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A Mutation Causing a Reduced Level of Expression of Six β4-Galactosyltransferase Genes Is the Basis of the Lec19 CHO Glycosylation Mutant†

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ABSTRACT: To identify factors required for the synthesis of complex glycans, we have isolated Chinese hamster ovary (CHO) cell mutants resistant to plant lectins. We previously identified Lec19 CHO cells as resistant to the Gal-binding lectins ricin, abrin, and modeccin and hypersensitive to the toxicity of other lectins that bind Gal, including L-PHA and E-PHA. Here we show that Lec19 cell extracts have a decreased ability to transfer Gal to simple sugar, oligosaccharide, and glycopeptide acceptors, particularly to biantennary, GlcNAc-terminated acceptors. $Ricin_{II}$−agarose lectin affinity chromatography, oligo-mapping, and monosaccharide analyses provided evidence that Lec19 *N*-glycans have fewer Gal residues than CHO *N*-glycans. MALDI-TOF mass spectra of *N*-glycans released from Lec19 cell glycoproteins by peptide *N*-glycanase F revealed species with the predicted masses of neutral *N*-glycans with few Gal residues. Such truncated species are essentially absent from CHO cell glycoproteins. However, the complement of fully galactosylated or sialylated bi-, tri-, and tetra-antennary *N*-glycans was largely equivalent in Lec19 and CHO cells. In addition, the coding region sequences of the β4GalT-1, -T-2, -T-3, -T-4, -T-5, and -T-6 genes were identical in CHO and Lec19 cells. However, Northern analyses revealed an ∼2−4-fold reduction in the level of transcripts of all six β4GalT genes in Lec19 cells. Since the recessive Lec19 phenotype is the result of a loss-of-function mutation, the combined data predict the existence of a trans-acting regulator of the steady-state level of transcripts that derive from these six mammalian β4GalT genes.

Many mammalian cell glycosylation mutants have been isolated using selections for resistance to plant lectins (*1*, *2*). Lectin resistant Chinese hamster ovary (CHO)[1] cell glycosylation mutants have been used to define glycosylation pathways, expression clone glycosyltransferase genes, engineer the glycosylation of recombinant glycoproteins, and identify complex glycan recognition specificities of plant and animal lectins (*3*). In addition, the mutants allow the identification of factors that regulate the expression of glycosyltransferase genes and other genes that affect glycosylation (*4*). Thus, it is important to understand the molecular basis of glycosylation change and to characterize the nature of the expressed glycans in each mutant.

In this paper, we describe the glycosylation defect in the Lec19 CHO cell mutant previously isolated by selection for ricin resistance from CHO cells mutagenized with *N*-methyl-*N*-ethylnitrosoguanidine (MNNG). Compared to parent CHO cells, Lec19 mutant cells are ∼10-fold more resistant to ricin and abrin and ∼2-fold hypersensitive to the agglutinins from *Phaseolus vulgaris* (L-PHA and E-PHA) and to wheat germ agglutinin (WGA) (*5*). Their lectin resistance phenotype is unique, and the *lec19* mutation behaves recessively in somatic cell hybrids formed between Lec19 and CHO cells (*5*). The Lec19 mutant belongs to a complementation group different from that of other ricin-resistant CHO glycosylation mutants (*5*) and thus has a novel genetic basis. Here we show that Lec19 cells bind reduced amounts of Gal-binding lectins, synthesize undergalactosylated *N*-glycans, and have reduced β4-galactosyltransferase (β4GalT) activity for a subset of acceptors. The phenotype is subtle with the largest affects being on biantennary *N*-glycans.

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[1] Abbreviations: β4GalT, β-1,4-galactosyltransferase; CHO, Chinese hamster ovary; VSV, vesicular stomatitis virus; Con A, concanavalin A; SNA, *Sambucus nigra*; Lotus, *Lotus floribunda*; GSII, *Griffonia simplicifolia* II; PWM, pokeweed mitogen; RCA_{II}, *Ricinus communis* agglutinin II; ECA, *Erythyinia corrigendum* agglutinin; PSA, pea lectin; MAAII, *Maackia aurensis* II; L-PHA, leukoagglutinin from *P. vulgaris*; DSA, *Datura stromonium*; E-PHA, erythroagglutinin from *P. vulgaris*; WGA, wheat germ agglutinin; Tomato, *Triticus lysopersicum*; sWGA, succinylated WGA; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; FBS, fetal bovine serum; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; RT−PCR, reverse transcription−polymerase chain reaction; GnGn, GlcNAc-terminated biantennary *N*-glycan; GnGnβ4Gn, GlcNAc-terminated triantennary *N*-glycan with a β-1,4-linked branch; GnGnGnGn, GlcNAc-terminated tetra-antennary *N*-glycan; HPTLC, high-performance thin-layer chromatography; GlcCer, glucosylceramide; LacCer, lactosylceramide; GM3, NeuNAcα2,3Galβ1, 4GlcCer; PNGase F, peptide *N*-glycosidase F.

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The regulation of mammalian glycoconjugate galactosylation is poorly understood. Upstream promoters, enhancers, and regulatory elements of mammalian β4GalT genes are in most cases either not identified or not well-characterized, nor have sequences that might lead to degradation or turnover of β4GalT gene transcripts been identified. Six β4GalT genes have been cloned from mammalian sources (*6*, *7*). All encode proteins with β4GalT activity that utilize the donor substrate UDP-Gal and transfer Gal in a β-1,4-linkage to GlcNAc or Glc. The *in vitro* acceptor specificity of each β4GalT enzyme has been examined (*7*−*13*). To understand *in vivo* functions, tissue expression patterns of each β4GalT are being determined. β4GalT-1, -T-3, -T-4, and -T-5 genes appear to be expressed ubiquitously, whereas the β4GalT-2 and -T-6 genes exhibit a more restricted expression pattern (*8*, *9*). Differential expression of β4GalT-1, -T-2, and -T-5 has been observed in subregions of mouse brain (*14*).

Previously, we showed that all six β4GalT genes are expressed in the Gat$^-$2 parental line of CHO cells (*15*). By characterizing independent CHO galactosylation mutants, we showed that β4GalT-1 is a key enzyme of galactosylation of *N*-glycans but is not involved in glycolipid synthesis in CHO cells, whereas β4GalT-6 contributes only to a small extent to the galactosylation of *N*-glycans and is also not involved in glycolipid synthesis (*15*). The Lec19 mutant described in this paper has a related but distinct galactosylation defect arising from the reduced expression of all six β4GalT genes.

EXPERIMENTAL PROCEDURES

Materials. D-[6-^{3}H]Glc (31.5 Ci/mmol), D-[6-^{3}H]GlcN (31.5 Ci/mmol), UDP-[6-^{3}H]Gal, and Con A-Sepharose were from Amersham Pharmacia Biotech. RCA_{II}−agarose and all FITC-labeled lectins were from Vector Laboratories. Bio-Gel P2 (45−95 mesh), the detergent compatible protein reagent, and AG1-X4 resin (200−400 mesh, Cl^- form) were from Bio-Rad. β-Galactosidase (*Diplococcus pneumoniae*) was from Roche Biosciences. NP-40, Triton X-100, neuraminidase (*Clostridium perfringens*), GlcNAc, Gal, UDP-Gal, human IgG, human fibrinogen, fetuin, and human α_1-acid glycoprotein, Galβ1,4GlcNAc, Galβ1,3GlcNAc, Galβ1,6GlcNAc, GlcCer, LacCer, and GM3 were from Sigma. GlcNAcβ-*O*-pNP, GlcNAcβ-*S*-pNP, and GlcNAcβ-*O*-benzyl were from Sigma. Galβ1,3(GlcNAcβ1,6)GalNAcα-*O*-pNP and Fucα1,3GlcNAcβ-*O*-CH_3 were from Toronto Research Chemicals, and PNGase-F was from Oxford Glycosystems. DNA molecular markers, fetal bovine serum, and α medium were from Life Technologies, Inc. Ecolume was from ICN Biomedicals.

Cell Lines and Cell Cultures. CHO cells were grown in suspension at 37 °C in complete medium (Life Technologies, Inc.) containing 10% fetal bovine serum. The isolation of the Lec19 CHO mutant termed Gat$^-$Lec19.10C from Gat$^-$2 parent CHO cells was described previously (*5*).

Fluorescence-Activated Cell Scanning (FACS). CHO and mutant Lec19 cells were washed with phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS containing 2% BSA and 0.02% azide (PBS/BSA). The washed cells (2×10^5) were incubated with FITC-labeled lectin (1 μg/mL) for 1 h at 4 °C on a rotator. After being washed with 3 mL of PBS, the cells were resuspended in 0.3 mL of PBS and analyzed on a Becton Dickson FACS Pstar Plus instrument. To remove sialic acid residues from cell surface glycoproteins, washed cells were incubated in PBS containing neuraminidase (10 milliunits/10^6 cells) from *C. perfringens* at 37 °C on a rotator. After 1 h, cells were washed once with PBS and resuspended in PBS/BSA. The cells were subsequently used for binding with FITC-labeled lectin as described above.

Preparation of Cell Extracts. Postnuclear supernatant from parent CHO and Lec19 cells was prepared by homogenization using a Balch homogenizer as previously described (*16*).

Preparation of Glycopeptides. Biantennary N-linked glycopeptides with no fucose and terminating with GlcNAc (GnGn) were isolated from human fibrinogen, and the corresponding glycopeptides with fucose [GnGn(F)] were prepared from human IgG. Triantennary N-linked glycopeptides (GnGnβ4Gn) were prepared from fetuin, and tetraantennary N-linked glycopeptides (GnGnGnGn) were prepared from α_1-acid glycoprotein as described previously (*15*). The glycopeptides were analyzed for purity by monosaccharide analysis and elution position from Dionex HPAEC-PAD.

β4-Galactosyltransferase Assay. Enzyme assays were carried out with microsomal membrane extracts in 1.5 mL Eppendorf tubes in a reaction volume of 50 μL as described previously (*15*). Acceptors were 1 μmol of GlcNAc, 2 μmol of Glc (with 0.4 mg/mL α-lactalbumin), 1 μmol of Fucα1, 3GlcNAcβ-*O*-CH_3, 50 nmol of GlcNAcβ-*O*-pNP, GlcNAcβ-*S*-pNP, GlcNAcβ-*O*-benzyl, and Galβ3(GlcNAcβ6)GalNAcα-*O*-pNP, or 0.11 μmol of GnGn, 0.11 μmol of GnGn(F), 0.01 μmol of GnGnβ4Gn, 0.05 μmol of GnGnGnGn, and 0.05 μmol of GlcCer. Reactions lacking acceptor were used to determine the extent of incorporation into endogenous acceptor and degradation of donor sugar. After the mixture had been incubated at 37 °C for 2 h, the reaction was stopped by adding 1 mL of cold water. Reaction mixtures containing simple sugar or glycopeptides were passed through a 1 mL column of AG1-X4 (Cl^- form) that was subsequently washed with 2 mL of water. Reaction mixtures with GlcNAcβ-*O*-pNP, GlcNAcβ-*S*-pNP, GlcNAcβ-*O*-benzyl, Galβ3-(GlcNAcβ6)GalNAcα-*O*-pNP, or GlcCer were passed through a Sep-Pak C_{18} cartridge (Waters), and the radiolabeled product was eluted with 50% aqueous methanol. Radioactivity was measured using a liquid scintillation counter. Oligosaccharide products were analyzed by HPAEC-PAD using a CarboPac PA-10 column (4 mm × 250 mm), which was eluted with 50 mM NaOH at 1 mL/min. Fractions (0.5 mL) were collected and counted.

Preparation and Lectin Affinity Chromatography of Radiolabeled Glycopeptides. Cells growing in suspension were infected with vesicular stomatitis virus (VSV) and subsequently cultured in α medium containing reduced glucose (0.5 mg/mL), 2% Nuserum (Collaborative Research), and 83 μCi of radiolabeled [^{3}H]GlcN per 10 mL as described previously (*17*). Virus was purified by gradient centrifugation and exhaustively digested with Pronase. The viral glycopeptides desalted on Bio-Gel P2 were fractionated on a 5 mL column of Con A-Sepharose as described previously (*17*) into branched and biantennary moieties. The Con A-bound fractions were desalted and fractionated on a 5 mL RCA_{II}−agarose column at 4 °C. Buffer containing 200 mM lactose was used to elute bound glycopeptides from the RCA_{II}−agarose column. Samples (0.5−1.0 mL) were mixed with

Table 1: Lectin Binding of CHO and Lec19 Cells

	mean fluorescence index before neuraminidase treatment			mean fluorescence index after neuraminidase treatment		
lectin[a]	CHO	Lec19	Lec19/CHO (%)	CHO	Lec19	Lec19/CHO (%)
no lectin	1.2	1.3	NA	1.2	1.3	NA
SNA	1.2	1.3	NA	1.2	1.3	NA
lotus	1.5	1.4	NA	1.4	1.4	NA
GSII	1.6	1.6	NA	1.8	1.6	NA
PWM	1.3	1.2	NA	1.4	1.5	NA
RCA_{II}	33 ± 16	23 ± 12	68	132 ± 21	117 ± 21	89
ECA	184	143	78	487	407	84
PSA	101 ± 17	85 ± 11	84	108 ± 12	88 ± 13	82
MAAII	47 ± 56	46 ± 1	99	25 ± 13	21 ± 9	84
L-PHA	236	219	93	278	293	106
DSA	83 ± 19	88 ± 28	106	79 ± 19	95 ± 29	121
E-PHA	206 ± 43	240 ± 17	117	219 ± 33	302 ± 39	138
WGA	412 ± 13	500 ± 11	122	246 ± 17	362 ± 18	147
Tomato	40 ± 15	51 ± 14	127	20 ± 2	31 ± 4	158
sWGA	30 ± 10	102 ± 18	341	17 ± 5	24 ± 0.4	140

[a] FITC-labeled lectins were incubated with CHO or Lec19 mutant cells as described in Experimental Procedures, and FACS analysis was performed. The mean fluorescence index (MFI) ± standard error of the mean were determined from four independent experiments.

Ecolume at a ratio of ~1:10 and counted in a scintillation counter.

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection of Glycopeptides. Con A-bound radiolabeled glycopeptides prepared from VSV G glycoprotein were desalted and desialylated by HCl treatment (0.01 M HCl for 2 h at 80 °C). The desialylated Con A-bound viral glycopeptides from CHO were digested with 5 milliunits of β-galactosidase (*D. pneumoniae*) in 50 mM sodium cacodylate buffer at pH 6.5 and 37 °C overnight. The glycopeptides desalted on a Bio-Gel P2 column were analyzed by HPAEC-PAD using a model PAD-2 detector and a CarboPac PA-100 (4 mm × 250 mm) pellicular anion-exchange column. Eluant 1 was 100 mM NaOH, and eluant 4 was 100 mM NaOH containing 1 M NaOAc. The column was eluted with eluant 4, the level of which was linearly increased to 10% over the course of 110 min, at a flow rate of 1 mL/min. Fractions (0.5 mL) were collected and counted.

Release of N-Linked Oligosaccharides by PNGase-F. N-linked oligosaccharides were released by treatment with PNGase-F as described previously (*15*).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). MALDI-TOF-MS was performed on a Voyager DE Biospectrometry Work Station (Perseptive Biosystem) equipped with delayed extraction as described previously (*15*). Oligosaccharide standards were used to achieve a two-point external calibration for mass assignment of ions. sDHB (2,5-dihydroxybenzoic acid and 5-methoxysalicilic acid) and 2,4,6-trihydroxyacetophenone matrices were used in the analysis of neutral and acidic oligosaccharides, respectively.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR). Reverse transcription was carried out using Superscript II reverse transcriptase with 2 μg of poly(A)$^+$ RNA, 0.5 μg of oligo dT primer$_{12-18}$, and 0.05 μg of random hexamer as described previously (*15*) using the following primers designed on the basis of proximal 5′-UTR and 3′-UTR sequences of the human, bovine, and mouse β4GalT genes: 5′-GTAGCCCACMCCCYTCTTAAAGC-3′ (forward primer) and 5′-AATGAGAGGGACCAGCCCAG-3′ (reverse primer) for β4GalT-1, 5′-GCTTGCGGGATGAGCAGACT-3′ (forward primer) and 5′-GGTCAGGCCTCTGGCAGGCA-3′ (reverse primer) for β4GalT-2, 5′-TGCCTCACATCTCTGCYCCC-3′ (forward primer) and 5′-GTTCAGTTCCCTCACATCCCTCT-3′ (reverse primer) for β4GalT-3, 5′-GCAGTGGCAAACATTACCTCAT-3′ (forward primer) and 5′-GTTGTGCTACTATGGAAGTCTCTA-3′ (reverse primer) for β4GalT-4, 5′-CCTGGCTGCAGCATGCGCGCCCGC-3′ (forward primer) and 5′-CCACTCTCTTCTGTCCTCCTGT-3′ (reverse primer) for β4GalT-5, and 5′-TCTGCGCTCAAGCGGATGATG-3′ (forward primer) and 5′-GCCATAGTCTTCGACTGGAGC-3′ (reverse primer) for β4GalT-6. The PCR mix contained primers (15 pmol), RT product (2 μL), 1 μL of 10 mM dNTPs, 0.5 μL of Taq DNA polymerase, 5 μL of 10× PCR buffer, and 3 μL of 25 mM $MgCl_2$ in a total volume of 50 μL. After the mixture had been heated at 94 °C for 3 min, annealing was carried out at 65 °C for 1 min and elongation at 72 °C for 2 min through 40 cycles. PCR products were purified using a QIAquick gel extraction kit (QIAGEN Inc.) and sequenced, either directly or after subcloning into the pCR2.1 vector using the Original TA Cloning Kit from Invitrogen.

Northern Blot Analysis. Total RNA from CHO or mutant cells was prepared using TRIzol Reagent (Life Technologies, Inc.), and poly(A)$^+$ RNA was prepared using an oligo d(T) column. Northern blots were generated as previously described (*15*) using coding region probes from the respective human β4GalT genes and reprobing with a GAPDH probe.

RESULTS

Altered Binding of Lectins to Lec19 Cells. The lectin resistance phenotype of Lec19 mutant cells suggested altered cell surface galactosylation of glycoproteins (*5*). To investigate this directly, the lectin binding properties of Lec19 cells were compared to those of parental CHO cells by FACS analysis (Table 1). Lec19 cells bound less *Ricinus communis* agglutinin II (RCA_{II}) and *Erythinia corriagendum* (ECA) lectins but more Tomato lectin (LTL), wheat germ agglutinin (WGA), and the erythroagglutinin from *P. vulgaris* (E-PHA) than parent CHO cells. Mutant cells bound succinylated WGA (sWGA) ~3.5-fold better than parent cells. In contrast, the bindings of pea lectin (PSA), *Maackia aurensis* agglutinin

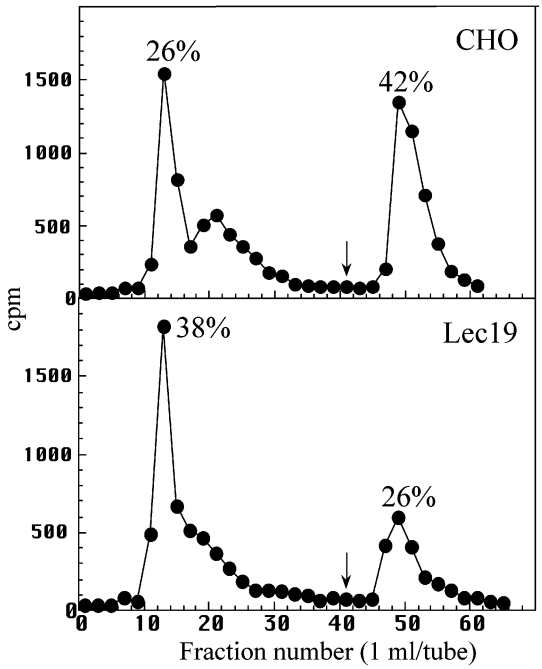

FIGURE 1: RCA_{II} lectin affinity chromatography of glycopeptides. Pronase-digested glycopeptides from CHO/VSV and Lec19/VSV labeled with [^{3}H]glucosamine were separated on Con A-Sepharose. The Con A-bound glycopeptide fraction with biantennary *N*-glycans was desalted and fractionated on an RCA_{II}−agarose column at 4 °C. The arrows mark elution with 200 mM lactose.

II (MAA_{II}), the leukoagglutinin from *P. vulgaris* (L-PHA), and *Datura stramonium* (DSA) were similar in both cell lines. Neither parent CHO nor Lec19 cells bound *Sambucus nigris* (SNA), *Lotus floribundum* (LFA), *Griffonia simplicifolium* II (GS_{IID}), or pokeweed mitogen (PWM). The increased level of binding of Lec19 cells to WGA and sWGA suggests an increase in the number of exposed GlcNAc residues (*18*), and an increased level of E-PHA binding is also indicative of terminal GlcNAc or a bisecting GlcNAc (*17*). The bisecting GlcNAc is transferred to *N*-glycans by *N*-acetylglucosaminyltransferase III [GlcNAc-TIII (*19*)]; however, this activity was not detected in extracts from the mutant, and reverse transcriptase PCR showed that Lec19 cells have no *Mgat3* gene transcripts (data not shown). Oversialylation was not the basis of the Lec19 phenotype since neuraminidase changed lectin binding to Lec19 and parent CHO cells equivalently (Table 1). The combined data suggested a loss of Gal residues accompanied by an increase in the level of exposure of GlcNAc on cell surface glycans.

A Reduced Level of Galactosylation of Biantennary N-Glycans in Lec19 Cells. To investigate the galactosylation of *N*-glycans, uniformly radiolabeled Pronase glycopeptides of the G glycoprotein of VSV grown in parent or Lec19 cells were subjected to serial lectin affinity chromatography. Con A-Sepharose chromatography showed no difference in the proportion of branched (∼20%) and biantennary (∼80%) complex *N*-glycans between parent and mutant-derived VSV glycopeptides (data not shown). However, when the Con A-bound, biantennary *N*-glycans were fractionated on an RCA_{II}−agarose column at 4 °C, 42% of the parent CHO/VSV biantennary *N*-glycans bound to the RCA_{II}−agarose column, compared to only 26% of the Lec19/VSV biantennary *N*-glycans (Figure 1). Con A-bound biantennary *N*-glycans were also analyzed by HPAEC-PAD (Figure 2).

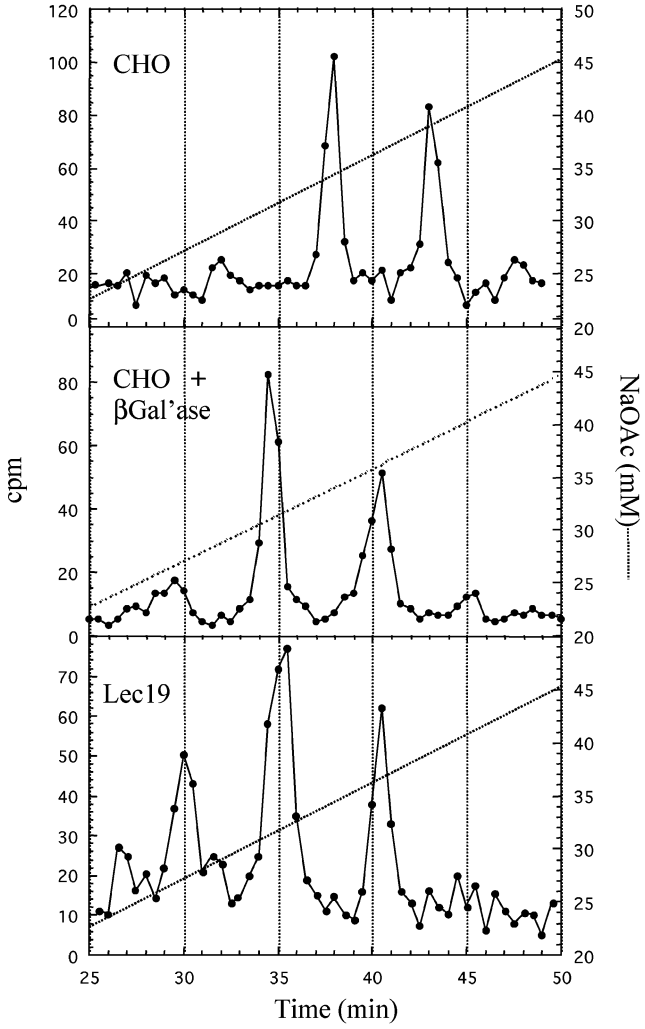

FIGURE 2: HPAEC-PAD analysis of biantennary viral glycopeptides. Pronase-digested viral glycopeptides from CHO/VSV and Lec19/VSV labeled with [^{3}H]glucosamine were separated on Con A-Sepharose into branched and biantennary species. The biantennary glycopeptides were desalted on Bio-gel P2, treated with 0.01 M HCl to remove sialic acid, and desalted again. One portion of the desialylated glycopeptides from CHO cells was analyzed directly by HPAEC-PAD, and the other portion was subjected to β-galactosidase treatment before analysis by HPAEC-PAD.

N-Glycans from parent CHO/VSV eluted in two major peaks at 37.5 and 43 min on a PA-10 column. These species correspond to a biantennary species terminating in Gal (GG) at 43 min and a species containing a core Fuc (GGFuc) at 37.5 min. In contrast, the major Lec19 biantennary *N*-glycans eluted earlier at 35.5 and 40.5 min, consistent with the loss of a Gal residue from GGFuc and GG, respectively. The species at 30 min in Lec19 corresponds to a biantennary *N*-glycan lacking Gal (G0) and the species at ∼26.5 min to G0 with a Fuc. When CHO/VSV *N*-glycans were digested with *D. pneumoniae* β-galactosidase, a profile similar to that of Lec19 was generated in terms of the major species (biantennary *N*-glycans with one Gal residue). Clearly, the β-galactosidase treatment of CHO/VSV *N*-glycans was partial since very little G0 was generated. The combined data in Figure 2 indicate that the major Lec19/VSV biantennary *N*-glycans contain a single Gal residue and ∼25% contain no Gal residues. A G0 species was not present in CHO/VSV biantennary *N*-glycans.

Table 2: β4GalT Activities of CHO and Lec19 Cells

acceptor	specific activity[a] [nmol h^{-1} (mg of protein)$^{-1}$] CHO	Lec19	Lec19/CHO (%)	*P* value
GlcNAc	23.7 ± 1.1	19.1 ± 2.4	81	<0.05
Glc (with α-lactalbumin)	10.0 ± 0.5	8.8 ± 0.4	88	<0.01
Fucα1,3GlcNAcβ-*O*-CH_3	0.8	0.8	98	
GlcNAcβ-*O*-pNP	3.4 ± 0.5	3.5 ± 0.3	106	
GlcNAcβ-*S*-pNP	4.9 ± 0.4	4.6 ± 1.1	94	
GlcNAcβ-*O*-benzyl	6.8 ± 0.6	5.2 ± 0.6	77	<0.01
biantennary GnGn	7.6 ± 0.6	5.7 ± 0.6	76	<0.02
biantennary GnGn(F)	10.0 ± 1.2	9.3 ± 0.3	93	
triantennary GnGnGn	13.9 ± 1.4	12.5 ± 2.7	90	
tetra-antennary GnGnGnGn	13.3 ± 0.9	11.7 ± 0.2	89	<0.05
GlcNAcβ1,6GalNAcα-*O*-pNP	7.3 ± 0.6	7.7 ± 0.3	105	
Galβ1,3 GlcCer	0.23 ± 0.02	0.14 ± 0.01	61	<0.002

[a] The activity is the mean ± standard deviation of three or four different experiments performed in duplicate. Significant differences of <0.05 are indicated.

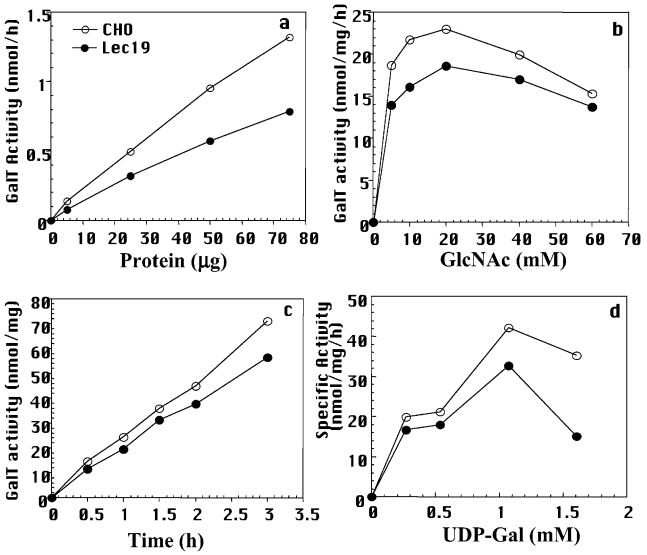

FIGURE 3: β4GalT activities of CHO and Lec19 cell extracts. β4GalT activities were measured (a) with increasing concentrations of protein, (b) with increasing concentrations of GlcNAc, (c) for different times, and (d) with increasing concentrations of UDP-Gal. Assay conditions were as described in Experimental Procedures.

Lec19 Cell Extracts Have Reduced β4GalT Activity. A decreased level of galactosylation of Lec19 *N*-glycans may result from a defect in UDP-galactose synthesis or localization, or from transfer of Gal to glycoproteins. The unique lectin resistance phenotype of Lec19 cells and genetic complementation analyses (*5*) showed Lec19 to be distinct from Lec8 mutants that are defective in transport of UDP-Gal into the Golgi compartment (*20*, *21*), from the ldlD mutant that is defective in UDP-Gal biosynthesis (*22*), and from Lec20 mutants that lack β4GalT-1 (*15*). In addition, exogenous galactose at 50 μM did not rescue the ricin resistance of Lec19 mutant cells (data not shown). However, with GlcNAc as an acceptor, Lec19 cells had reduced activity compared to parent CHO cells under a variety of conditions (Figure 3). The reaction product was shown by HPAEC-PAD analysis with treatment with standards and β-galactosidase to be Galβ1,4GlcNAc.

Using the optimal assay conditions determined for transfer to GlcNAc, β4GalT activity was assayed using simple sugars, oligosaccharides, and glycopeptides (Table 2). Lec19 cell extract had a significantly ($P < 0.05$) reduced activity with a subset of acceptors: ~10% reduction with Glc and the tetra-antennary branched *N*-glycan terminating with GlcNAc (GnGnGnGn), ~20% reduction with GlcNAc and GlcNAcβ-*O*-benzyl, ~25% with the biantennary *N*-glycan GnGn, and ~40% with the glycolipid acceptor GlcCer. A reduced level of transfer of Gal to fucosylated, biantennary GnGn(F) and triantennary GnGnβ4Gn was less consistent, and there was no significant difference in transfer to core 2 *O*-glycan, Fucα1,3GlcNAcβ-*O*-pNP, GlcNAcβ-*O*-pNP, or GlcNAcβ-*S*-pNP between Lec19 and CHO cells. Lec19 extracts were most significantly defective in transferring Gal to biantennary *N*-glycans. In contrast, Lec19 cells had the same activity as CHO cells of an unrelated glycosyltransferase, *N*-acetylglucosaminyltransferase I (GlcNAc-TI). The value for parent CHO extract GlcNAc-TI activity was 4.9 ± 0.8 nmol h^{-1} (mg of protein)$^{-1}$, whereas that of Lec19 cells was 5 ± 0.8 nmol h^{-1} (mg of protein)$^{-1}$.

MALDI-TOF-MS Analysis of N-Glycans Released from CHO and Lec19 Glycoproteins. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been used for both structural characterization and relative quantitation of neutral and sialylated *N*-glycans in a mixture (*23*−*25*). To examine galactosylation of the complement of major *N*-glycans in Lec19 cells, *N*-glycans were released from parent and mutant CHO cellular glycoproteins by PNGase-F and analyzed by MALDI-TOF mass spectrometry. The spectra of CHO and Lec19 neutral *N*-glycans were very similar (Figure 4a). Peaks were assigned to known neutral *N*-glycan structures on the basis of their mass (Table 3). Both cells synthesized a similar complement of oligomannosyl structures. As expected, Lec19 *N*-glycans contained a higher proportion of undergalactosylated, biantennary species (peaks 2, 3, 5, and 7 in Figure 4a) and a significant proportion of undergalactosylated triantennary species (peak 9). In contrast, the number of tetra-antennary *N*-glycans (peak 16) was comparatively low in the mutant (Figure 4a and Table 3). However, fully galactosylated bi-, tri-, and tetra-antennary *N*-glycans were present in similar proportions in both cell lines. The results are consistent with the β4GalT data in Table 2 indicating that Lec19 cells are most affected in the galactosylation of biantennary *N*-glycans,

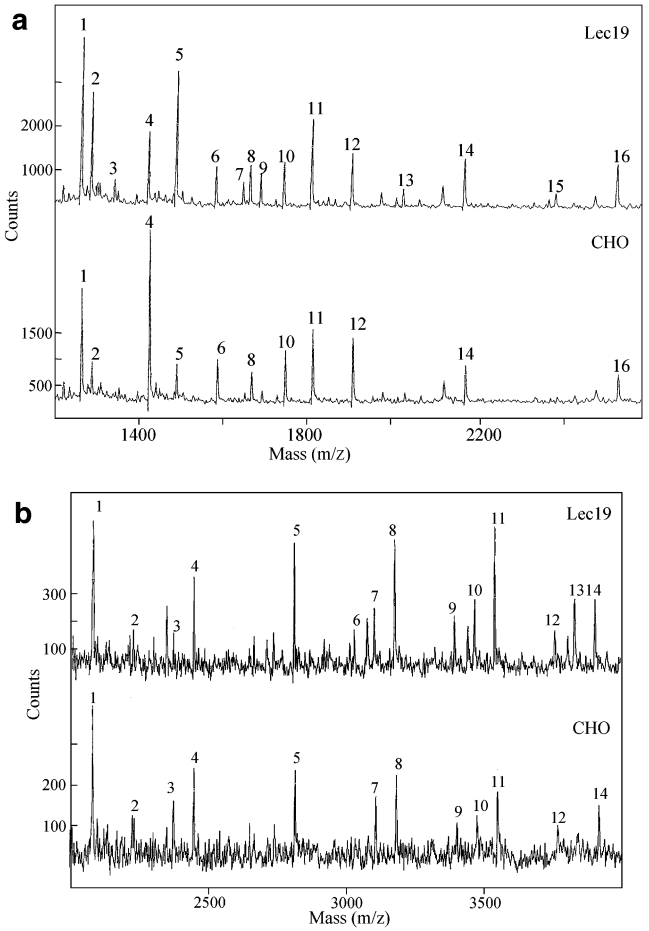

FIGURE 4: MALDI-TOF-MS of N-linked oligosaccharides from cellular glycoproteins of CHO and Lec19 cells. N-linked oligosaccharides from cellular glycoproteins of CHO and Lec19 cells were released by PNGase F and analyzed by MALDI-TOF-MS as described by Lee et al. (*15*): (a) neutral *N*-glycans in the positive ion mode and (b) sialylated *N*-glycans in the negative ion mode.

although the predominant species lacking Gal was GnGnF (peak 5) rather than GnGn as expected from *in vitro* β4GalT assays. The defect is much more subtle than in Lec20 CHO mutants that completely lack β4GalT-1. The latter express the full gamut of undergalactosylated bi-, tri-, and tetra-antennary neutral *N*-glycans and only small amounts of fully galactosylated species (*15*). Neither Lec19 (Figure 4a and Table 3) nor Lec20 CHO mutants (*15*) express neutral *N*-glycans with more than four *N*-acetyllactosamine groups.

In the case of sialylated *N*-glycans, there were very few differences between CHO and Lec19 spectra (Figure 4b). All of the sialylated *N*-glycans from both cell types were fully galactosylated. Interestingly, species containing five, six, and seven *N*-acetyllactosamine units were represented among the sialylated *N*-glycans. This result contrasts with sialylated Lec20 *N*-glycans synthesized in the absence of β4GalT-1 which contained only one to three *N*-acetyllactosamine units (*15*). The significant differences between Lec19 and CHO sialylated *N*-glycans were a relative increase in the level of $SG_6Gn_6M_3Gn_2F$ and $S_2G_6Gn_6M_3Gn_2F$ (peaks 11 and 13, respectively) and other multiantennary species with a single sialic acid among Lec19 *N*-glycans (Table 4 and Figure 4b). It appears that Lec19 cells can synthesize the same complement of fully galactosylated species as CHO cells but in smaller amounts as reflected by the relative preponderance of species with a single sialic acid. As observed previously (*15*), it seems that sialylation does not commence until galactosylation of *N*-glycans is complete and that sialic acids are added sequentially, since species with one sialic acid predominate over those with two or more.

The Lec19 Mutant Expresses Six β4GalT Genes of Unchanged Sequence at a Reduced Level. To investigate the molecular basis of the reduced β4GalT activity in Lec19 cell extracts, the coding region of each of the six β4GalT genes known to transfer Gal to GlcNAc or Glc in the synthesis of *N*-glycans or glycolipids was sequenced. The amino acid sequences of the CHO β4GalTs were found to be 82−95% identical with those of the corresponding human β4GalTs (Figure 5). The β4GalT-1 coding sequence from Lec19 is not shown as it was identical to the sequence found in parent CHO cells (Pro^-5 and Gat^-2) and reported previously (*15*). In addition, not a single nucleotide difference between Lec19 and CHO was found in cDNAs encoding any of the other β4GalT genes (see the accession numbers in the legend of Figure 5). However, when expression levels of β4GalT gene transcripts were compared by Northern analyses, the Lec19 mutant had ~3−4-fold less β4GalT-1, -T-4, and -T-5 transcripts than the parent CHO (Figure 6). Quantitative RT−PCR using the Taqman method gave an ~2-fold reduction in the level of β4GalT-1 transcripts when compared with that of GAPDH (Y. Hong, unpublished observations). β4GalT-2, -T-3, and -T-6 transcript levels were also decreased in Lec19 cells by ~1.5−2-fold. Thus, the undergalactosylation of *N*-glycans and the reduced β4GalT activity of Lec19 cells appear to result from the reduced level of expression of all six β4GalT genes.

DISCUSSION

Molecular characterization of the Lec19 CHO mutant has provided insight into several facets of the control of glycosylation in a mammalian cell. First, it is apparent that Lec19 cells have a defect in the galactosylation of *N*-glycans that mainly affects biantennary structures. Whereas neutral and sialylated biantennary *N*-glycans of the VSV G glycoprotein and cellular glycoproteins from Lec19 have reduced Gal content, multiantennary and polylactosamine-containing *N*-glycans are synthesized in roughly equivalent amounts in parent and Lec19 cells. Since it has been shown that all six human β4GalTs can transfer Gal to *N*-glycans and can form polylactosamines (*12*, *13*), it is of interest that the effect in Lec19 glycoproteins is mainly restricted to the undergalactosylation of biantennary *N*-glycans. In contrast to Lec19, *N*-glycans in Lec20 mutant CHO cells that lack β4GalT-1 contain the full spectrum of undergalactosylated complex *N*-glycans. Thus, the other β4GalTs that are normally expressed in Lec20 cells cannot fully compensate for the loss of β4GalT-1, though they do cause the synthesis of a small proportion of fully galactosylated *N*-glycan species in Lec20. The unique phenotype of Lec19 CHO cells thus appears to result primarily from the reduced level of galactosylation of biantennary *N*-glycans on cell surface glycoproteins. A change in galactosylation like this could be used by cells to modulate interactions with Gal-binding lectins such as selectins or galectins *in vivo*. The differences in lectin resistance between Lec19 and Lec20 mutants certainly reveal marked consequences of specific changes in galactosylation. For example, the Lec20 mutant that lacks

Table 3: Major Neutral *N*-Glycans Released from CHO and Lec19 Glycoproteins

peak	observed mass for Lec19[a] ([M + Na]$^+$) (Da)	predicted mass for Lec19 ([M + Na]$^+$) (Da)	predicted *N*-glycan[b]	CHO	Lec19
		High-Mannose *N*-Glycans			
1	1257.6	1258.1	M_5Gn_2	+++	++++
4	1419.6	1420.2	M_6Gn_2	++++	++
6	1581.5	1582.4	M_7Gn_2	+	+
10	1743.7	1744.5	M_8Gn_2	+	+
12	1905.5	1906.7	M_9Gn_2	++	++
		Biantennary *N*-Glycans			
2	1282.5	1283.2	GnM_3Gn_2F	+	+++
3	1339.5	1340.2	$Gn_2M_3Gn_2$	−	(+)[c]
5	1485.5	1486.3	$Gn_2M_3Gn_2F$	+	+++
7	1647.5	1648.5	$GGn_2M_3Gn_2F$	−	(+)[c]
8	1663.6	1664.5	$G_2Gn_2M_3Gn_2$	+	+
11	1809.5	1810.6	$G_2Gn_2M_3Gn_2F$	++	++
		Triantennary *N*-Glycans			
9	1688.5	1689.5	$Gn_3M_3Gn_2F$	−	+
13	2029.0	2029.7	$G_3Gn_3M_3Gn_2$	−	(+)[c]
14	2174.7	2175.9	$G_3Gn_3M_3Gn_2F$	+	+
		Tetra-Antennary *N*-Glycans			
15	2393.7	2394.8	$G_4Gn_4M_3Gn_2$	−	(+)[c]
16	2539.9	2541.3	$G_4Gn_4M_3Gn_2F$	+	+

[a] Masses were taken from the Lec19 spectrum in Figure 4a. Among different spectra, species with the same peak number had a mass within 0.5−1 Da of that given in the table. [b] *N*-Glycans were predicted on the basis of the mass and composition of known neutral. *N*-Glycan structures (G, galactose; Gn, *N*-acetylglucosamine; M, mannose; F, fucose). [c] Peaks were present, but in small amounts.

Table 4: Major Sialylated *N*-Glycans Released from CHO and Lec19 Cells

peak	observed mass for Lec19[a] ([M − H]$^-$) (Da)	predicted mass for Lec19[a] ([M − H]$^-$) (Da)	predicted *N*-glycan[b]	CHO	Lec19
		Biantennary *N*-Glycans			
1	2076.3	2077.9	$SG_2Gn_2M_3Gn_2F$	+++	+++
2	2221.4	2223	$S_2G_2Gn_2M_3Gn_2$	(+)[c]	(+)[c]
3	2368.9	2369.1	$S_2G_2Gn_2M_3Gn_2F$	+	(+)[c]
		Triantennary *N*-Glycans			
4	2441.4	2443.2	$SG_3Gn_3M_3Gn_2F$	++	++
6	3025.6	3026	$S_3G_3Gn_3M_3Gn_2F$	−	(+)[c]
		Tetra-Antennary *N*-Glycans			
5	2807.1	2808.5	$SG_4Gn_4M_3Gn_2F$	++	+++
7	3098.6	3099.8	$S_2G_4Gn_4M_3Gn_2F$	+	+
9	3390.1	3391.1	$S_3G_4Gn_4M_3Gn_2F$	(+)[c]	+
		Polylactosamine *N*-Glycans			
8	3172.2	3173.9	$SG_5Gn_5M_3Gn_2F$	++	+++
10	3463.8	3465.1	$S_2G_5Gn_5M_3Gn_2F$	(+)[c]	+
11	3537.3	3539.2	$SG_6Gn_6M_3Gn_2F$	+	+++
13	3828.7	3830.1	$S_2G_6Gn_6M_3Gn_2F$	−	++
12	3756.1	3758.4	$SG_7Gn_7M_3Gn_2$	(+)[c]	(+)[c]
14	3903.2	3904.5	$SG_7Gn_7M_3Gn_2F$	+	++

[a] Masses were taken from the Lec19 spectrum in Figure 4b. Among different spectra, species with the same peak number had a mass within 0.5−1 Da of that given in the table. [b] *N*-Glycans were predicted on the basis of the mass and composition of known sialylated *N*-glycan structures (G, galactose; Gn, *N*-acetylglucosamine; M, mannose; F, fucose). [c] Peaks were present, but in small amounts.

only β4GalT-1 (*15*) is 7-fold more resistant to E-PHA than CHO cells, while the Lec19 mutant is 2-fold more sensitive than CHO cells to the toxicity of this Gal-binding lectin (*5*).

Another interesting feature of the Lec19 mutant is that, unlike Lec20 mutants, Lec19 extracts had a decreased ability to transfer Gal to GlcCer (Table 2). Previously, it was suggested that β4GalT-5 synthesizes LacCer in CHO cells because neither β4GalT-1 nor β4GalT-6 was found to be responsible for the synthesis of the major CHO glycolipids LacCer and GM3 (*15*). Sequence comparisons and acceptor specificities also suggest that β4GalT-5 is most related to β4GalT-6, a putative LacCer synthase. Consistent with this possibility, we show here that Lec19 mutant cells with reduced β4GalT-5 gene transcripts also have reduced *in vitro* β4GalT activity for GlcCer (~60% compared to parent cell extract). However, a corresponding increase in the amount of GlcCer was not observed in thin layer chromatograms of glycolipids extracted from Lec19 cells (J. Lee, unpublished observations). Thus, it appears that *in vivo*, the remaining activity of β4GalT-5 may be sufficient to synthesize normal amounts of GM3 and LacCer in Lec19 cells. Another possibility is β4GalT-3, which has been shown to generate LacCer *in vitro* (*7*).

The altered galactosylation characteristic of Lec19 CHO cells is apparently due to the decreased level of expression of all six β4GalT genes known to be responsible for the transfer of Gal to GlcNAc or Glc on glycoconjugates (Figure 6). This decreased level of expression is specific since the

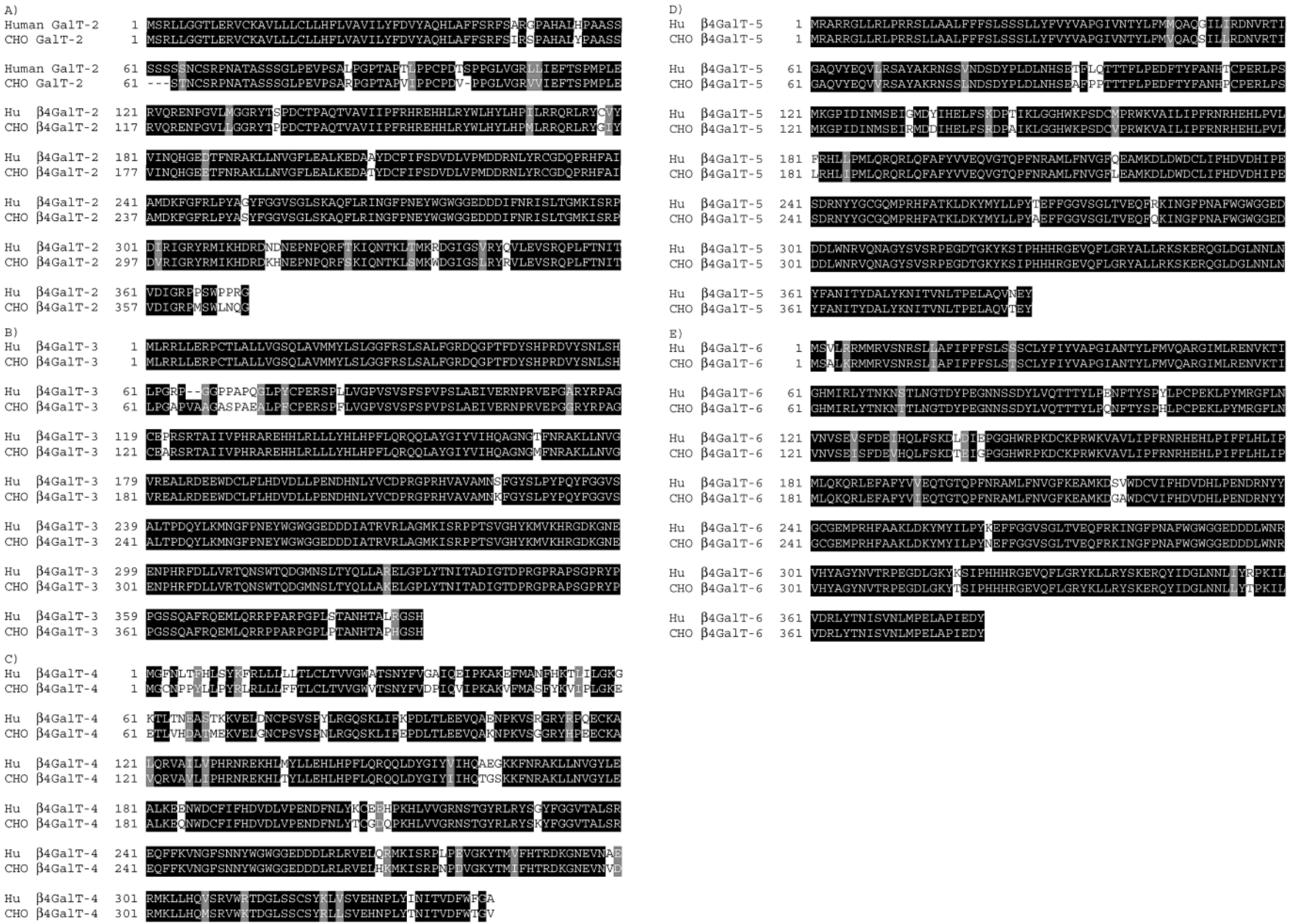

FIGURE 5: Clustal W analysis of CHO β4GalTs. CHO β4GalT gene coding amino acid sequences are from 81 to 95% identical to the corresponding human β4GalTs. Identical residues are highlighted in black, and similar residues are highlighted in gray. The hamster nucleotide sequences have been deposited in GenBank with the following accession numbers: AF318896 for β4GalT-1, AY117536 for β4GalT-2, AY117537 for β4GalT-3, AY117538 for β4GalT-4, AY117539 for β4GalT-5, and AF506839 for β4GalT-5.

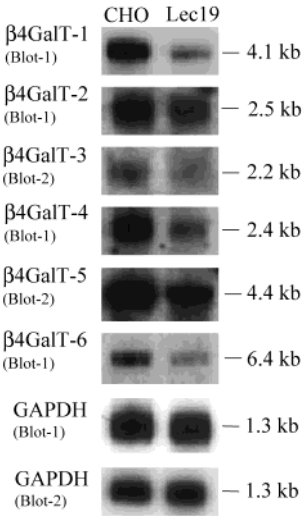

FIGURE 6: Northern analysis of CHO and Lec19 RNA. Northern blots containing 2 μg of poly(A)$^+$ RNA were sequentially hybridized with ~800 bp probes corresponding to the six human β4GalT genes and then to a probe for GAPDH as described previously (*15*). Each blot was exposed to X-ray film at −80 °C for ~5 days. The CHO (Gat$^-$2) data from these blots were previously published in ref *15*.

activity of another Golgi transferase (GlcNAc-TI) is equivalent in CHO and Lec19 cells, and GAPDH transcript levels also do not differ. Sequencing of gene transcripts revealed identical coding region sequences for each β4GalT in parent and Lec19 cells. Thus, no mutations or polymorphisms were identified, and it is clear that genetic variation does not occur frequently during culture of CHO cells, as previously observed (*26*).

A key question is how the Lec19 mutation arose given that the six β4GalT genes map to different chromosomal locations in humans and rodents. A likely molecular basis would seem to be the loss of a trans-acting factor that regulates transcription of the six β4GalT genes, or that controls the stability, transport, localization, or translation of the respective β4GalT gene transcripts. The acquisition by Lec19 cells of a negative regulator of β4GalT gene expression that reduces the level of transcription of all six β4GalT genes, or degrades their transcripts, is less likely because the *lec19* mutation behaves recessively (*5*). In contrast, the loss of a transcriptional activator for the six β4GalT genes is possible and would predict the existence of a conserved upstream sequence in the promoter region of the respective β4GalT genes. However, little is known at present about functional β4GalT gene promoters, nor have the 5′-UTR and 3′-UTR regions of β4GalT gene transcripts yet been identified. The transcript half-life has recently been reported for β4GalT-1 in one cell type (*27*), and a similar study in CHO versus Lec19 cells would be of interest. Cloning the factor responsible for the Lec19 phenotype by cDNA library complementation of the Lec19 defect would be most direct but complicated by the fact that overexpression of a large number of different factors involved in galactosylation (including any of the six β4GalT genes) could potentially rescue the Lec19 phenotype by mechanisms unrelated to the primary mutation. Once mammalian genomes are more fully decoded, a likely molecular genetic basis of the *lec19* CHO mutation may be revealed. Meanwhile, our analysis of the altered glycosylation in the Lec19 CHO mutant has identified a novel mechanism by which galactosylation may be regulated in mammals.

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