A Family of Human β4-Galactosyltransferases

CLONING AND EXPRESSION OF TWO NOVEL UDP-GALACTOSE:β-N-ACETYLGLUCOSAMINE β1,4-GALACTOSYLTRANSFERASES, β4Gal-T2 AND β4Gal-T3

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BLAST analysis of expressed sequence tags (ESTs) using the coding sequence of the human UDP-galactose:β-N-acetylglucosamine β1,4-galactosyltransferase, designated β4Gal-T1, revealed a large number of ESTs with identical as well as similar sequences. ESTs with sequences similar to that of β4Gal-T1 could be grouped into at least two non-identical sequence sets. Analysis of the predicted amino acid sequence of the novel ESTs with β4Gal-T1 revealed conservation of short sequence motifs as well as cysteine residues previously shown to be important for the function of β4Gal-T1. The likelihood that the identified ESTs represented novel galactosyltransferase genes was tested by cloning and sequencing of the full coding region of two distinct genes, followed by expression. Expression of soluble secreted constructs in the baculovirus system showed that these genes represented genuine UDP-galactose:β-N-acetylglucosamine β1,4-galactosyltransferases, thus designated β4Gal-T2 and β4Gal-T3. Genomic cloning of the genes revealed that they have identical genomic organizations compared with β4Gal-T1. The two novel genes were located on 1p32-33 and 1q23. The results demonstrate the existence of a family of homologous galactosyltransferases with related functions. The existence of multiple β4-galactosyltransferases with the same or overlapping functions may be relevant for interpretation of biological functions previously assigned to β4Gal-T1.

During the last decade, more than 40 mammalian glycosyltransferases have been cloned and characterized (1, 2). The initial strategy for cloning glycosyltransferases was cumbersome purification of labile enzyme proteins followed by screening of cDNA libraries with antibodies or DNA probes based on amino acid sequence information (3–11). The introduction of transfection cloning by Lowe and co-workers (12) resulted in a marked increase in the cloning of novel glycosyltransferase genes (13–17). A third successful approach has taken advantage of conserved sequences in glycosyltransferases that share donor and/or acceptor substrates. Thus, searches for novel members of homologous glycosyltransferase gene families utilizing conserved sequence motifs for RT-PCR cloning with degenerate primers have resulted in the identification and cloning of a number of novel genes (18, 19).

One part of the human genome project is the establishment of a data base of expressed sequence tags (ESTs), which currently has over 700,000 unique sequences. ESTs represent short 5′- and 3′-sequences (200–500 bp) of cDNA clones from a large variety of human and animal organs (20). The EST data base is now estimated to contain sequence information from more than half the human genes; it therefore provides a unique source for identifying novel members of homologous gene families (21). The EST data base has recently been successfully utilized in searches for novel glycosyltransferase genes of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family, where several novel members of this homologous gene family have been isolated by identification of ESTs with sequence similarities (22, 23).

A number of homologous families of glycosyltransferase genes exist. The largest family identified so far is the sialyltransferase family, for which at least 11 distinct members have been identified (24). Sequence motifs shared between sialyltransferases are part of the catalytic domain, suggesting that conservation of these sequences is related to functional requirements (25). Additional homologous glycosyltransferase gene families include the α2- and α3/4-fucosyltransferase families (26), the α3-GalNAc-transferase family (27), the β6-GlcnAc-transferase family (28), the ceramide galactosyltransferase family (29), and the polypeptide GalNAc-transferase family (30). Analysis of the sequence similarities within these glycosyltransferase gene families reveals that conserved sequences are generally limited to short sequence stretches in the putative catalytic domains, which are located in the central or C-terminal portions of enzymes that are type II transmembrane proteins. An additional characteristic is that cysteine residues in these areas are conserved in spacing (23, 30). Cysteine residues are important for intramolecular disulfide bonding as well as for the catalytic activity of glycosyltransferases (31–34).

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† The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; EST, expressed sequence tags; bp, base pair(s); Cer, ceramide.

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The UDP-galactose:β-N-acetylgalcosaminyl transferase (designated β4Gal-T1; GenBank™/EMBL Data Bank accession number X14085) was the first glycosyltransferase to be isolated and cloned (3–5, 7, 35), and early searches for homologous genes by low stringency Southern hybridization suggested that this gene was unique. Characterization of β4-Gal-transferase activities from different sources, however, indicated that distinct activities exist (36, 37). Emerging evidence now reveals that several β4-galactosyltransferase genes may exist. Shaper et al. (38) have identified two different chick cDNA sequences, which have 65 and 48% sequence similarity to human β4Gal-T1. Both chick cDNAs were shown to encode catalytically active β4-Gal-transferases. Thus, the β4Gal-T1 gene is likely to be part of a homologous gene family with recognizable sequence motifs, and this is supported by a large number of human ESTs with sequence similarities to β4Gal-T1 in EST data bases. Two independent groups have analyzed β4-Gal-transferase activities in mice homozygously deficient in β4Gal-T1 (39, 40). Both studies showed residual β4-Gal-transferase activity, providing clear evidence for the existence of additional β4-gal-transferases. Uehara and Muramatsu (41) have identified and cloned a mouse cDNA that was shown to encode a protein with low β4-galactosyltransferase activity when expressed in Escherichia coli. This putative β4-galactosyltransferase exhibited little or no sequence similarity to β4Gal-T1, indicating that the gene is unlikely to represent an evolutionarily related member of a galactosyltransferase family. Since the gene was expressed in E. coli, it makes evaluation of the kinetic parameters uncertain as this expression system has been shown to be poor for glycosyltransferases (42).

In this study, we used available human EST sequence information to identify novel genes homologous to β4Gal-T1. The β4Gal-T1 gene was found to be highly represented in the EST data base by ESTs with nearly identical sequences, but in addition, a number of ESTs with similar sequences were identified, and these had shared motifs with conserved cysteine residues previously shown to be functionally important for β4Gal-T1. The full coding sequences of two of these genes were established, and expression demonstrated that these genes represented active UDP-Gal-β-GlcNAc β4,1-Gal-transferase.

**EXPERIMENTAL PROCEDURES**

**Identification of Genes Homologous to β4Gal-T1—**Data base searches were performed with the coding sequence of the human β4Gal-T1 sequence (43) using the BLASTn and tBLASTn algorithms against the dbEST data base at the NCBI. The BLASTn algorithm was used to identify ESTs representing the query gene (identities of ≥95%), whereas tBLASTn was used to identify non-identical, but similar EST sequences. ESTs with 50–90% nucleotide sequence identity were regarded as different from the query sequence. The results of tBLASTn searches were evaluated by visual inspection after elimination of ESTs regarded as identical to the query sequence (>95% nucleotide sequence identity). ESTs with several apparent short sequence motifs and cysteine residues arranged with similar spacing were selected for further sequence analysis. Initially, the identified ESTs (5–sequence) were used in BLASTn searches of the dbEST data base to search for overlapping ESTs (95–100% identity in at least 30 bp) (see Fig. 1). If new ESTs were identified, the procedure was repeated, and sequences were merged. In addition, all identified ESTs were analyzed in the Unigene data base to confirm that they were from the same gene transcript and to select cDNA clones with the longest inserts as well as to identify additional ESTs with a non-overlapping 5′-sequence. Composites of all the sequence information for each set of ESTs were compiled and analyzed for sequence similarity to human β4Gal-T1. EST cDNA clones with the longest inserts (see Fig. 1) were obtained from Genome Systems Inc.

Cloning and Sequencing of the Full Coding Sequence of β4Gal-T2—Two partly overlapping ESTs were identified (see Fig. 1). Sequencing of the inserts revealed an open reading frame that potentially encoded a sequence similar to that of β4Gal-T1, but the 5′-sequence was shorter and without an initiation codon. Further 5′-sequence was obtained by 5′-rapid amplification of cDNA ends using human fetal brain Marathon cDNA (CLONTech) in combination with reverse primers EBER102 (5′-GAAATCAGCCTTAATCAGGC) and EBER104 (5′-TCCTACCTCGCTGAGTAAAGG) for 35 cycles at 95 °C for 45 s, 55 °C for 15 s, and 68 °C for 3 min using the Expand kit enzyme (Boehringer Mannheim). The products from the 5′-rapid amplification of cDNA ends were cloned into the BamHI/Hind site of pBluescript19, and multiple clones were sequenced. The entire sequence was confirmed by sequencing genomic P1 clones. The complete sequence contained an open reading frame of 1191 bp potentially encoding a protein with a type II domain structure (see Fig. 2), with an overall sequence identity of ~63% to β4Gal-T1.

Cloning and Sequencing of the Full Coding Sequence of β4Gal-T3—One EST clone (184081) with a 1980-bp insert was identified by its 3′-EST sequence in Unigene (National Center for Biotechnology Information) (see Fig. 1). Sequencing of the insert revealed an intact open reading frame of 1179 bp potentially encoding a protein with a type II domain structure (see Fig. 3), with an overall sequence identity of ~54% to β4Gal-T1.

Expression of β4Gal-T2 and β4Gal-T3 in Sf9 Cells—An expression construct designed to encode amino acid residues 56–397 of the β4Gal-T2 gene was prepared by RT-PCR with mRNA from the Colo205 cell line using the primer pair EBER100FOR (5′-TACCTTGTACGCTTACCCCG) and EBER114 (5′-GAAACAGGCGCCGTCGC) with BamHI restriction sites (see Fig. 2). An expression construct designed to encode amino acid residues 23–393 of β4Gal-T3 was prepared by RT-PCR with RNA from the MKN45 gastric carcinoma cell line using the primer pair EBER200FOR (5′-CAGTGGTTACCTGCTAAGG) and EBER214 (5′-TACGGCACGACGATTCG) with BamHI restriction sites (see Fig. 3). The PCR products were cloned into the BamHI site of pVL1193 (Pharmingen), and the construct was sequenced to verify correct insertion and sequence. A full-length coding expression construct of β4Gal-T3 was prepared by RT-PCR with MKN45 RNA using the primer pair EBER200FUL (5′-CAAGAGATGGTCGCCGTCGC) and EBER214 (see Fig. 4), and the product was cloned into the BamHI site of pVL1193 (Pharmingen). Plasmids pAcGP67-β4Gal-T2-sol, pAcGP67-β4Gal-T3-sol, and pVL-β4Gal-T3-full were cotransfected with BaculoGold™ DNA (Pharmingen) as described previously (19). Recombinant baculovirus was obtained after two successive amplifications in Sf9 cells grown in serum-containing medium, and titers of virus were estimated by titrations on cell plates with monitoring of enzyme activities. Controls included pAcGP67-GalNAc-T3-sol (19). Standard assays were performed in 50 μl of total reaction mixture containing 25 mM Tris (pH 7.5), 10 mM MgCl2, 0.25% Triton X-100, 100 μM UDP-[14C]Gal (2300 cpm/ nmol; Amersham Corp.), and varying concentrations of acceptor substrates (Sigma) (see Table I for structures). The soluble constructs were assayed with 50–20 μl of culture supernatant from infected cells, whereas the full-length construct was assayed with 1% Triton X-100 homogenates of washed cells. Bovine milk β4,1-Gal-transferase (Sigma) was used as control. Assays used for determination of Km of acceptor substrates were modified to include 200 μM UDP-[14C]Gal, and assays for donor substrate Km were performed with 2 mM (for β4Gal-T3 and bovine milk Gal-transferase) or 0.25 mM (for β4Gal-T2) benzyl-β-GlcNAc.

Characterization of the Product Formed with β4Gal-T3—Soluble β4Gal-T3 was partially purified by sequential DEAE and S-Sepharose chromatographies from serum-free medium as described previously (44). Terminal glycosylation of LeCer (see Table III for definitions of Svensserholm nomenclature) was performed in a reaction mixture consisting of 3 milliunits of β4Gal-T3 (specific activity determined with benzyl-β-GlcNAc), 250 μg of LeCer, 25 mM Tris (pH 7.4), 10 mM MgCl2, 0.25% Triton CF-54, and 3.5 μmol of UDP-Gal in a final volume of 100 μl. The LeCer substrate was prepared from sialoylactolectotetrosylceramide of bovine erythrocytes (45) by desialylation with 5% HAc in 2-propanol/hexane/H2O (55:25:20, v/v/v; lower phase) at 80 °C for 4.5 h, isolation of the nLeCer product by passage through DEAE-Sephadex A-25 (form II) in CHCl3/MeOH/H2O (30:60:8, v/v/v), and treatment of the product with jack bean mannosidase as described previously (46, 47). The LeCer product was evaluated by high performance TLC and 1H NMR (47). The glycosylation of LeCer with β4Gal-T3 was monitored by high performance TLC and run for 24 h until completed. The crude reaction mixture was taken up in ~3 ml of 0.1 M NaCl, sonicated thoroughly, applied to a 1 ml disposable octadecylsilica cartridge (Bakerbond, J. T. Baker Inc.), washed through with 4 ml each of 0.1 M NaCl.
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NaCl and H2O, and then eluted with 4-ml portions of H2O containing increasing amounts of MeOH from 10 to 90%, followed by 100% MeOH. Following concentration of each fraction, an assay by high performance TLC analysis showed that the glycosphingolipid product was eluted as a single band with 100% MeOH, whereas the detergent eluted at 80% MeOH. The purified product was deuterium-exchanged by dissolving in CDCl3/CD3OD (2:1), evaporating thoroughly under dry nitrogen (repeating twice), and then dissolving in 0.5 ml of MeSO-d6 and 2% D2O (48). One-dimensional 1H NMR spectroscopy was performed on a Bruker AMX-500 spectrometer (temperature, 308 K; spectral width, 5000 Hz acquired over 16,000 data points; relaxation delay, 2 s; and solvent suppression by presaturation pulse). NMR spectra of both the substrate and product were interpreted by reference to spectra of relevant standards acquired previously under identical conditions (46, 47, 49).

Northern Analysis—Human multiple tissue Northern blots were obtained from CLONTECH. The soluble expression construct of β4Gal-T2 and the full coding construct of β4Gal-T3 were used as probes. Probes were random prime-labeled using [α-32P]dCTP (Amersham Corp.) and an oligonucleotide labeling kit (Pharmacia Biotech Inc.). The blots were probed overnight at 42 °C and washed twice for 10 min each at room temperature with 2 × SSC and 1% Na4P2O7; twice for 20 min each at 65 °C with 0.2 × SSC, 1% SDS, and 1% Na4P2O7; and once for 10 min with 0.2 × SSC at room temperature.

Genomic Cloning and Characterization of the Organization of β4Gal-T2 and β4Gal-T3—A human foreskin genomic P1 library (DuPont Merck Pharmaceutical Co. Human Foreskin Fibroblast P1 Library) was screened using the primers EBER100 (5′-TGAAGGGAGATGC-GCCCTATGAC) and EBER102 for β4Gal-T2 and EBER200 (5′-CCCT-GCGTGATAGAGGATGGG) and EBER202 (5′-ATCTCAGCTACTG-GTCAGG) for β4Gal-T3. Three clones for β4Gal-T2 (DPMC-HFF10638:515:G9, DPMC-HFF10639:516:G4, and DPMC-HFF10640:924:A11) and three clones for β4Gal-T3 (DPMC-HFF1-0288-D2, DPMC-HFF1-0838-A8, and DPMC-HFF1-1486-H6) were obtained from Genome Systems Inc. DNA from P1 phages was prepared as recommended by Genome Systems Inc. The entire coding sequence of each gene was sequenced in full using automated sequencing (ABI377, Perkin-Elmer) with dye terminator chemistry. Intron/exon boundaries were determined by comparison with the cDNA sequences optimizing for the gt/ag rule (50).

In Situ Hybridization to Metaphase Chromosomes—Fluorescence in situ hybridization was performed on normal human lymphocyte metaphase chromosomes using essentially the procedures as described previously (51). Briefly, P1 DNA was labeled with biotin-14-dATP using the bioNICK labeling system (Life Technologies, Inc.). The labeled DNA was precipitated with ethanol in the presence of herring sperm DNA. A total of 300 ng of P1 DNA was precipitated with 50 × human Cot1 DNA (Life Technologies, Inc.) and dissolved in 12 ml of hybridization solution (2 × SSC, 10% dextran sulfate, 1% Tween 20, and 50% formamide (pH 7.0)). Prior to hybridization, the probe was heat-denatured at 80 °C for 10 min, chilled on ice, and incubated at 37 °C to allow re-annealing of highly repetitive sequences. After denaturation of the slides, probe incubations were carried out under a 18 × 18-mm coverslip in a moist chamber for 45 h. Immunochemical detection of the probe was achieved using fluorescein isothiocyanate-labeled avidin (Vector Laboratories, Inc.) and several successive steps with rabbit anti-fluorescein isothiocyanate- and mouse anti-rabbit fluorescein isothiocyanate-conjugated antibodies.

RESULTS

Identification and Cloning of Human β4Gal-T2 and β4Gal-T3—The strategy outlined in Fig. 1 produced two novel genes with significant sequence similarity to β4Gal-T1 (Figs. 2 and 3). Additionally, two genes with significant similarities in the 3′-region were identified (data not shown). Multiple sequence alignment analysis of β4Gal-T1, the two novel human β4Gal-T enzymes, two homologous sequences from chick (38), and a snail β4GlcNAc-transferase (52) is shown in Fig. 4. The β4Gal-T1 gene shows high sequence similarity (65%) throughout the coding region to one of the chick genes (GenBank™/EMBL Data Bank accession number U19890), and β4Gal-T2 shows high similarity (72%) to the second chick gene (GenBank™/EMBL Data Bank accession number U19899), suggesting that these may represent species-associated members of related gene families. The amino acid sequence similarities among the three human β4-Gal-transferrases and the snail β4GlcNAc-transferase are limited to the central regions; there were no significant similarities in the NH2-terminal regions. Human
β4Gal-T1 is closest in sequence to human β4Gal-T2 (52% identity), more distant from human β4Gal-T3 (44% identity), and most distant from snail β4-GlcNAc-transferase (34% identity). Several sequence motifs in the putative catalytic domains are conserved among all the sequences. More important, four cysteine residues are conserved in all coding sequences (Fig. 4).

**Fig. 2.** Nucleotide sequence and predicted amino acid sequence of human β4Gal-T2. The amino acid sequence is shown in single-letter code. The hydrophobic segment representing the putative transmembrane domain is double-underlined (Kyte and Doolittle (85), window of 8). Three potential N-glycosylation sites are indicated by asterisks. The locations of the primers used for RT-PCR preparation of the expression construct are single-underlined.

### β4Gal-T3

β4Gal-T1 is closest in sequence to human β4Gal-T2 (52% identity), more distant from human β4Gal-T3 (44% identity), and most distant from snail β4-GlcNAc-transferase (34% identity). Several sequence motifs in the putative catalytic domains are conserved among all the sequences. More important, four cysteine residues are conserved in all coding sequences (Fig. 4).
The predicted coding region of β4Gal-T2 has three potential initiation codons, and the most 5' is in agreement with Kozak's rule (53) (Fig. 3). All three initiation codons are relatively far from the sequence encoding the transmembrane segment: 25, 30, and 37 residues, respectively. The predicted coding sequence depicts a type II transmembrane glycoprotein with three potentially different long N-terminal cytoplasmic domains, a transmembrane segment of 21 residues, and a stem region and catalytic domain of 339 residues, with three potential N-linked glycosylation sites (Fig. 2). A 39-untranslated region without polyadenylation signals was included in the EST cDNA clones sequenced. The 39-ESTs (STsG4681) were linked to chromosome 1 between D1S2861 and D1S211 microsatellite markers at 73–75 centimorgans (National Center for Biotechnology Information).

Expression of β4Gal-T2—Expression of a soluble construct of β4Gal-T2 in Sf9 cells resulted in a marked increase in galactosyltransferase activity using the benzyl-β-GlcNAc acceptor substrate compared with uninfected cells or cells infected with control constructs for polypeptide GalNAc-transferases or histo-blood group A and O genes (Table I) (19, 54). Analysis of the substrate specificity of the soluble β4Gal-T2 activity showed that only benzyl-β-GlcNAc, and not benzyl-α-GlcNAc or benzyl-α-GalNAc, was an acceptor substrate. Free glucose was not an acceptor, but in the presence of increasing concentrations of α-lactalbumin, incorporation rates similar to those for bovine milk β4-Gal-transferase were observed (Fig. 5A). Differences in the concentration of α-lactalbumin needed to achieve maximum activity with Glc were observed, with 400 μg/ml required for β4Gal-T2 and only 100 μg/ml for the bovine milk enzyme. The activities of both β4Gal-T2 and the bovine milk enzyme with GlcNAc were inhibited by α-lactalbumin, but β4Gal-T1 was overall more sensitive to inhibition (Fig. 5B). The apparent $K_m$ for benzyl-β-GlcNAc was 160 μM, and the $K_m$

### Fig. 4. Multiple sequence analysis (ClustalW) of human β4Gal-T1, β4Gal-T2, and β4Gal-T3; two chick sequences; and the snail β4-GlcNAc-transferase. Introduced gaps are shown as dashes, and aligned identical residues are boxed (black for all sequences, dark gray for five sequences, and light gray for four sequences). β4GlcNACT; β4-GlcNAc-transferase.
for UDP-Gal using benzyl-β-GlcNAc was 11 μM (Table II). Bovine milk β4-galactosyltransferase showed higher a Ki for UDP-Gal, in agreement with previous studies (37, 55–58), and the measured Ki for GlcNAc was similar to that determined in some studies (59, 60), but 5–10-fold higher compared with other studies (55–58). As shown in Fig. 6, β4Gal-T2 was inhibited at high concentrations of both benzyl-β-GlcNAc and free N-acetylglucosamine to a higher degree than bovine milk β4-Gal-transferase and β4Gal-T3 (61). β4Gal-T2 showed strict donor substrate specificity for UDP-Gal and did not utilize UDP-GalNAc or UDP-GlcNAc with the acceptor substrates tested (data not shown).

**Expression of β4Gal-T3**—Expression of the soluble construct of β4Gal-T3 in Sf9 cells produced a marked increase in Gal-transferase activities with the benzyl-β-GlcNAc acceptor substrate compared with uninfected cells or cells infected with an irrelevant construct were subtracted. Background values obtained with uninfected cells or cells infected with an irrelevant construct were subtracted. The background rates were not higher than 0.5 nmol/min/ml.

| Substrate concentration | β4Gal-T2a | β4Gal-T3a | Bovine β4-Gal-transferasea |
|-------------------------|-----------|-----------|---------------------------|
| GlcNAc                  | 1.4       | 3.0       | 3.0                       |
| Bzl-β-GlcNAc            | 6.8       | 2.3       | 63                        |
| α-Nph-β-GlcNAc          | 0.4       | 1.3       | 0                         |
| β-β-GlcNAc              | 0.4       | 1.0       | 0.0                       |
| 4-Me-lumb-β-GlcNAc      | 0.8       | 2.0       | 0                         |
| Bzl-α-GalNAc            | 5.8       | 7.8       | 246                       |
| β-GlcNAc-(1–3)-β-Gal-1-OMe | 8.5     | 11.3      | 264                       |
| Bzl-2-(2-β-GlcNAc)-α-GlcNAc | 9.9     | 2.8       | 535                       |
| 4-Me-lumb-β-GalNAc      | ND        | ND        | 0                         |
| Bzl-α-GalNAc            | ND        | ND        | 0                         |
| 4-Me-lumb-β-Gal         | ND        | ND        | 0                         |
| α-Nph-β-Gal            | ND        | ND        | 0                         |

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![Graph A](file)

**Fig. 5. α-Lactalbumin modulation of β4-galactosyltransferase activities.** A, activities with glucose in the presence of increasing amounts of α-lactalbumin. The results are presented relative to the activities obtained with 40 mM glucose. B, activities with GlcNAc in the presence of increasing amounts of α-lactalbumin. The results are presented relative to the activities obtained with 2 mM (for the bovine milk enzyme and β4Gal-T3) or 0.25 mM (for β4Gal-T2) benzyl-β-GlcNAc (Bz-β-GlcNAc). Purified bovine milk enzyme or medium from Sf9 cells expressing secreted forms of either β4Gal-T2 or β4Gal-T3 was used as the enzyme source. ▲, bovine milk Gal-transferase; ■, β4Gal-T2; ●, β4Gal-T3.
control construct for GalNAc-T3 (Table I). A similar increase in activity was found in Sf9 cell homogenates after expression of the full-length construct of β4Gal-T3 (data not shown). The substrate specificity was similar to that of β4Gal-T2. β4Gal-T3 was largely insensitive to α-lactalbumin and did not efficiently utilize glucose (Fig. 5). The apparent $K_m$ for benzyl-β-GlcNAc was 580 μM, and the $K_m$ for UDP-Gal using benzyl-β-GlcNAc was 84 μM (Table II). β4Gal-T3 showed strict donor substrate specificity for UDP-Gal. Analysis of the specificity of β4Gal-T3 with glycolipid substrates revealed that Le$\alpha$Cer was efficiently utilized, whereas Glcβ1-Cer and nLe$\alpha$Cer were poor substrates (Table III). This was in contrast to the activities found with the bovine milk β4-Gal-transferase that efficiently utilized both Le$\alpha$Cer and nLe$\alpha$Cer.

Structural characterization of the product formed with Le$\alpha$Cer by 1H NMR showed that β4Gal-T3 forms the same linkage as β4Gal-T1. One-dimensional 1H NMR spectroscopy showed that the product was a single glycosphingolipid with a spectrum virtually identical to that of nLe$\alpha$Cer acquired previously under identical conditions (49) and distinct from that of Le$\alpha$Cer (46). In the downfield region of the spectrum (Fig. 7), four distinct β-anomeric resonances ($^{3}J_{1,2} = 7-9$ Hz) were observed at chemical shifts of 4.169 ppm ($^{3}J_{1,2} = 7.4$ Hz), 4.212 ppm ($^{3}J_{1,2} = 7.7$ Hz), 4.263 ppm ($^{3}J_{1,2} = 6.8$ Hz), and 4.663 ppm ($^{3}J_{1,2} = 8.3$ Hz). These were all within ±0.004 ppm of those previously observed (71) for I-1, IV-1, II-1, and III-1, respectively, of nLe$\alpha$Cer and thus clearly consistent with a β1–4 linkage of the terminal Gal residue. Under these conditions, the resonance for H-1 of the terminal Galβ1-3 of Le$\alpha$Cer is found at 4.140 ppm ($^{3}J_{1,2} = 7.3$ Hz), whereas that of the β-GlcNAc III-1 to which it is linked is found at 4.780 ppm ($^{3}J_{1,2} = 7.9$ Hz) (46).

**Northern Analysis of β4Gal-T2 and β4Gal-T3—**Northern analysis with mRNA from 16 human adult organs showed a single transcript of both genes of 2.2 kb (Fig. 8). β4Gal-T2 was expressed weakly in several adult organs, with the highest expression in prostate, testis, ovary, intestine, and muscle. β4Gal-T3 appeared to be more strongly expressed than β4Gal-T2, but with a similar pattern, with the exception of placenta, where strong expression of T3 was also found.

**Genomic Organization and Chromosomal Localizations—**The coding regions of β4Gal-T2 and β4Gal-T3 were found in seven and six exons, respectively (Fig. 9). Human β4Gal-T1 and mouse βGal-T1 are encoded in six exons (62, 63). The first putative coding exon of β4Gal-T2 encodes the first potential initiation codon and only eight amino acid residues of the N-terminal sequence of the longest form (Fig. 9), and it is

![Fig. 6. Differential inhibition of β4-Gal-transferase activities by high acceptor substrate concentrations.](image_url)

**Table II**

| Substrate       | β4Gal-T2 | β4Gal-T3 | Bovine β4-Gal-transferase |
|-----------------|----------|----------|--------------------------|
|                 | $K_m$ μM | $V_{max}$ pmol/min | $K_m$ μM | $V_{max}$ pmol/min | $K_m$ μM | $V_{max}$ pmol/min |
| UDP-Gal         | 0.011    | 51.9     | 0.084                    | 128.7    | 0.034            | 146.0 |
| Bzl-β-GlcNAc$^a$| 0.16     | 88.0     | 0.58                     | 112.5    | 0.53             | 88.2  |
| β-GlcNAc        | 2.65     | 59.4     | 13.1                     | 121.7    | 9.6              | 113.6 |

$^a$ Bzl, benzyl.
possible that the most 5'-initiation codon is not used. An intron in the 5'-untranslated region of β4Gal-T3 was found in a similar position as the most 5'-intron in β4Gal-T2 (Fig. 10). Comparison of the intron/exon boundaries of β4Gal-T1, β4Gal-T2, and β4Gal-T3 revealed that the five introns in the coding regions of the three genes are placed identically (Fig. 10). The central coding exons of all three genes are of nearly identical length, and variation in lengths of coding regions may be attributable to different initiation and stop codons in the first and last coding exons.

Human β4Gal-T1 was previously localized to chromosome 9p13 (64). The β4Gal-T2 and β4Gal-T3 genes were localized to chromosomes 1p32-33 and 1q23 by fluorescence in situ hybridization (Fig. 11). Nearly identical localizations were obtained from the EST mapping. No specific hybridization signals were observed at other chromosomal sites. For each gene, a total of 20 cells in metaphase were analyzed.

**DISCUSSION**

The data presented here demonstrate that the human β4Gal-T1 gene is a member of a large family of homologous glycosyltransferase genes and that at least three of the members of this family, β4Gal-T1, β4Gal-T2, and β4Gal-T3, encode β1,4-galactosyltransferases with similar kinetic parameters. The EST strategy used in this study has successfully been applied to other homologous gene families (22, 23, 65). The short sequence information of 200–400 bp obtained from a single EST, which may contain sequencing artifacts, may not be considered strong evidence for a particular gene sequence of interest. However, in our experience, the reliability of EST

### Table III

| Substrate specificities with glycolipid acceptors | β4Gal:T3 | Bovine milk β4Gal-transferase |
|-------------------------------------------------|---------|-----------------------------|
| Acceptor substrate                              |         | nmol/h/ml | nmol/h/mg |
| GlcCer (Glcβ1-Cer)                              | 0.38    | 40.3 | ND^a |
| LacCer (Galβ1-4Glcβ1-Cer)                       | ND      | ND | ND^a |
| Gbα (Galα1-4Galβ1-4Glcβ1-Cer)                   | ND      | ND | ND |
| Gbα (GalNAcβ1–3Galα1–4Galβ1–4Glcβ1-Cer)         | ND      | ND | ND |
| Gbα (GalNAcβ1–4Galβ1–4Glcβ1-Cer)                | ND      | ND | ND |
| Gbα (GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1-Cer)     | ND      | ND | ND |
| Gbα (Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1-Cer) | ND | ND | ND |
| Lcβ (GalNAcβ1–3Galβ1–4Glcβ1-Cer)                | 9.7     | 297 | (386)^a |
| nLcβ (Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1-Cer)        | ND      | ND | ND |
| nLcβ (GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1-Cer) | 0.93    | 333 | (58)^a |

^a Assayed using 100 μg of Triton CF-54/100-μl reaction mixture (enzyme source medium from infected Sf9 cells).

^b Assayed using 100 μg of taurodeoxycholate/100-μl of reaction mixture (enzyme source, Sigma).

^c Assayed using 100 μg of Triton CF-54/100 μl of reaction mixture.

^d ND, not detectable.
sequences is very high, and the finding of similar protein sequence motifs and conserved cysteine residues similar to those of the query sequence strongly indicates the identification of a novel homologous gene. The establishment of the Unigene data base further confirms this by identifying other ESTs from the same gene from which more sequence can be compiled by direct comparison of sequences or with available insert sizes of ESTs. Thus, with the two genes identified in this study, most of the coding region can be identified from merged EST sequences, and a more detailed assessment of the authenticity of the putative homologous gene can be made. One drawback of the EST cloning strategy is that genes with long 3'-untranslated sequences may not be represented, and this has been a feature associated with some glycosyltransferase genes (8, 10, 66). A strong point of the EST strategy is that it offers access to expressed sequences from many different adult and fetal tissues, leading to the possibility of identifying genes with very restricted expression patterns.

Some evidence suggested that β4Gal-T1 was a member of a homologous gene family. One distant member of this family was found to use UDP-GlcNAc and not UDP-Gal (52), indicating that members may have different donor substrate specificities and functions. Interestingly, UDP-Gal:β-GlcNAc β1,3-galactosyltransferases have no sequence similarity with β4Gal-T1 (GenBank™/EMBL Data Bank Accession number E07739).3 Several features of the two identified genes suggested that they represented genuine glycosyltransferase genes. The genes had higher sequence similarities to β4Gal-T1 than to a homologous β4-GlcNAc-transferase from snail (52). Four cysteine residues were conserved in all sequences, and these have been found to be involved in intramolecular disulfide bonding and catalysis for β4Gal-T1 (31, 32). Finally, the genomic organizations of both genes were shown to be identical to that of β4Gal-T1, with the positions of five intron/exon boundaries conserved (Fig. 10). The last finding strongly suggests that this gene family arose through complete gene duplication late in evolution. Furthermore, the genomic organization of the homologous snail β4-GlcNAc-transferase gene was recently shown to be organized similarly, with conservation of the same five intron positions, but additionally, two exons were found as well (Fig. 10) (67). More important, the two chick β4-Gal-transferases identified by Shaper et al. (38) were also found to have genomic organizations identical to that of the β4Gal-T1 gene. The three β4-Gal-transferase genes have different chromosomal locations (64). Several large glycosyltransferase gene families have now been found to have different chromosomal locations (23, 24), but some are also clustered in one region (26, 28, 66).

The β4Gal-T1 gene is different from most other known glycosyltransferase genes in having two translational initiation sites controlled by two different promoters (35, 68). The two isoforms of β4Gal-T1 differ by having a short (11 residues) or long (24 residues) cytoplasmic sequence, and studies indicate that the short form primarily localizes to the Golgi apparatus and the long form primarily to the cell membrane (69). A number of biological roles have been attributed to a cell membrane form of β4Gal-T1 (70). The β4Gal-T2 gene, showing the highest similarity to β4Gal-T1, also resembles β4Gal-T1 in

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having three initiation codons, potentially yielding isoforms with 25-, 30-, or 37-residue cytoplasmic sequences. A sequence motif (L/E/Q)RXC is found in the cytoplasmic sequences of the human and chick genes immediately preceding the hydrophobic putative transmembrane signal sequence (Fig. 4). Since the major parts of the N-terminal regions of these genes show little or no sequence similarities, this conserved motif may be important. Bendik et al. (71) have shown that the β4-Gal-transferase appears to exist as a high molecular weight aggregate, and this motif could potentially play a role in such aggregation. Aoki et al. (34) showed that a cysteine residue in the hydrophobic putative transmembrane sequence was important for Golgi retention, and this cysteine is conserved in β4Gal-T2, but not β4Gal-T3 or the snail β4-GalNAc-transferase (Fig. 4). The implications of this are not known at present.

The fine specificity of the three β4-Gal-transferases for different types of glycoconjugates and branch points of oligosaccharide structures needs to be determined in comparative assays with recombinant forms of the enzymes. Here, we have correlated the activities of recombinant soluble β4Gal-T2 and β4Gal-T3 with purified bovine milk enzyme, which potentially contains other β-Gal-transferase activities than β4Gal-T1. The substrate specificities with monosaccharides and simple derivatives were similar (Table I). β4Gal-T2 had a lower \( K_m \) for UDP-Gal than both bovine milk β4-Gal-transferase and β4Gal-T3 (Table II) and also a lower \( K_m \) for the best acceptor substrates (benzyl-β-GalNAc and GlcNAc) used in this study. At high concentrations of acceptor substrates (benzyl-β-GalNAc and GlcNAc), all three enzymes showed inhibition of activities, but β4Gal-T2 was the most sensitive (Fig. 6). Inhibition of β4Gal-T1 was previously noted at concentrations in excess of 20–30 mM (61), but in the present study, this was seen at higher concentrations.

The substrate specificity of β4Gal-T1 is modulated by α-lactalbumin (72, 73). A similar modulation of the substrate specificity was found for β4Gal-T2, whereas β4Gal-T3 was largely insensitive (Fig. 5). Interestingly, mice rendered deficient in β4Gal-T1 were shown not to produce lactose (39, 40). This suggests that β4Gal-T2 is not expressed in lactating mammary glands. Differences in sensitivity to α-lactalbumin modulation of putative members of the β4-Gal-transferase family have previously been observed. Two distinct porcine trachea β4-Gal-transferases have been separated, and one of these was shown to be insensitive to α-lactalbumin, similar to β4Gal-T3 (36). The homologous β4-GlcNAc-transferase identified in snail is apparently not sensitive to α-lactalbumin (74); however, another putative member of this enzyme family, a β4-GalNAc-transferase, was found to be modulated by α-lactalbumin (75).

As β4Gal-T1 and β4Gal-T2 were most similar in sequence and seemed to have similar properties, the specificity of β4Gal-T3 was studied in more detail. Analysis of β4Gal-T3 and bovine milk β-Gal-transferase with glycolipid substrates showed that both enzymes utilize Lc\(_3\)Cer efficiently, and characterization of the product formed by β4Gal-T3 using \(^1\)H NMR clearly established the expected product as being nLc\(_4\) with a Gal \( \beta \) to Lac linkage, whereas β4Gal-T3 poorly utilized nLc\(_4\)Cer, which may suggest that β4Gal-T1 is involved in all steps of the synthesis of polyn-\( N \)-acetylactosamine chain extension, whereas β4Gal-T3 may be mainly involved in the synthesis of the first \( N \)-acetylactosamine unit. Differential expression of β4-Gal-transferases may be part of the regulation of polyn-\( N \)-acetylactosamine chain extension, which appears to be developmentally regulated (76, 77).

The differences found in the kinetic properties of the three
β-Gal-transferases may relate to past findings of β-Gal-transferase activities in different organs and in B cells of rheumatoid arthritis patients. Furukawa et al. (37) showed that liver β4-Gal-transferase activity was near 20-fold higher with asialo-agalactotransferrin compared with asialo-agalacto-IgG, whereas the activity found in T and B cells showed only a 4–5-fold difference with the two substrates. The β4-Gal-transferase activity in B cells of rheumatoid arthritis patients appears to be similar to that in B cells of healthy controls with several substrates, including asialo-agalactotransferrin (37) and β-GlcNAc-phenylisothiocyanate-bovine serum albumin (78), but different with asialo-agalacto-IgG (37). Furthermore, the $K_m$ for UDP-Gal of β4-Gal-transferase activity in B cells of rheumatoid arthritis patients was 2-fold higher (35.6 μM) than that in normal B cells (17.6 μM) (37). Finally, the activity in B cells with asialo-agalactotransferrin was more sensitive to α-lactalbumin inhibition than the activity with asialo-agalacto-IgG. It is intriguing that β4Gal-T2 has a lower $K_m$ for UDP-Gal than the other two enzymes and that this is close to that found in normal B cells (37). In addition, β4Gal-T2 was slightly less sensitive to α-lactalbumin inhibition, in agreement with the activity with asialo-agalacto-IgG found in normal B cells. A number of studies have concluded that there was no change in β-Gal-transferase activity in B cells of rheumatoid arthritis patients (79, 80). With the new insight into the β-Gal-transferase family, which includes at least two additional genes, it is possible that the contradictory findings of Furukawa et al. (37) can be explained by a model with two β-Gal-transferases with different kinetic parameters expressed in normal B cells and a selective down-regulation of one in B cells of rheumatoid arthritis patients. β4Gal-T2 may be a candidate for such a second enzyme, and studies are now in progress to determine this. β4Gal-T2 and β4Gal-T3 were differentially expressed in human organs (Fig. 6). In contrast, β4Gal-T1 is considered to be more widely expressed, and extensive analysis of the regulatory elements indicated that this gene is a ubiquitously expressed housekeeping gene (81). However, a detailed comparative analysis of the expression patterns of the three enzymes at the level of individual cell types is not available at present. Two additional members of this gene family have been cloned, and expression is ongoing; and it is possible that these may represent β4-Gal-transferases with different expression patterns. Analysis of β4-Gal-transferase activities in extracts of organs from mice deficient in β4Gal-T1 showed that only 5% activity remain in liver and spleen (40); however, 30% activity was left in brain, although the total activity in this organ was low (39). Interestingly, expression of β4Gal-T2 and β4Gal-T3 could not be detected in liver, but β4Gal-T3 was weakly expressed in spleen and brain. Studies of β4-Gal-transferase activities by Shur and co-workers (39) have generally been performed with 20–30 mM N-acetylglucosamine, based on kinetic properties of cell-surface β4-Gal-transferase activity in mouse sperm and embryonic carcinoma cell lines (6, 61). In this study, we show that all three β4-Gal-transferases are inhibited, but that β4Gal-T2 is inhibited at lower concentrations than both bovine milk β4-Gal-transferase and β4Gal-T3 (Fig. 6). Thus, analysis of galactosyltransferase activities at high acceptor substrate
concentrations may be misleading for the actual activity present. Since mice deficient in β4Gal-T1 developed normally before birth and only exhibited reduced growth and lethality after birth, it is likely that other β4-Gal-transferases besides β4Gal-T1 are functioning in fetal life. Analysis of serum glycoproteins of 10-week-old mice deficient in β4Gal-T1 showed a marked decrease in galactosylation (40), suggesting that the major β4-Gal-transferase involved in the synthesis of these glycoproteins is β4Gal-T1. In contrast, Lu et al. (39) found that despite a lack of detectable β4-Gal-transferase activity in salivary glands, a normal level of Galβ1-4GlcNAc terminating chains in salivary gland extracts was found by lectin analysis and α2,6-sialylation. It is therefore likely that another β4-Gal-transferase besides β4Gal-T1 is active in salivary glands.

It is clear that the single step of forming the Galβ1-4GlcNAcβ linkage is performed by a family of β4-Gal-transferases and that each member of this family may play a distinct role in different cells. A similar finding was made for the initiation step of GalNAc and Man-O-glycosylation in animals and yeast. Large families of homologous enzymes with distinct but overlapping specificities and different expression patterns perform a reaction previously considered to be maintained by a single enzyme (23, 54). The α2-fucosyltransferases (82), α3/4-fucosyltransferases (83), and some of the sialyltransferases (24) are other examples of this phenomenon of partial redundancy. The findings that the genes in most glycosyltransferase families have diverged considerably and show only 30–50% sequence similarities combined with differential expression patterns suggest that each member of these gene families plays a distinct role. The seemingly similar enzyme activities determined in in vitro assays may not fully reveal the in vivo functions of these enzymes.

From the present status of cloning of glycosyltransferases, one may predict that there is no requirement for primary sequence similarities between glycosyltransferases utilizing the same donor or acceptor substrates per se. Analysis of the known glycosyltransferase gene families shows that these have common specificities for the nucleotide of the donor substrate and the anomeric configuration of the linkage formed, whereas the acceptor substrate and the linkage positions formed seem to be more variable. The sialyltransferases all utilize CMP-NeuAc and form α-anomeric linkages, but they form linkages to several different acceptors (Gal, GalNAc, GlcNAc, and NeuAc) and at different positions (C-3, C-6, and C-8) (24). Although all members of this gene family share some sequence similarity, it is apparent that members forming the same linkage type have higher sequence similarity. The α1,3/4-fucosyltransferase family utilizes GDP-Fuc and forms either α1–3 or α1–4 linkages to GlcNAc (84). The α3-GalNAc-transferase family utilizes UDP-Gal or UDP-GalNAc and forms α1–3 linkages to Gal (27). The β6-GlcNAc-transferases use different acceptor substrates (28).

The polypeptide GalNAc-transferase family generally utilizes UDP-GalNAc, but one member (GalNAc-T2) was recently found also to utilize UDP-Gal (23, 44). Finally, the snail β4-GlcNAc-transferase homologues to the β4-Gal-transferase family utilize UDP-GlcNAc, but form the same linkage. Therefore, specific functions of a newly identified homologous gene in a glycosyltransferase family are not easily predicted even though homologous glycosyltransferases recognize the same nucleotide of the donor substrate and form the same anomeric configuration of linkages.

The existence of multiple β4-Gal-transferases has implications for interpretation of past studies of the expression pattern and biological functions previously assigned to β4Gal-T1; this is what was learned from analysis of mice deficient in β4Gal-T1. Detection of gene expression of β4Gal-T1 by Northern or in situ hybridization is likely to be representative of genuine β4Gal-T1 expression, but analysis of enzyme activities in extracts or body fluids may represent all or any of the β4-Gal-transferases, and knowledge of the kinetic parameters of each of the enzymes is required to ascertain that all activities are measured. Immunodetection with antibodies prepared against purified rather than recombinant β4-Gal-transferases may likewise be directed toward any or several of the enzymes in the family.

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Note Added in Proof—A fourth human β4-galactosyltransferase was recently cloned and expressed. (Sato, T., Furukawa, K., Bakker, H., Van den Eijnden, D. H. (1997) Glycoconj. J. 14, (suppl.) 46). This gene showed lower sequence similarity to β4 Gal-T1 than the two genes reported here, and the expressed enzyme was not olaclotribulin-sensitive.

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