Nalge Nunc International, Rochester, NY), incubated overnight, and then fixed with methanol at −20°C for 6 min. The fixed cells were blocked with 3% BSA/phosphate-buffered saline (PBS) for 30 min at room temperature, incubated with primary antibody diluted in 3% BSA/PBS for 1 h at room temperature, and washed three times with 3% BSA/PBS. The cells were then incubated with an appropriate secondary antibody and 25 μg/ml fluorescein isothiocyanate (FITC)-conjugated GS-II (*Griffonia simplicifolia* lectin II; EY Laboratories, San Mateo, CA) for 1 h at room temperature and washed twice with 3% BSA/PBS, twice with PBS, and once with water, and then

TABLE I. **PCR primers used in this work.**

Primer	Sequence
UGT(F)	AAAAGCTGCGGAATTCCAACATGGCAGCGGTTGGGGCTGGT
CST(R)	GGCTCGAGCGGCCGCTCACACACCAATAACTCTCTCCTTTG
UC-8(F)	CTGTCTACCTCTACAGCCTTCCCAGACAAGACACTACATCCATCCAAC
UC-8(R)	GTTGGATGGATGTAGTGTCTTGTCTGGGAAGGCTGTAGAGGTAGACAG
UC-7(F)	CTACTGGTGGCTGTGGTTGTCAAGTACACAGACAACATCATGAAAGG
UC-7(R)	CCTTTCATGATGTTGTCTGTGTACTTGACAACCACAGCCACCAGTAG
UC-6(F)	GTACCGCCGTGGCCACCCGTGGTTTTTTCTATGGTTACACATATTATG
UC-6(R)	CATAATATGTGTAACCATAGAAAAAACCACGGGTGGCCACGGCGGTAC
UC-HA(R)	GGCTCGAGCGGCCGCTCATGCGTAGTCAGGGACGTCGTAAGGGTACACACCAATAACTCTCTC
CST(F)	CTAAAAGCTGCGGAATTCCATGGCTGCCCCGAGAGACAATGTC
UGT(R)	GGCTCGAGCGGCCGCTCACTTCACCAGCACTGACTTTGGCA
CU-0(F)	GCGAATTCACCATGGCTGCCCCGAGAGACAATGCTCACAGGCGCCTGAAGTACA
CU-1(F)	CAGACAAAGAACTCTACTTTTCAACGACTGCTGTGGTCATGGCGGAAG
CU-1(R)	CTTCCGCCATGACCACAGCAGTCGTTGAAAAGTAGAGTTCTTTGTCTG
CU-2(F)	GCAGCAGTGTACCAGGTGACCTACCAGCTGAAGATCCTGACCAC
CU-2(R)	GTGGTCAGGATCTTCAGCTGGTAGGTCACCTGGTACACTGCTGC
UGT-HA(F)	GCGAATTCAACATGTACCCTTACGACGTCCCTGACTACGCAGCGGTTGGGGCTGGT
UGT-HA(R)	CTGGCGGCCGCTTAGGCGTAGTCAGGGACGTCGTAAGGGTACTTCACCAGCACTGACTTT

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mounted with Permafluor (IMMUNOTECH-A COULTER COMPANY, Marseille, France). Fluorescence labeling was visualized under a Carl Zeiss laser scanning confocal microscope (LSM510).

Selective permeabilization of cells for examination of N- and C-terminal topology was carried out as described previously (*8*). Briefly, the cells were first fixed with 3.7% formaldehyde at room temperature for 30 min, then incubated at 4°C for 10 min with 200 U/ml of streptolysin O (SLO) which had been preactivated by treating with 10 mM dithiothreitol at 0°C for 10 min, and the cells were further incubated in 10 mM dithiothreitol in PBS at 37°C for 20 min. After washing twice with PBS, indirect immunofluorescence was performed as described above.

Antibodies—Rabbit anti-hUGT1 antibody was prepared as described before (*17*). Rat anti-HA monoclonal antibody (clone 3F10) was purchased from Roche Diagnostics (Basel, Switzerland). Rabbit anti-α-mannosidase II antibody was kindly provided by Dr. K. Moremen (University of Georgia, Athens, GA) (*20*). Rabbit anti-calnexin antibody was a kind gift from Dr. H. Taira of Iwate University. Secondary antibodies used for indirect immunofluorescence were as follows: Cy3-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA), FITC-conjugated goat anti-rat IgG antibody (ICN Pharmaceuticals, Costa Mesa, CA), Alexa594 (a substitute for texas red)-conjugated goat anti-rat IgG antibody, Alexa488 (a substitute for FITC)-conjugated goat anti-rabbit IgG antibody, and Alexa546 (a substitute for tetramethylrhodamine)-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). The secondary antibodies used for Western blot analysis were horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody and HRP-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blot Analysis—Cells were lysed in lysis buffer (0.2% Nonidet P-40/10 mM HEPES-Tris, pH 7.4/10 mM KCl/0.1 mM EDTA/2 μg/ml each of aprotinin, pepstatin A and leupeptin A/0.5 mM phenylmethanesulfonyl fluoride), left on ice for 30 min, and then centrifuged at 10,000$\times g$ for 10 min. The supernatant was used as a cell lysate and the amount of protein in the lysate was determined using BCA reagent (Pierce Chemical, Rockford, IL). Cell lysates (50 μg) were fractionated by 10% SDS-PAGE and electrotransferred to a Hybond-P (poly(vinylidene difluoride)) membrane (Amersham Pharmacia Biotech. AB, Uppsala, Sweden) with a semi-dry blotting system (EB-150; ADVANTEC-TOYO, Tokyo). After blocking with 5% skim milk and 0.2% Tween 20 in 20 mM Tris-HCl (pH 7.6) and 137 mM NaCl for 1 h, the membrane was incubated for 1 h with an appropriate primary antibody diluted in the same solution, followed by incubation with an appropriate secondary antibody for 1h. The proteins recognized by the antibodies were detected using a RENAISSANCE Western Blot Chemiluminescence Reagent Plus Kit (NEN Life Science Products, Boston, MA).

Northern Blot Analysis—Poly(A)$^+$ RNA was extracted from cells transfected with appropriate expression plasmids using QuickPrep Micro mRNA Purification Kits (Amersham Pharmacia Biotech. AB). Poly(A)$^+$ RNA (2.5 μg from plasmid-transfected cells or 1 μg from Lec8/hUGT1 cells) was fractionated by electrophoresis in a 1.3% agarose gel containing formaldehyde and blotted onto a Hybond-N$^+$ (nylon) membrane (Amersham Pharmacia Biotech. AB) by capillary blotting. The full-length-hUGT1 open reading frame was amplified by PCR using hUGT1 cDNA and suitable primers, and then ^{32}P-labeled using an RTG DNA Labeling Kit (Amersham Pharmacia Biotech. AB). Prehybridization and hybridization (2 h) were carried out in ExpressHyb solution (Clontech Laboratories, Palo Alto, CA) as recommended by the manufacturer. Radioactivity was captured and visualized with Fuji BAS2000 (Fuji Photo Film, Tokyo). Reprobing was performed following the instructions of the manufacturer using β-actin cDNA (Clontech Laboratories).

RESULTS

Orientation of the N- and C-Termini of hUGT1 Relative to the Golgi Membranes—To ascertain the structural similarity between hUGT1 and hCST, we first examined the membrane topology of the N- and C-termini of hUGT1.

An HA-tag was introduced at either the N-terminus (hUGT1-nHA; Fig. 1, A-F) or the C-terminus (hUGT1-cHA; Fig. 1, G-L) of hUGT1 cDNA, and then Lec8 cells were transfected with these HA-tagged hUGT1 cDNAs. Two days after transfection, cells were treated with SLO for selective permeabilization of their plasma membrane, or with SLO and Triton X-100 for non-selective permeabilization of cellular membrane systems. The permeabilized cells were then examined for α-mannosidase II and for expression of the HA-tagged hUGT1 by indirect immunofluorescence.

The cells were positively stained with anti-α-mannosidase II antibody only when they were non-selectively permeabilized (Fig. 1, F and L as compared with Fig. 1, C and I). This is because the cognate epitope of α-mannosidase II is located in the lumenal space of the Golgi apparatus, and the Golgi membrane is impermeable to the antibody after selective permeabilization by SLO treatment (*8*). In contrast, both N- and C-terminally HA-tagged hUGT1 were stained with anti-HA antibody similarly under the conditions of selective permeabilization (Fig. 1, B and H) and of non-selective permeabilization (Fig. 1, E and K). This clearly indicates that both the N- and C-termini of hUGT1 face toward the cytosol. We showed previously that the C-terminus of hCST faces the cytosol (*8*), and more recently, the C- and N-termini of murine CST have been reported to face the cytosol (*18*). It has also been established that hUGT1 and hCST show similar hydropathy profiles (*2*). The present results further substantiate their structural similarity.

Synthesis of Chimeric cDNAs between hUGT1 and hCST—To examine the role of cytoplasmic ends and transmembrane helices of hUGT1, we synthesized chimeric DNAs with hUGT1 and hCST sequences as illustrated in Fig. 2 by a sequential PCR method. UC-8, UC-7, and UC-6 are chimeras in which C-terminal portions of hUGT1 are replaced by hCST sequences with an HA-tag attached to their C-terminus. CU-0, CU-1, and CU-2 are chimeras in which N-terminal portions of hUGT1 are replaced by hCST sequences. The figure also illustrates an eight-time transmembrane model for hUGT1 and hCST as predicted with the aid of the TMAP program (*6*), together with a 10-time transmembrane model recently proposed for murine CST (*18*). For convenience, we describe our results in terms of

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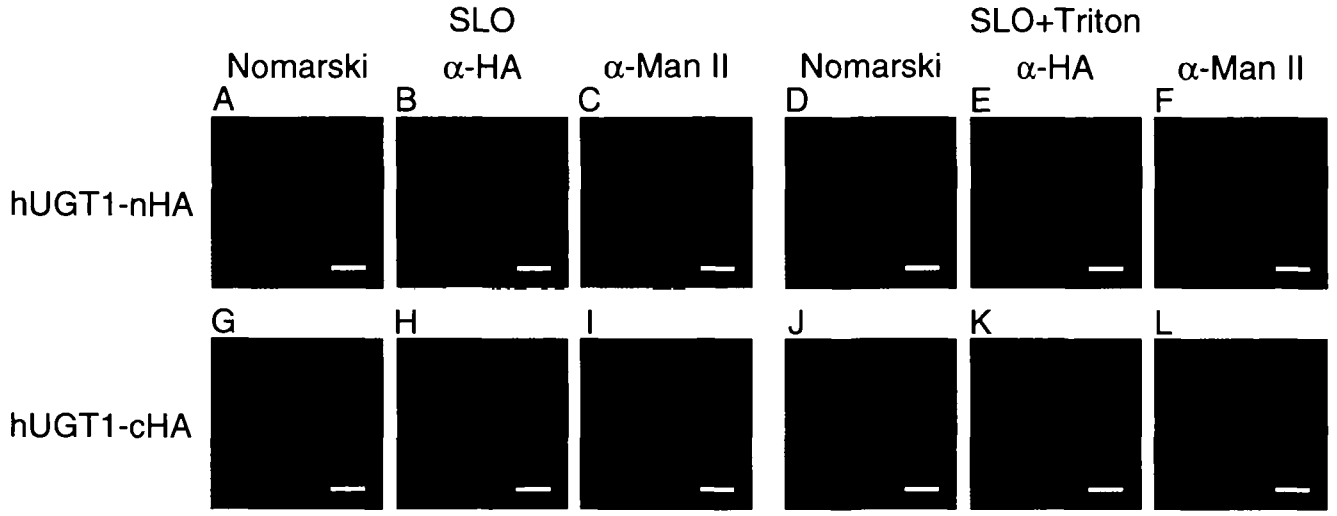

Fig. 1. **The membrane topology of the N- and C-termini of the hUGT1 protein.** Lec8 cells were transfected with hUGT1-nHA (A-F) or hUGT1-cHA (G-L). Two days after transfection the cells were treated with SLO (A-C, G-I) or SLO and Triton X-100 (0.1%) (D-F, J-L), and doubly stained with rat anti-HA monoclonal antibody (1:100), which was detected with FITC-conjugated goat anti-rat IgG antibody (1:100; B, E, H, K), and rabbit anti-α-mannosidase II antibody (1:1,000), which was detected with Alexa546-conjugated goat anti-rabbit IgG antibody (1:200; C, I, F, L). Nomarski images are shown in A, D, G, and J. Bar, 20 μm.

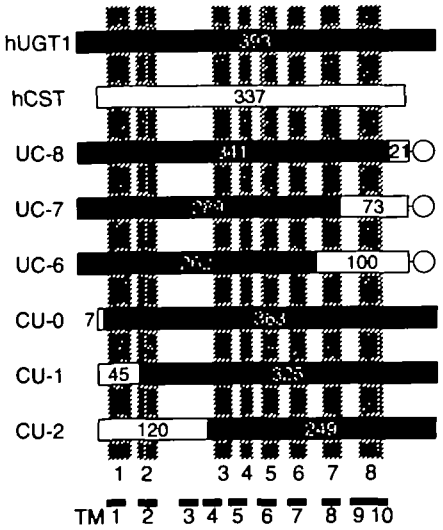

Fig. 2. **A schematic representation of chimeric constructs.** Black and white boxes represent the portions contributed by hUGT1 and hCST, respectively. The numbers in the boxes indicate the number of amino acid residues constituting each portion. The circles represent HA tags. Shaded boxes (1-8) indicate the positions of putative membrane-inserted helices. Solid bars at the bottom indicate the positions of the 10 transmembrane helices (TM1-TM10) proposed recently for murine CST.

the eight-time membrane-spanning model in this report.

Assessment of UGT Activity of Chimeric Proteins—To examine whether the chimeric proteins possessed UGT activity, we developed a quick test utilizing their transient expression in Lec8 cells, and show a representative analysis in Fig. 3. UGT-deficient Lec8 cells bound GS-II lectin, which recognizes terminal GlcNAc residues in cell-surface glycoconjugates, while CHO cells did not bind the lectin (*8*). This could be easily visualized by using FITC-conjugated GS-II as shown in Fig. 3, A-D and E-H. GS-II did not bind to Lec8/hUGT1, a stable hUGT1 cDNA transformant of Lec8, in which hUGT1 proteins were detected by staining with anti-hUGT1 antibody (Fig. 3, I-L). This reflects the recovery of parental phenotype as a result of the expression of hUGT1 in these cells. When a one-to-one mixture of Lec8 and Lec8/hUGT1 cells was examined (Fig. 3, M-P), half of the cells which expressed hUGT1 (Lec8/hUGT1 cells) were not stained by GS-II, while those not stained by anti-hUGT1 antibody (Lec8 cells) were positively stained by GS-II. Thus, there is no complication due to any diffusible factor in this assessment. Finally, when Lec8 cells were transfected with hUGT1 cDNA (Fig. 3, Q-T), and examined under conditions of transient expression, the cells expressing hUGT1 protein, which were stained by anti-hUGT1 antibody (about 70-80% of the total population), were not stained by GS-II. Transient expression of an active chimeric transporter would give a result similar to Fig. 3, Q-T, while GS-II binding would persist if an expressed chimeric protein had no UGT activity.

Effects of C-Terminal Substitution by hCST Sequences—To examine the roles of the C-terminal cytoplasmic tail and two transmembrane helices of hUGT1, we constructed three chimeric molecules in which segments of various lengths from the C-terminus of hUGT1 were replaced by hCST sequences.

When UC-8 cDNA, in which the C-terminal 52 amino acids (the C-terminal tail) of hUGT1 was substituted by the corresponding part of hCST (21 amino acids long), was introduced into Lec8 cells (Fig. 4, A-D), the chimeric protein was detected in about 70% of the cells, and GS-II was not bound by the cells expressing the protein. This indicates that UC-8 protein has UGT activity. Transfection of Lec8 cells with UC-7 also produced cells that expressed UGT-active chimeric proteins but did not bind GS-II (Fig. 4, E-H). The amount of the chimeric proteins seemed somewhat less than that in UC-8-transfected cells, but the result clearly demonstrated that the UC-7 chimera is active in UDP-Gal transport. Therefore, the C-terminal 104

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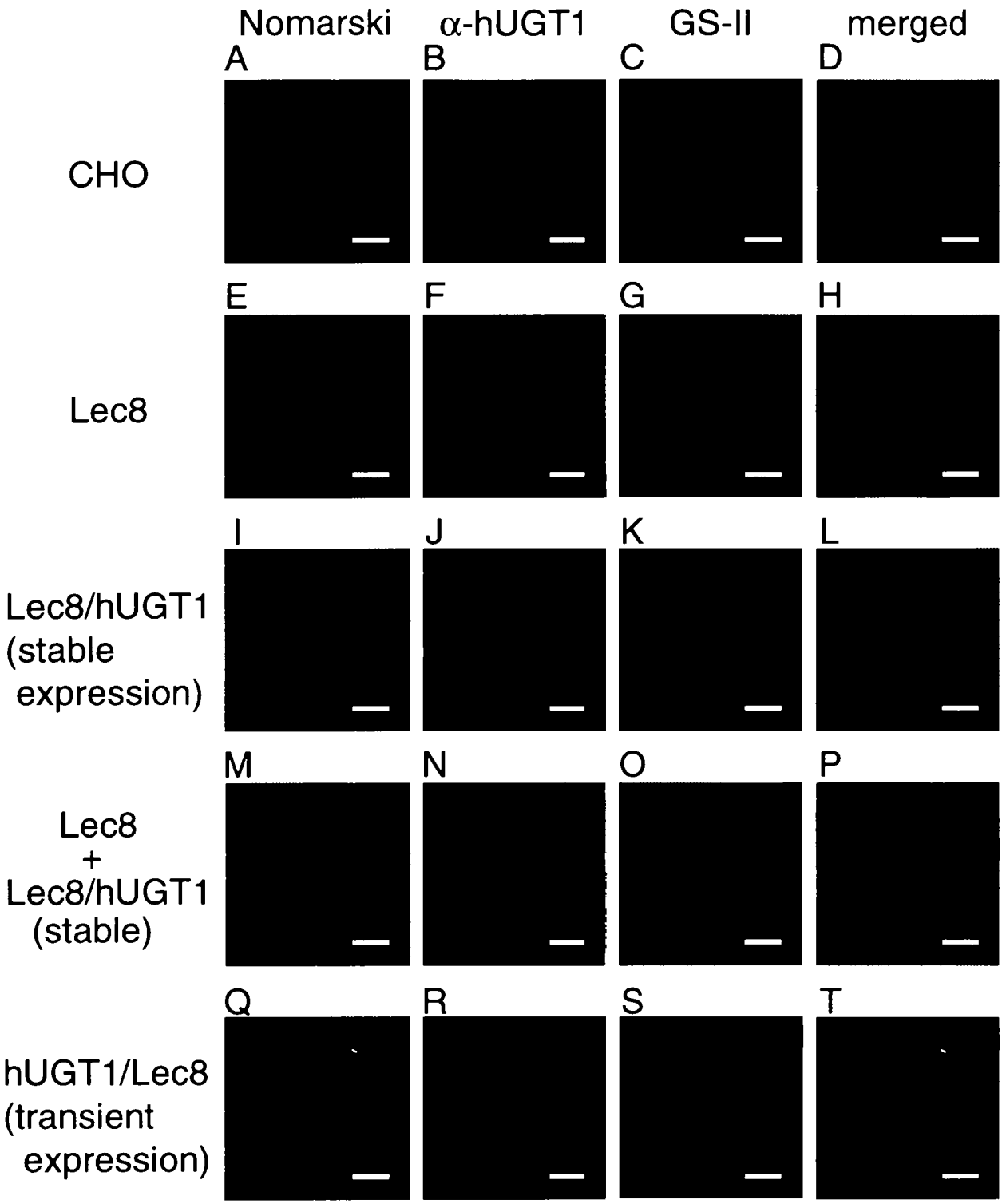

Fig. 3. **Detection of hUGT1-expression and *in situ* assessment of the UGT activity.** CHO cells (A-D), Lec8 cells (E-H), Lec8/hUGT1 cells (I-L), and a 1:1 mixture of Lec8 and Lec8/hUGT1 cells (M-P) were doubly stained with rabbit anti-hUGT1 antibody (1:100), which was detected with Cy3-conjugated goat anti-rabbit IgG antibody (1:200; B, F, J, N), and FITC-conjugated GS-II (C, G, K, O). Nomarski images are shown in A, E, I, and M. In D, H, L, and P, the three images on the left of each panel are merged. Lec8 cells were transfected with hUGT1 cDNA, and 2 days after transfection they were examined for expression of hUGT1 (R) and binding of FITC-conjugated GS-II (S) as described above. A Nomarski image of the cells (Q) and a merged image obtained from Q to S (T) are also shown. Bar, 20 μm.

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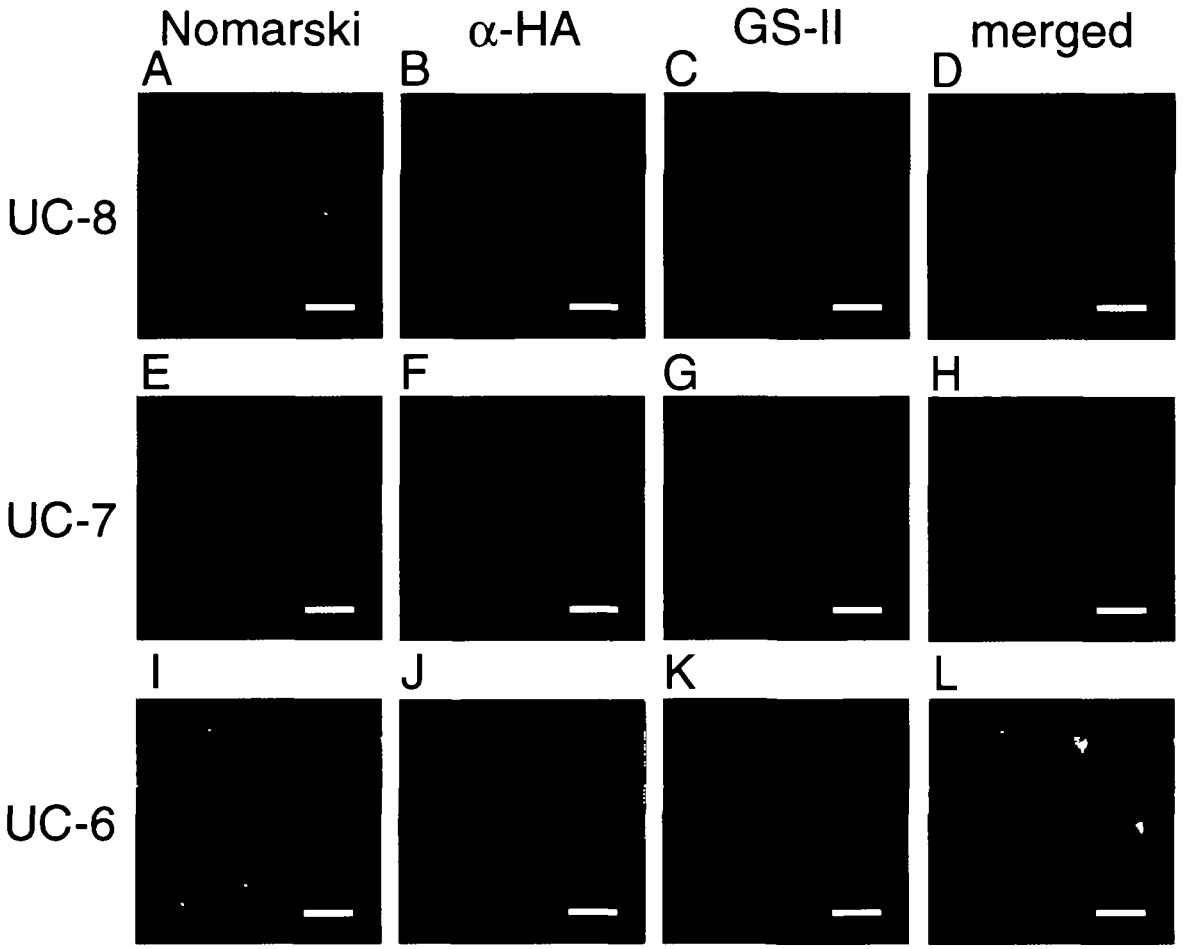

Fig. 4. **Expression and activity of chimeras: effects of C-terminal substitution of hUGT1 by hCST sequences.** Lec8 cells were transfected with chimeric cDNAs UC-8 (A-D), UC-7 (E-H), and UC-6 (I-L). Two days after transfection, the cells were doubly stained with rat anti-HA monoclonal antibody (1:100), which was detected with Alexa594-conjugated goat anti-rat IgG antibody (1:100; B, F, J), and FITC-conjugated GS-II (C, G, K). Nomarski images are shown in A, E, and I. In D, H, and L, the three images on the left of each panel are merged. Bar, 20 μm.

amino acids of hUGT1 (consisting of the C-terminal tail, the eighth helix, and the seventh loop) can be replaced by the corresponding portion of hCST (73 amino acids long) without impairing the UGT activity.

On the other hand, when Lec8 cells were transfected with chimera UC-6, cells expressing the chimeric protein were intensely labeled with FITC-GS-II (Fig. 4, I-L). This indicates that the UC-6 chimera is inactive in UDP-Gal transport. The chimeric protein was detected in only a few cells, and moreover, the chimeric proteins were not localized in the Golgi apparatus but were instead more diffusely distributed throughout the cytoplasm.

Effects of N-Terminal Substitution by hCST Sequences—Subsequently, we examined the effect of substituting N-terminal segments of hUGT1 by hCST segments to assess the roles of the N-terminal cytoplasmic region and two transmembrane helices of hUGT1. In CU-0, CU-1, and CU-2 chimeras, the N-terminal 30, 68, and 144 amino acids of hUGT1 (which represent the N-terminal cytoplasmic region, the N-terminal cytoplasmic region, the first helix, and the first loop, and the N-terminal cytoplasmic region, the first and the second helices, and most of the second loop, respectively) were replaced by the N-terminal 7, 45, and 120 amino acids of hCST, respectively (Fig. 2). Since these chimeric molecules retain the hUGT1 sequence at their C-termini, they can be detected by anti-hUGT1 antibody which was raised against the C-terminal peptide of hUGT1 protein (*17*). As shown in Fig. 5, CU-0 was expressed efficiently, and was active in UDP-Gal transport (Fig. 5, A-D). On the other hand, cells expressing CU-1 (Fig. 5, E-H) or CU-2 (Fig. 5, I-L) were detected very infrequently, and the cells expressing these chimeric proteins remained defective in UDP-Gal transport as judged by GS-II binding. The CU-1 and CU-2 proteins were not localized in the Golgi apparatus, but were instead distributed more diffusely throughout the cytoplasm. These results indicate that the N-terminal cytoplasmic region of hUGT1 may be replaced by hCST sequences without loss of the transport activity, but conservation of the first helix and/or the first loop is critical for the UGT function.

Western Blot Analysis of the Expression of Chimeric Proteins—Since some of the chimeric proteins were detected much more infrequently than others, the amounts and apparent molecular weights of chimeric proteins were examined by Western blot analysis (Fig. 6). Lec8 cells were transfected with chimeric cDNAs in the same way as

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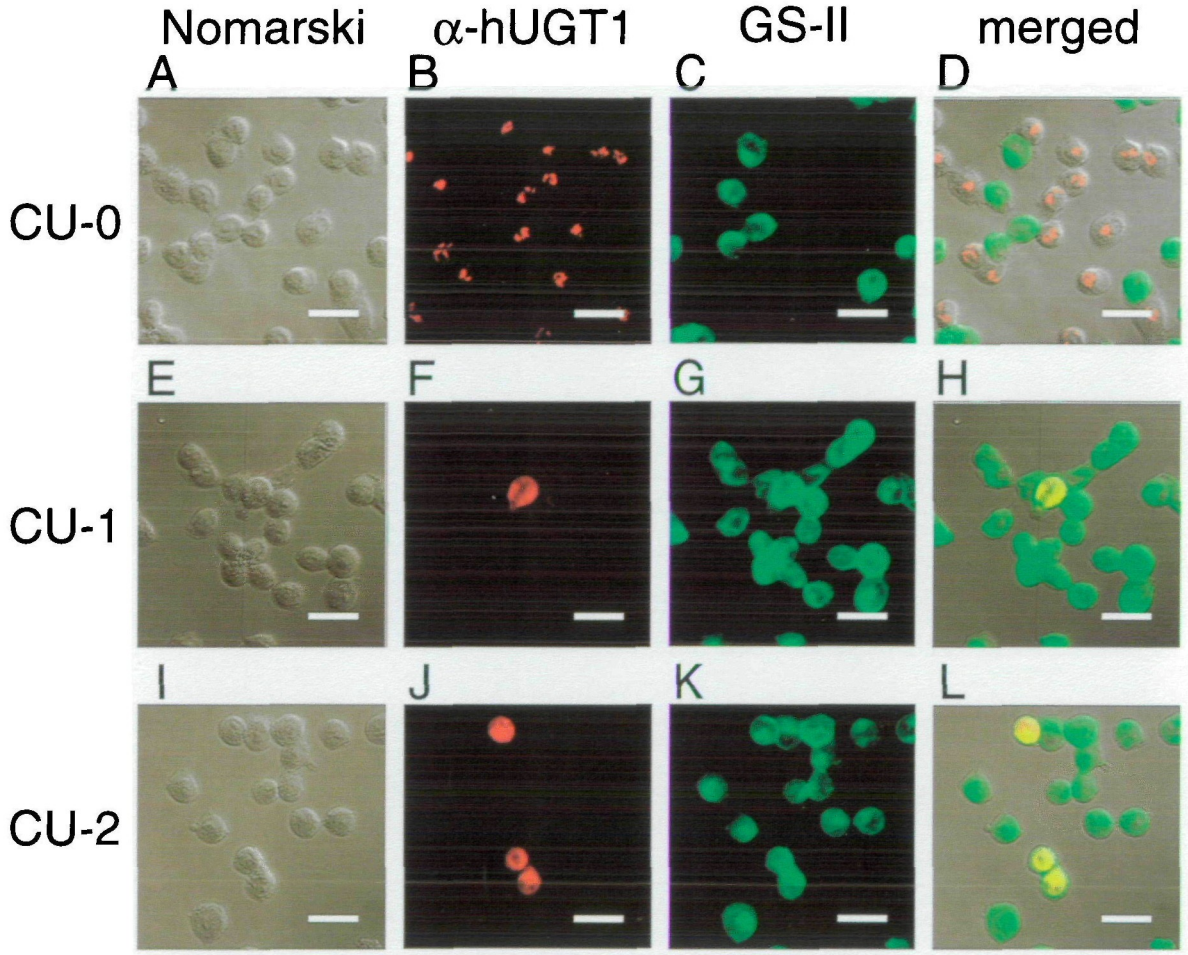

Fig. 5. **Expression and activity of chimeras: effects of N-terminal substitution of hUGT1 by hCST sequences.** Lec8 cells were transfected with chimeric cDNAs CU-0 (A-D), CU-1 (E-H), and CU-2 (I-L). Two days after transfection, they were doubly stained with rabbit anti-hUGT1 antibody (1:100), which was detected with Cy3-conjugated goat anti-rabbit IgG antibody (1:200; B, F, J), and FITC-conjugated GS-II (C, G, K). Nomarski images are shown in A, E, and I. In D, H, and L, the three images on the left of each panel are merged. Bar, 20 μm.

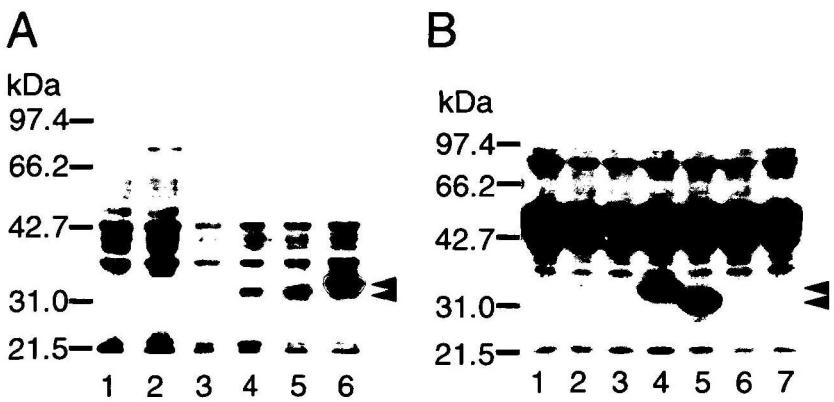

Fig. 6. **Western blot analysis of the expression of chimeric proteins.** (A) Lec8 cells were transfected with vector alone (lane 3), UC-6 (lane 4), UC-7 (lane 5), or UC-8 (lane 6), and were collected and lysed 3 days after transfection. After SDS-PAGE and blotting onto a Hybond-P membrane, chimeric proteins were detected with rat anti-HA monoclonal antibody (1:5,000) and HRP-conjugated goat anti-rat IgG antibody (1:10,000). Lane 1, CHO cells. Lane 2, Lec8 cells. The positions of molecular size markers are shown on the left, and those of chimeric proteins are indicated by arrowheads to the right of the panel. (B) Lec8 cells were transfected with vector alone (lane 3), hUGT1 cDNA (lane 4), CU-0 (lane 5), CU-1 (lane 6), or CU-2 (lane 7), and collected and lysed 2 days after transfection. The chimeric proteins were detected with rabbit anti-hUGT1 antibody (1:2,000) and HRP-conjugated goat anti-rabbit IgG antibody (1:10,000). Lane 1, CHO cells. Lane 2, Lec8 cells. The positions of molecular size markers are shown on the left, and those of hUGT1 and chimeric proteins are indicated by arrowheads to the right of the panel.

described in the preceding sections, and the cells were collected and lysed 2 or 3 days after transfection. After SDS-PAGE, UC-8, UC-7, and UC-6 proteins in cell lysates were detected with anti-HA antibody (Fig. 6A), and CU-0, CU-1, and CU-2 proteins with anti-hUGT1 antibody (Fig. 6B). The expression of chimeric proteins was confirmed with all the chimeras examined, but the amount of protein differed widely from one chimera to another. The amount of

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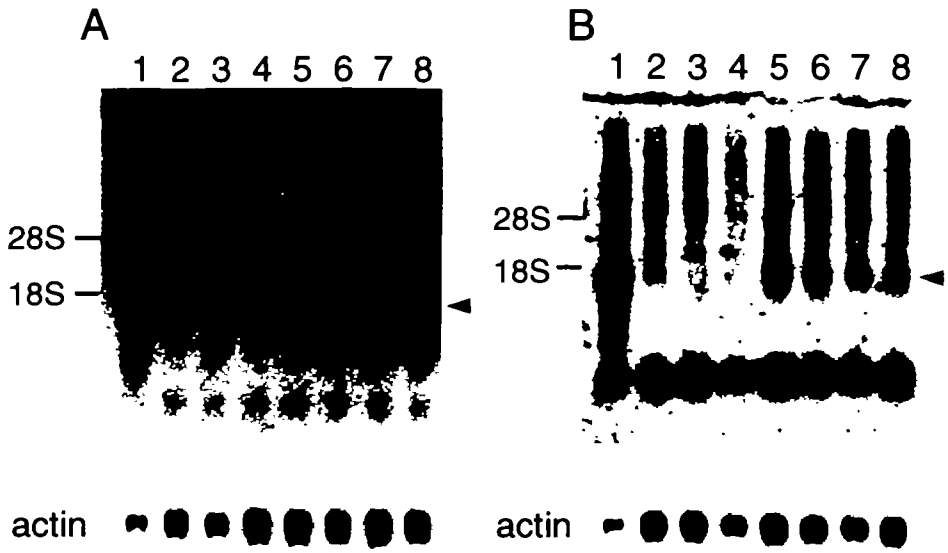

Fig. 7. **Northern blot analysis of chimera mRNAs in transfected cells.** (A) (upper panel) Lec8 cells were transfected with vector alone (lane 4), hUGT1 cDNA (lane 5), UC-6 (lane 6), UC-7 (lane 7), or UC-8 (lane 8), and collected and lysed 3 days after transfection. The samples were analyzed as described in "MATERIALS AND METHODS." Lane 1, Lec8/hUGT1 cells. Lane 2, CHO cells. Lane 3, Lec8 cells. The positions of rRNAs are shown on the left, and the position of hUGT1 and chimera mRNA is indicated to the right of the panel. (lower panel) The membrane shown above was reprobed for β-actin mRNA. (B) (upper panel) Lec8 cells were transfected with vector alone (lane 4), hUGT1 cDNA (lane 5), CU-0 (lane 6), CU-1 (lane 7), or CU-2 (lane 8) and collected and lysed 2 days after transfection. Lane 1, Lec8/hUGT1 cells. Lane 2, CHO cells. Lane 3, Lec8 cells. The positions of rRNAs are shown on the left, and the position of hUGT1 and chimera mRNA is indicated to the right of the panel. (lower panel) The membrane shown above was reprobed for β-actin mRNA.

UC-7 protein was less than that of UC-8 protein. This is consistent with the result of indirect immunofluorescence shown in Fig. 4. The amount of UC-6 protein was even less than that of UC-7 protein. This is in line with the fact that UC-6 protein was detected in only a very small number of transfected cells. A point of interest to be noted here is that the apparent molecular weights of UC-6 and UC-7 are definitely smaller than the apparent molecular weight of UC-8 protein. The reason for this is unclear at present, but it may reflect some structural difference among these chimeric proteins, since the lengths of these chimeras are identical. The apparent molecular weights of UC-6 and UC-7 appeared to be larger than the apparent molecular weight of hCST (*8*). This is consistent with the fact that UC-6 and UC-7 contain a longer N-terminal region than hCST.

As for the N-terminally substituted chimeric proteins, the amount of CU-0 was almost the same as that of hUGT1, while the amounts of CU-1 and CU-2 were much less than the amount of hUGT1. This is again consistent with infrequent occurrence of transfected cells expressing these chimeras. The decrease in the apparent molecular weights of these chimeras as compared to the apparent molecular weight of hUGT1 may be attributed to the shortening of the N-terminal region.

Northern Blot Analysis of Chimera mRNAs—To determine if the low expression level of several chimeric proteins was due to their low transcription efficiency, we examined the amounts of chimera mRNAs in chimeric cDNA-transfected cells by Northern blot analysis. The cells transfected with chimeric cDNAs were collected 2 or 3 days after transfection, and poly(A)$^+$ RNA was extracted and analyzed. As shown in Fig. 7, mRNAs for the six chimeric molecules and hUGT1 were present in almost the same amounts. This suggests that the reduction in the amounts of chimeric proteins in CU-1-, CU-2-, UC-6-, and UC-7-transfected cells are likely due to reduced post-translational stability of the chimeric proteins, although the possibility of inefficient translation cannot be totally excluded.

Intracellular Localization of the UC-6 Chimeric Protein—Chimeras whose expression was limited to a small fraction of transfected cells, in particular, UC-6, CU-1, and CU-2, appeared not to be localized in the Golgi apparatus (Figs. 4 and 5). Thus, we next examined the intracellular localization of hUGT1 and UC-6 proteins in detail. Lec8 and CHO cells were transfected with hUGT1 and UC-6 cDNAs to transiently express the respective products, and the localization of the cDNA products was compared with the distribution of calnexin, an endoplasmic reticulum (ER) marker protein (*21*) (Fig. 8). UC-6, whenever it was detected, appeared to be co-localized with calnexin in the ER region in both Lec8 (Fig. 8, A-D) and CHO cells (Fig. 8, I-L). Human UGT1, previously shown to be localized in the Golgi apparatus (*17*), was distributed differently from calnexin (Fig. 8, E-H and M-P). Most if not all of the chimeric proteins seemed to be retained in the ER, and this may be relevant to their low expression level.

DISCUSSION

Complementary DNAs for various nucleotide-sugar transporters have been cloned in recent years, but currently only limited information is available about the structural basis of their function and intracellular sorting. In the present study we constructed chimeric molecules between hUGT1 and hCST, and analyzed their expression and UDP-Gal-transporting function. Chimeras between structurally similar but functionally distinct proteins are useful in analyzing the significance of submolecular domains, since modification of the functions of the proteins in question may be studied without gross structural alterations. For instance, Kasahara *et al.* made chimeric proteins between two yeast sugar transporters, Gal2 and Hxt2, and identified amino acid residues responsible for the determination of substrate specificity (*22, 23*). Human UGT1 and hCST show substantial similarity in amino acid sequence and hydropathy profile (*2, 7*). Their N- and C-termini have also been shown to be oriented toward the cytosol (*8, 18*, this

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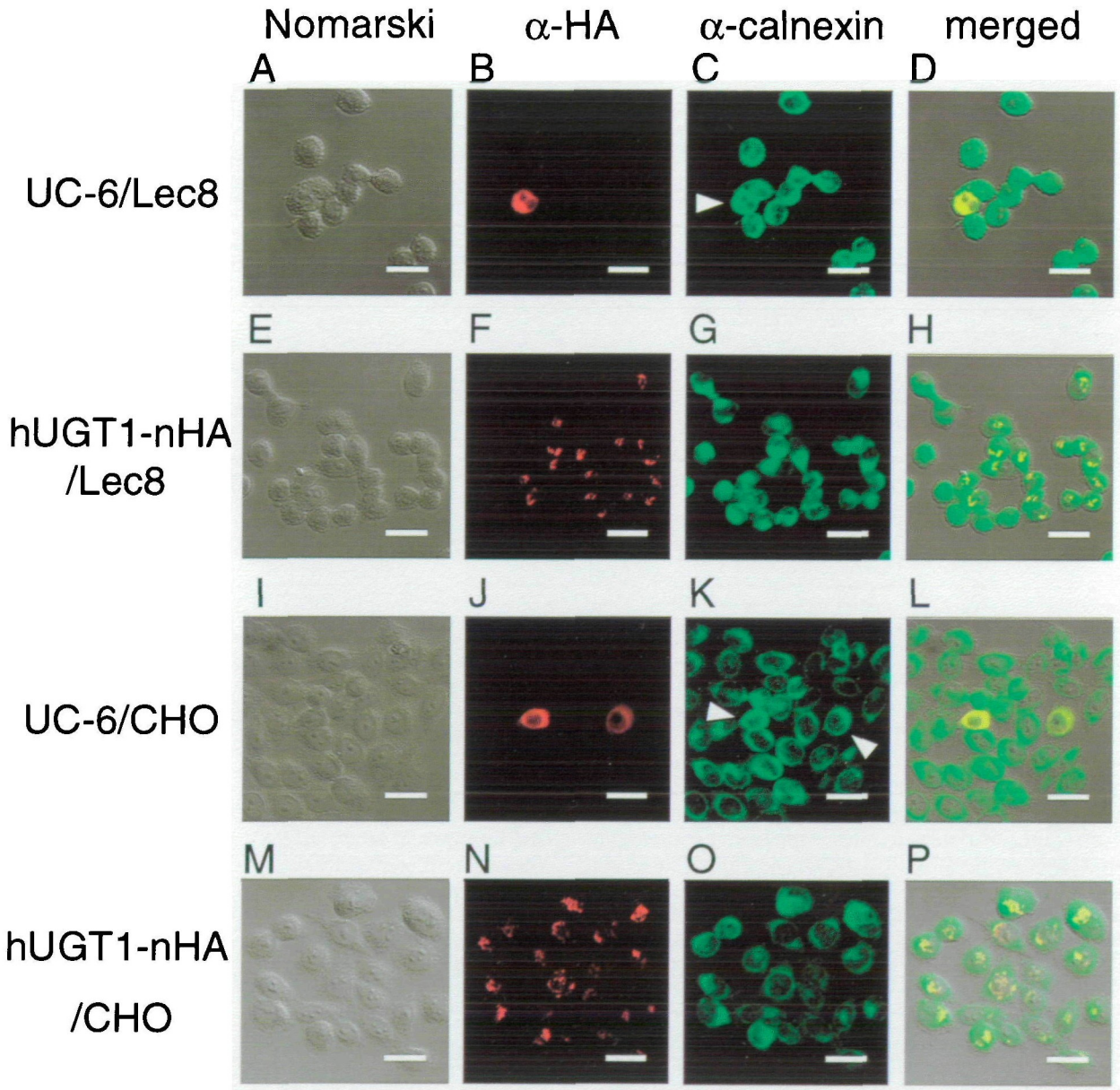

Fig. 8. **Intracellular localization of UC-6 chimera.** Lec8 cells (A-H) and CHO cells (I-P) were transfected with UC-6 (A-D, I-L) or hUGT1-nHA (E-H, M-P). Two days after transfection, cells were doubly stained with rat anti-HA monoclonal antibody (1:100), which was detected with Alexa594-conjugated goat anti-rat IgG antibody (1:300; B, F, J, N), and rabbit anti-calnexin antibody (1:400), which was detected with Alexa488-conjugated goat anti-rabbit IgG antibody (1:300; C, G, K, O). Nomarski images are shown in A, E, I, and M. In D, H, L, and P, the three images on the left of each panel are merged. The cells stained in B and J are indicated by white arrowheads in C and K, respectively. Bar, 20 μm.

work). Based on these similarities, we chose this pair as the parent molecules for chimera construction in the present study. To quickly assess whether a given chimeric protein retains the UGT activity or not, we developed a simple test using a transient expression system. This method, based on *in situ* detection of chimeric proteins and examination of GS-II-binding to the chimera-expressing cells, enabled us to easily test the function and localization of chimeric proteins at the same time and on the same specimen.

Either the N- or C-terminal cytoplasmic segment of hUGT1 could be replaced by the corresponding part of hCST (CU-0 and UC-8) without affecting the UGT activity, indicating that these regions of hUGT1 are not involved in the determination of substrate specificity. This is consistent with our recent finding that most amino acid differences between human and murine UGTs are scattered within these relatively short stretches, and that most of these stretches may be deleted without affecting the murine UGT activity (*25*). The C- and N-terminal cytoplasmic regions are likely not essential for UGT activity, and therefore may be replaced by the corresponding portions of hCST. This is also consistent with the fact that we have not so far found any functional distinction between the hUGT1 and hUGT2 isoforms which are produced from a single gene by alternative splicing and are identical except that the C-terminal five amino acids of the former are replaced by a

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distinct eight-amino acid stretch in the latter (*7*).

The exchange of the eighth (the most C-terminus-proximal) helix of hUGT1 by that of hCST similarly resulted in a chimeric protein which retained the UGT activity (UC-7). This indicates that this helix, which corresponds to two helices, TM9 and TM10, of a recently proposed model for murine CST (*18*), is not responsible for the determination of substrate specificity. This seems reasonable in view of the fact that the region replaced in this chimeric molecule shows a relatively low degree of conservation among UGTs of various species (*25*).

Three out of six chimeric proteins (UC-6, CU-1, and CU-2) were detected only in a very small number of transfected cells, although the mRNAs for these chimeras were synthesized normally. It was also noted that their protein products were mainly distributed in the ER region. It is likely that the chimeric proteins failed to be folded correctly, and were degraded in the ER by a quality-control system. Eckhardt *et al.* also reported deletion mutants of murine and hamster CST exhibiting abnormal localization, and suggested that the mutant proteins might be degraded in the ER (*24*). The present results suggest that the first and seventh transmembrane helices are important for the correct folding and transport of the protein to the Golgi membrane. Several additional chimeras in which longer stretches from the N- or C-terminus of hUGT1 than CU-2 or UC-6 were replaced by hCST sequences were also tested, but in none of these instances were we able to observe cells efficiently expressing pertinent chimeric proteins.

Initially, we had expected that some of the chimeric proteins might acquire CMP-sialic acid transport activity concomitantly with the loss of UDP-Gal transport activity, but none of the six chimeric proteins used in this study showed any CMP-sialic acid transport activity (data not shown). It is therefore likely that the replaced regions, from the N-terminus to the second loop and from the seventh helix to the C-terminus, are not involved in the determination of substrate specificity. It is interesting that 5 out of 10 "substrate-specific" residues noted through alignment of nucleotide-sugar transporter sequences (*9*) are located in the first and seventh helices, whose substitution led to an impairment of stable expression of hUGT1/hCST chimeras in Lec8 cells and of correction of the mutant phenotype. In contrast, the regions which can be replaced by hCST, that is, the eighth helix and N- and C-terminal cytoplasmic regions, do not contain any "substrate-specific" residues. The significance of these residues in substrate recognition as well as in other aspects related to the transporter function, including the maintenance of stable protein structure through interaction with other parts of the molecule, is an intriguing subject for future studies.

It was rather unexpected that replacement of only one transmembrane helix by another with substantial similarity affected the intracellular distribution of the chimeric protein so greatly as we observed in the case of the CU-1 chimera. Practically nothing is known yet concerning the structural requirements or mechanisms of sorting and targeting of multiple-membrane-spanning Golgi proteins. It is therefore important that the intracellular distribution of nucleotide-sugar transporter proteins was found to be highly sensitive to presumably minimal structural perturbances. With this fact in mind, we are currently investigating the effects of one-by-one substitution of putative transmembrane helices of hUGT1 by corresponding helices of hCST.

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