

Activity, Splice Variants, Conserved Peptide Motifs, and Phylogeny of Two New α 1,3-Fucosyltransferase Families (FUT10 and FUT11)*

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We report the cloning of three splice variants of the *FUT10* gene, encoding for active α -L-fucosyltransferase-isoforms of 391, 419, and 479 amino acids, and two splice variants of the *FUT11* gene, encoding for two related α -L-fucosyltransferases of 476 and 492 amino acids. The *FUT10* and *FUT11* appeared 830 million years ago, whereas the other α 1,3-fucosyltransferases emerged 450 million years ago. FUT10-391 and FUT10-419 were expressed in human embryos, whereas FUT10-479 was cloned from adult brain and was not found in embryos. Recombinant FUT10-419 and FUT10-479 have a type II *trans*-membrane topology and are retained in the endoplasmic reticulum (ER) by a membrane retention signal at their NH₂ termini. The FUT10-479 has, in addition, a COOH-ER membrane retention signal. The FUT10-391 is a soluble protein without a *trans*-membrane domain or ER retention signal that transiently localizes to the Golgi and then is routed to the lysosome. After transfection in COS7 cells, the three FUT10s and at least one FUT11, link α -L-fucose onto conalbumin glycopeptides and biantennary *N*-glycan acceptors but not onto short lactosaminyl acceptor substrates as do classical monoexonic α 1,3-fucosyltransferases. Modifications of the innermost core GlcNAc of the *N*-glycan, by substitution with ManNAc or with an opened GlcNAc ring or by the addition of an α 1,6-fucose, suggest that the FUT10 transfer is performed on the innermost GlcNAc of the core chitobiose. We can exclude α 1,3-fucosylation of the two peripheral GlcNAcs linked to the trimannosyl core of the acceptor, because the FUT10 fucosylated biantennary *N*-glycan product loses both terminal GlcNAc residues after digestion with human placenta α -N-acetylglucosaminidase.

Fucosyltransferases are globular type II *trans*-membrane Golgi-resident proteins that catalyze the transfer of α -L-fucose from GDP-Fuc onto *N*- and *O*-linked glycans, free oligosaccharides, or lipids (1) or directly onto proteins (2). These fucosylations are involved in a variety of biological processes, including selectin-mediated leukocyte-endothelial adhesion, lymphocyte homing, ABO blood group histocompatibility, notch receptor signaling (3), embryo-fetal development, and host-microbe interactions (4). Changes in the glycosylation pattern of proteins may interfere with cellular functions and may thus lead to health disorders, such as cancers or rare autosomal recessive diseases, such as congenital disorders of glycosylation, characterized by glycosylation deficiencies (5).

Fucosyl residues in mammals are found linked to an oligosaccharide acceptor in α 1,2-, α 1,3-, α 1,4-, and α 1,6-orientations (6) or directly to serine or threonine as protein-*O*-fucosylations (7–9). The α 1,2-fucosyltransferases (FUT1, FUT2, and Sec1) (10) and the α 1,3/4-fucosyltransferases (FUT3–FUT7 and FUT9), resulting mainly from two rounds of whole genome duplication (11), are implicated in terminal fucosylations (12, 13), and they are encoded by monoexonic genes (1). The α 1,6-fucosyltransferase encoded by the *FUT8* gene transfers α -L-fucose onto the innermost asparagine-linked GlcNAc of the chitobiose disaccharide unit of glycoproteins (14, 15). Previously, we found that α 1,2-fucosyltransferases, α 1,6-fucosyltransferases, and protein-*O*-fucosyltransferases share three main conserved peptide motifs (16) and constitute a new superfamily.

Human α 1,3/4-fucosyltransferases and their genetic expression are developmentally regulated (17, 18). We have previously shown that *FUT4* and *FUT9* genes are derepressed early in human embryogenesis, whereas *FUT6* and *FUT3* appear sequentially after the 8th week of development (19). This suggests that during development, the Le^x or CD15 antigen (generated by FUT4 or FUT9) appears earlier than the sialyl-Le^x (made by FUT5, FUT6, or FUT7) or the type 1 Lewis structures (Le^a and Le^b antigens, made by FUT3 or FUT5). Le^x has been found mainly in undifferentiated rapidly dividing cells (20), whereas sialyl-Le^x is more abundant in differentiated cells (17). This is particularly interesting because these glycotopes are implicated in embryo-fetal development, selectin-dependent leukocyte recruitment, and lymphocyte homing (21).

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Expression of Two New FUT Families, FUT10 and FUT11

A few years ago, Renkonen and co-workers (22), using the *Drosophila* genome-wide bioinformatics approach to identify the proteome involved in α -L-fucosylated glycan metabolism, identified a *Drosophila* fucosyltransferase and two human orthologous genes, encoding for the fucosyltransferases FUT10 and FUT11. Due to the presence of the two main conserved motifs (23), they were assumed to be α 1,3-fucosyltransferases, but their activity has not been experimentally demonstrated yet in any species, including humans (22), mice (24),⁵ flies (26–28), or honeybees (29). Four insect α 1,3-fucosyltransferases (Fuc-TA, Fuc-TB, Fuc-TC, and Fuc-TD) were first identified in *D. melanogaster* (26–28). The Fuc-TA is a core α 1,3-fucosyltransferase (28), the Fuc-TB is orthologous to human FUT10 and FUT11, Fuc-TC is probably involved in the synthesis of Le^x (29), and no activity has yet been found for the Fuc-TD.

In this work, we cloned three new active splice variants of the human *FUT10* gene, two in the embryo and one in the adult. We investigated their subcellular distribution and their fucosyltransferase activity. In addition, we report an α 1,3-fucosyltransferase activity for FUT11, with an acceptor pattern similar to FUT10.

EXPERIMENTAL PROCEDURES

Fucosyltransferase Assays—Transfected COS7 cells were homogenized on ice in 2% Triton X-100, and protein concentration was measured with the Bio-Rad Bradford protein microassay. Each fucosyltransferase assay was performed, unless otherwise stated, in a total volume of 65 μ l, containing 15 μ g of cell protein extract, 65 mM cacodylate buffer (pH 7.25), 10 mM L-fucose, 7 μ M GDP-[¹⁴C]L-fucose (29×10^4 dpm/test at 300 mCi/mmol; Amersham Biosciences), and 5 μ l/test of a 1 mg/ml solution of acceptor substrate (Table 1). For α 1,3-fucosyltransferase assays, we used the conditions already described for the α 1,6-fucosyltransferase FUT8 (30), and the activities were compared in the absence or presence of 20 mM MnCl₂ as cofactor. The S.D. values of the mean value activities correspond to 10 independent tests for each enzyme. The mean of three independent enzymatic experiments is shown when there is no S.D. For kinetic studies, we used 25 μ g of homogenate proteins, 3 μ M GDP-[¹⁴C]L-fucose (125×10^3 dpm/test at 300 mCi/mmol) plus 125 μ M cold GDP-Fuc (kindly provided by Claudine Augé, Paris-XI University of Sciences, Orsay, France).

The reactions were generally continued for 4 h at 37 °C, stopped by the addition of 3 ml of cold water, and centrifuged, and the supernatant was applied to a conditioned Sep-Pak C18 reverse-phase chromatography cartridge (Waters, Milford, MA) attached to a 10-ml syringe (19, 31). The retained hydrophobic acceptors were separated from the unreacted GDP-[¹⁴C]fucose and its hydrolysis products by washing with 25 ml of H₂O and then eluted with two 5-ml fractions of methanol and counted with 10 ml of Instagel-Plus (Hewlett-Packard, Evry, France) in a liquid scintillation β -counter (LS-6500; Beckman). The transfer of [¹⁴C]fucose was expressed in pmol/h/mg of protein, unless otherwise stated.

Preparation of α 1,3- and α 1,6-Fucosyltransferase Acceptors—The biotin-labeled natural biantennary-glycoasparagine oligo-

saccharide acceptor was obtained from a pool of human plasma proteins by Pronase digestion followed by gel purification, desialylation, degalactosylation, and binding to biotin (BGA-biotin), as already described (30) (Table 1).

Synthetic short amphiphilic oligosaccharide acceptors were synthesized as 8-methoxycarbonyl octyl glycosides also called oligosaccharide-O-grease (Ogr).⁶ These acceptors were kindly provided by Ole Hindsgaul and Monica Palcic (Carlsberg Laboratory, Copenhagen, Denmark) (Table 1).

Biantennary or other *N*-glycan-related acceptors, synthesized as biotinylated monomeric probes (0987-BM, 0988-BM, 0989-BM, 0990-BM, and chitobiose-BM), were bought from Lectinity Corp. (Moscow, Russia). They are synthesized with an elongated spacer linked to biotin (32), giving them the possibility of being adsorbed on Sep-Pak C18 cartridges as described for the above mentioned hydrophobic acceptors.

The biantennary derivative, where the innermost GlcNAc residue is substituted by an *N*-acetyl-mannosamine residue (referred as 7OS-ManNAc-BM), was obtained from free 7OS by alkali 2-epimerization followed by 1-amination, chloroacetylation, ammonolysis, and final biotinylation (32).

The “opened” amino-alditol-GlcNAc derivative of biantennary OS (referred as 7OS-amino-alditol-GlcNAc-BM) was obtained by reductive amination of free 7OS with NH₃ in the presence of sodium cyanoborohydride, followed by biotinylation (32) (Table 1).

Preparation of the α 1,6-Fucosylated 0989-BM Acceptor with the FUT8 Fucosyltransferase—The innermost GlcNAc residue of the 0989-BM acceptor (50 μ g) was fucosylated in the α 1,6-position with the recombinant FUT8 enzyme. The reaction conditions were as follows: 65 μ l containing 80 μ g of protein from the homogenate of COS7 cells transfected with FUT8 cDNA, 65 mM cacodylate buffer (pH 7.25), 10 mM L-fucose, 300 μ M cold GDP-L-fucose, and 5 μ g/test of the 0989-BM acceptor substrate (Table 1). After 4 h at 37 °C, the reaction is more than 97% complete, and the retained Fuc α 1,6-0989-BM product was purified as described above. The transfer of the [¹⁴C]fucose in position 3 of Fuc α 1,6-0989-BM by the different FUT10 isoforms was performed as described above with a 16-h incubation at 37 °C. The activity was expressed as dpm/reaction.

Conalbumin Glycopeptide Acceptor Preparation for Fucosyltransferase Assay—Glycopeptides were prepared from 100 μ g of chicken egg white conalbumin (P-7786; Sigma) after digestion with 7 units of Pronase (5 mg/ml; P-5147; Sigma) for 24 h at 37 °C, in 0.1 M Tris/HCl, 4 mM CaCl₂, pH 8.0, buffer. The reaction was stopped by heating for 5 min at 100 °C. Conalbumin glycopeptides were recovered from the supernatant, after a 10-min centrifugation at 4000 rpm, column-desalted, dried, and then used as a source for potential glycopeptide acceptors for α (1,3)- or α (1,6)-fucosyltransferase activities, as described above.

Another fucosylation reaction was performed as described above, using 100 μ g of native chicken egg white conalbumin.

⁵ S. K. Patnaik (2007) *Nature Precedings*, available on the World Wide Web.

⁶ The abbreviations used are: Ogr, oligosaccharide-O-grease; BSA, bovine serum albumin; cds, coding sequence; ER, endoplasmic reticulum; GFP, green fluorescent protein; MYA, millions of years ago; ORF, open reading frame; PBS, phosphate-buffered saline; TMD, trans-membrane domain; NAG, β -N-acetylglucosaminidase; contig, group of overlapping clones.

TABLE 1Acceptors used for the α 1,3- and α 1,6-fucosyltransferase activities

Acceptor names	Acceptor structures
BGA-biotin	GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc β 1AspNHCO-biotin GlcNAc β 1,2Man α 1,3
0989-BM	GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin GlcNAc β 1,2Man α 1,3
0988-BM	Gal β 1,4GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin Gal β 1,4GlcNAc β 1,2Man α 1,3
0987-BM	NeuAc α 2,6Gal β 1,4GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin NeuAc α 2,6Gal β 1,4GlcNAc β 1,2Man α 1,3
0990-BM	Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin Man α 1,3
H-type-2-Ogr	Fuc α 1,2Gal β 1,4GlcNAc β O(CH ₂) ₈ COOCH ₃
LacNAc-type-2-Ogr	Gal β 1,4GlcNAc β O(CH ₂) ₈ COOCH ₃
H-type-1-Ogr	Fuc α 1,2Gal β 1,3GlcNAc β O(CH ₂) ₈ COOCH ₃
LacNAc-type-1-Ogr	Gal β 1,3GlcNAc β O(CH ₂) ₈ COOCH ₃
chitobiose-BM	GlcNAc β 1,4GlcNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin
OP-392-Ogr	Man α 1,6 Man β O(CH ₂) ₈ COOCH ₃
OP-395C-Ogr	GlcNAc β 1,6 Man α 1,6Man β O(CH ₂) ₈ COOCH ₃ GlcNAc β 1,2
SHT-2156-Ogr	GlcNAc β 1,2Man α 1,6 Man β O(CH ₂) ₈ COOCH ₃ Man α 1,3
7OS-ManNAc-BM	GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4ManNAc β 1NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin GlcNAc β 1,2Man α 1,3
7OS-amino-alditol- GlcNAc-BM	GlcNAc β 1,2Man α 1,6 Man β 1,4GlcNAc β 1,4GlcNAc(ol)-NHCOCH ₂ NHCO(CH ₂) ₅ NH-biotin GlcNAc β 1,2Man α 1,3

After terminating the fucosyltransferase incubation, Pronase was added, and the reactions were incubated overnight at 37 °C. The resulting [¹⁴C]fucosylglycopeptides (fractions 9–15) were separated from unreacted GDP-[¹⁴C]fucose and liberated [¹⁴C]fucose (fractions 18–26) on a Biogel P2 column (27 × 1 cm; Bio-Rad) equilibrated with 100 mM acetic acid. The resulting [¹⁴C]fucosyl-conalbumin glycopeptides were quantitated with a liquid scintillation β -counter.

TLC—A fucosyltransferase assay using the homogenate derived from cells transfected with the FUT10-419 construct

was performed for 6 or 16 h at 37 °C with the BGA-biotin acceptor. The radioactive oligosaccharide product was purified on a Sep-Pak C18 cartridge and eluted with methanol. After lyophilization, two-thirds of the radioactive product was digested overnight at 37 °C with 0.6 units of β -N-acetylglucosaminidase (NAG) from human placenta (Sigma) in 0.1 M citrate/phosphate buffer, pH 5.5. Subsequent to clean-up using Sep-Pak cartridges as described above, standards and NAG-treated ¹⁴C-fucosylated acceptor molecules were resolved on silica-coated plastic TLC sheets (Merck) devel-

TABLE 2

Oligonucleotide primer sequences used in this study for amplifications and sequencing

Primers ^a	Sequence 5'–3'	Positions ^b
F10-1s	5'–CATGCTGCTTCTCTCGATGCCAAGC–3'	Ex 2, 19–44
F10-8s	5'–GTTCTTCCATGTGAAGTTGTCTCGGG–3'	Ex 2, 180–205
F10-479s	5'–GTTAGCCCTCTGTCACTCAGCCAGG–3'	Ex 2, 239–263
F10-391s	5'–TAGAAGGTCATGGTTGAGCTGGG–3'	Ex 4, 55–60/Ex 5, 1–17
F10-4as	5'–GAGCATAATGGGGTAGCTGTCCAATTCC–3'	Ex 5, 147–174
F10-7s	5'–ATCCAGCCTCTATGGATGCCGATGGC–3'	Ex 6, 397–422
F10-as2	5'–GAATCTATAGCTAGATCTCTCCCTCC–3'	Ex 6, 505–528
F10-GFPas	5'–GAGATGGCCAGCCTGCTGGCTCT–3'	Ex 6, 860–882
F10-12as	5'–GCAGGAGAGCAGAGAATCAAGATGG–3'	Ex 6, 860–894
F10-13as	5'–CACTGATGAGGTGGCAACAACAGAAGG–3'	Ex 6, 907–933
F10-K1GFPas	5'–TTCGGTCCCTTGAATACTAGGCCCC–3'	Ex 7, 206–230
F11-ints	5'–CGCCACACTCCGGTAGACTCC–3'	Ex 1, 740–753/Ex 2, 1–7
F11-intas	5'–GAGTGATTGTTCGCATCCAGTCC–3'	Ex 2, 226–249

^a s, sense; as, antisense.^b Ex, exon.

oped in *n*-propyl alcohol/acetic acid/water (3:3:2 for 24 h) (33). Radioactive components were visualized by fluorography after spraying the TLC plates with En³hance (PerkinElmer Life Sciences). Standard oligosaccharides separated under the same conditions were Man₅GlcNAc₂ (34) and ¹⁴C-labeled Gal₂GlcNAc₂Man₃GlcNAc₂, which was generated by incubating GlcNAc₂Man₃GlcNAc₂ (Dextra Laboratories) with UDP-[¹⁴C]galactose and bovine milk galactosyltransferase (Sigma).

RNA Isolation and Northern Blot Analysis—Embryos aged from 50 to 70 days were obtained from legal abortions and stored at –80 °C as already described (19). Total RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Contaminating DNA was removed by digestion with RNase-free DNase I (10 units/μg of RNA from Roche Applied Science) for 15 min at room temperature, followed by 15 min of inactivation at 70 °C and purification of the RNA by phenol/chloroform extraction. Embryonic poly(A)⁺ mRNAs were double purified using oligo(dT)-cellulose (type 3; Sigma) chromatography. Poly(A)⁺ RNAs (4 μg/lane) were denatured and fractionated with 1.2% phosphate-agarose gel electrophoresis, transferred to Hybond-N membranes (Amersham Biosciences), and immobilized by baking at 80 °C for 2 h. Prehybridization and hybridization were performed for 16 h at 42 °C in a buffer containing 50% formamide, 5× SSC, 1× PE, 250 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate with the *cds*-FUT10 probe of 350 bp, obtained by PCR using primers sense F10-8s and antisense F10-4as (Table 2). The blots were first washed at low stringency: 2 × 15 min (in 2× SSC, 0.1% SDS) at 42 °C, followed by a single wash of 15 min (2× SSC, 0.1% SDS) at 50 °C and autoradiographed. A last 15 min wash in (0.5× SSC, 0.1% SDS) at 60 °C was performed, and another autoradiography was made. The films were developed after 3 days at –80 °C.

Construction of cDNA Libraries—Poly(A)⁺ mRNAs (1 μg) from a single 50-day embryo and from an adult brain were used to initiate the first strand cDNA synthesis. They were reverse transcribed at 42 °C for 90 min, using the oligo(dT)-cDNA synthesis primer (52-mer) and 200 units of the Superscript-II RNase H-reverse transcriptase from the Superscript first strand cDNA synthesis system kit (Invitrogen). The embryonic and adult cDNA libraries were stored at –20 °C until used. The PCRs were carried out with primers specific for FUT10 (Table

2), the KlenTaq mixture (Clontech), and 1 μl of cDNA templates from the 50-day embryo or from adult brain cDNA libraries for the first PCR and 1 μl of the first PCR product diluted 1:10 for the second PCR. The same amplification program with the Advantage cDNA amplification kit mix (BD Clontech, Palo Alto, CA) was used. All PCRs were performed in 50 μl, with 1× KlenTaq buffer, a 0.2 μM concentration of each primer, 1 unit of KlenTaq DNA polymerase, and 0.2 mM dNTP with the touch-down-RACE program: initial denaturation 94 °C for 90 s, followed by five cycles of 94 °C for 30 s and 72 °C for 4 min, 5 cycles of 94 °C for 30 s and 70 °C for 4 min, and 25 cycles of 94 °C for 30 s and 68 °C for 4 min.

Cloning of the FUT10 Transcripts—Four FUT10 cDNA isoforms (FUT10-357, FUT10-391, FUT10-419, and FUT10-479) were amplified by a double PCR using the embryo and the adult cDNA libraries as templates. The FUT10-357 was a truncated variant of FUT10-391, lacking the conserved motifs I and II. It was devoid of enzyme activity and was not further analyzed. The primer associations F10-1s and F10-13as or F10-1s and F10-as2 were used for the first PCR, and distinct combinations of nested primers as F10-8s and F10-12as or F10-8s and F10-as2 were used for the second PCR (Table 2). With these two nested primer combinations, we obtained a broad PCR product of 1400 bp in the embryo cDNA with F10-8s and F10-12as and a product of 1800 bp from the adult cDNA library, with F10-8s and F10-as2 primers. These final PCR products were gel-purified and cloned into the TA cloning vector PCR3.1 (Eukaryotic TA cloning kit; Invitrogen), and 20 plasmid clones were PCR-selected from each positive ligation and sequenced. To generate the FUT10-GFP-tagged transcripts, we amplified the three selected FUT10 constructs: FUT10-391 with primers F10-391s and F10-GFPas, FUT10-419 with primers F10-8s and F10-GFPas, and FUT10-479 with primers F10-479s and F10-K1GFPas, in the presence of the high fidelity Hotstart DNA polymerase (Accuprime-pF_x DNA polymerase; Invitrogen). The PCR products were gel-purified and inserted into the mammalian pcDNA3.1-GFP vector (TOPO-CT-GFP-cloning kit; Invitrogen). The resulting GFP-COOH-tagged plasmids were selected by PCR, sequenced, and called FUT10-391-GFP, FUT10-419-GFP, and FUT10-479-GFP.

Isolation of FUT11 cDNA Clones—The IMAGE clone 40005868 (BC100994) in a pCR-Blunt-TOPO vector encoding for a 476-amino acid protein and the IMAGE clone 5271548

(BC036037) in a pBluescriptR vector, encoding for a 492-amino acid protein, were obtained from Gene-service Ltd. (Cambridge, UK). The IMAGE clones were digested with EcoRI (NEB-Biolabs, Ozyme, Saint Quentin Yvelines, France). Two DNA fragments of 1730 and 1660 bp, corresponding to FUT11-476 and FUT11-492 cDNA, respectively, were purified and subcloned into the PCR3.1-TA cloning vector (Invitrogen). The presence of the inserts was verified by PCR with Taq polymerase (MBI Fermentas, Euromedex, Strasbourg, France) and the internal primers F11-ints and F11-intas (Table 2). The correct orientation of FUT11-476 and FUT11-492 plasmids was checked with BamHI and EcoRI.

DNA Sequencing—The different FUT10, FUT11, and pcDNA3.1-FUT10-GFP expression vectors were sequenced in both directions (Biofidal SARL, Vaulx-en-Velin, France). All of the full-length isoform constructs devoid of PCR errors were selected and used for transfection analysis.

Transient Expression of FUT10, FUT11, and GFP-tagged-FUT10 cDNA Constructs in COS7 Cells—cDNA transcripts were inserted into PCR3.1 (TA-cloning System; Invitrogen) and transiently transfected into COS7 cells (20 μ g of plasmid) with DEAE-dextran (19). After 12, 18, 24, or 48 h, the cells were washed with phosphate-buffered saline (PBS) and harvested for fucosyltransferase assays or indirect immunofluorescence microscopy.

Immunofluorescent Localization of the GFP-tagged FUT10 Fusion Proteins— 2×10^5 cells were seeded on glass coverslips in 35-mm cell culture Petri dishes 24 h before transfection. Five μ g of the GFP-tagged cDNA constructs were transfected into COS7 cells. After 12, 18, 24, or 48 h of growth, they were washed with PBS and then fixed for 15 min with 2% paraformaldehyde in PBS. To quench residual paraformaldehyde, cells were incubated for 20 min in 50 mM NH_4Cl in PBS. Thereafter, they were permeabilized with 0.075% saponin, 0.1% bovine serum albumin (BSA) in PBS for 15 min, followed by 1 h of incubation with primary antibodies at room temperature. The GFP-tagged FUT10 recombinant proteins were visualized with a Leica DMR epifluorescence microscope, with a PLAN-APO $\times 63/1.32$ -0.6 oil objective lens and an HC-PLAN $\times 10/25$ ocular lens (Leica Microsystems, Wetzlar, Germany). Images were captured with a LEI-750 CE digital camera and LIDA volume 54 Leica software and further processed with Adobe Photoshop 5.0 (Adobe, San Jose, CA).

Double immunofluorescence experiments were conducted using a Golgi-specific mouse monoclonal anti-giantin antibody kindly given by Hans-Peter Hauri (35) (1:1000 in PBS plus 1% BSA) or an endoplasmic reticulum (ER)-specific rabbit polyclonal anti-calnexin antibody (1:200 in PBS plus 1% BSA; StressGen, Assay Designs, Inc., Ann Arbor, MI) or the lysosomal specific mouse monoclonal anti-Lamp-1 (BB6) antibody, kindly given by Sven Carlsson (1:1000 in PBS plus 1% BSA), and the GFP-tagged-FUT10 recombinant protein. After washing the cells three times in PBS plus 0.1% BSA, the anti-giantin and the anti-Lamp-1 antibodies were revealed with conjugated anti-mouse Ig-Cya3 red fluorochrome, diluted 1:200 (Jackson Laboratories, L'Arbresle, France), and the anti-calnexin antibody with anti-rabbit Ig-Cya3, diluted 1:200 (Jackson Laboratories). The secondary labeled antibodies were incubated for 1 h

at room temperature, and the reaction was stopped by washing three times in PBS. The coverslips with the labeled cells were mounted on slides with Mowiol and observed using a Leica DMR epifluorescence microscope.

Bioinformatics—Ten $\alpha 1,3$ -fucosyltransferase-like sequences from different species were retrieved from data banks by Psi-Blast (36) with the FUT10 and FUT11 human sequences. All of these sequences belong to the CAZY glycosyltransferase family 10. The systematic search for new FUT10 and FUT11 sequences was performed by TblastN using the expressed sequence tag and whole genome shotgun data banks, as previously described for sialyltransferases (37). Within each species, the DNA contigs for each new $\alpha 1,3$ -fucosyltransferase-like gene were made up with Cap3 (38). New complete protein sequences were analyzed for the presence of the NH_2 -terminal transmembrane domain (TMD) by PHD-htm (39) and for the presence of the $\alpha 1,3$ -fucosyltransferase conserved peptide motifs. The accession numbers of the animal sequences reconstructed *in silico* by these approaches and the human sequences used in the present paper are as follows: FUT10-like: *Homo sapiens* FUT10-479 AJ582015; FUT10-419 AJ512465; FUT10-391 AJ535838; FUT10-357 AJ535839; FUT10-428 AJ431184; *Bos taurus* AJ862259; *Rattus norvegicus* AJ879584, AJ880010; *Mus musculus* AJ880009; *Canis familiaris* AJ879585; *Gallus gallus* AJ535692; *Silurana tropicalis* AJ784815; *Danio rerio* AJ879586; FUT11-like: *H. sapiens* FUT11-492 BC036037, FUT11-476 BC100994; *R. norvegicus* AJ535753; *G. gallus* AJ535752; *S. tropicalis* AJ784816; *Xenopus laevis* AJ784717; *Takifugu rubripes* AJ606069; *Tetraodon nigroviridis* AJ783835; *Oryzias latipes* AJ879587; *Ciona intestinalis* BN000102; *Ciona savignyi* AJ784888; FUT10/11 like: *Drosophila yakuba* AJ880011, AM114003; *Drosophila erecta* AM113999; *Drosophila persimilis* AM114001; *Drosophila simulans* AM114002; *Drosophila pseudoobscura* AJ880012, AJ880013, AJ880014.

Related amino acids were grouped according to their properties based on a chemical alphabet comprising five groups: acidic or amide (Glu, Asp, Gln, and Asn); hydrophobic (Ile, Leu, Val, and Met); aromatic (Phe, Tyr, and Trp); basic (Arg, His, and Lys); and hydroxyl (Ser and Thr). The remaining four amino acids (Ala, Gly, Pro, and Cys) were analyzed separately. Amino acids of the same group were considered equivalent for the definition of conserved positions, and amino acids conserved at more than 50% in the different $\alpha 1,3$ -fucosyltransferases families were colored (Fig. 1).

Phylogeny—Protein and DNA alignments were performed by ClustalW (40) and saved in Pir format. The Pir alignment was used to select G-block-informative positions (41). By this computerized method, 206 amino acid positions in 14 G-blocks were selected for the phylogeny analysis. The ClustalW alignment of the G-block-selected positions was also used to count the percentage of FUT10- and FUT11-specific positions. The sequence lines of the ClustalW were then ordered by decreasing percentage (from 100 to 0%) of FUT10-specific positions and increasing percentage (from 0 to 100%) of FUT11-specific positions (Fig. 1). The sequences with more than 80% specific positions for either FUT10 or FUT11 can be ascribed to the corresponding family, whereas the sequences with equivalent proportions of specific positions ($50 \pm 10\%$) cannot be ascribed

Expression of Two New FUT Families, FUT10 and FUT11

Motifs			---	I	---	---	II	---	---	III	---	---	IV	---	---	V	---	FUT10	FUT11	
FUT10																				
M.musculus	83	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	307	100	0	
R.norvegicus	83	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	307	96	4	
H.sapiens	83	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<88>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	307	96	4	
B.taurus	67	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	291	96	4	
C.familiaris	56	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	280	96	4	
G.gallus	80	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	304	95	5	
X.laueis	88	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	312	91	9	
S.tropicalis	49	IVLW	WSPL	<33>	FYGT	DF	<12>	HDW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	FWR-PLKLGVPVYVG	273	91	9	
D.terio	68	IVVW	WSPL	<33>	FYGT	DF	<12>	HQW	ALFHEESP	<13>	FNHT	ATFSRSHSLPLTTQ	<89>	YKF	ILAFENAVCDYITEK	LWR-PLKLGVPVYVG	292	82	18	
FUT11																				
C.savagnyi	24	VILW	WQDN	<44>	HYGT	DF	<12>	HLW	ALLHEESP	<13>	FNFS	STFRKNSDPLTTQ	<90>	YKF	ILAFENAVCDYITEK	FTR-PLHVGSPVIYVG	253	19	81	
C.intestinalis	44	VILW	WQDN	<46>	HYGT	DF	<12>	HLW	ALLHEESP	<13>	FNFS	STFRKNSDPLTTQ	<90>	YKF	ILAFENAVCDYITEK	FTR-PLHVGSPVIYVG	273	15	85	
T.nigroviridis	87	IVLW	WSAG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNHT	ATFSRSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PLHVGSPVYVG	317	15	85	
O.latipes	80	IVLW	WSAG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNHT	ATFSRSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PLHVGSPVYVG	310	14	86	
H.sapiens	77	VLLW	WSPG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNLT	STFSRSHSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PMHLGAVPVYVG	303	12	88	
R.norvegicus	73	VLLW	WSPG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNLT	STFSRSHSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PMHLGAVPVYVG	307	10	90	
M.musculus	73	VLLW	WSPG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNLT	STFSRSHSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PMHLGAVPVYVG	303	9	91	
G.gallus	90	IVLW	WSGS	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNLT	STFSRSHSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PMHLGAVPVYVG	319	9	91	
S.tropicalis	117	IVLW	WSEN	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNHT	ATFSRSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PMHLGAIPYVG	345	8	92	
X.laueis	58	IVLW	WSEN	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNHT	ATFSRSDYPLTTQ	<92>	IKF	HLAFENGLCPDYITEK	LWR-PMHLGAIPYVG	289	7	93	
T.rubripes	36	IVLW	WSAG	<37>	FYGT	DF	<12>	QW	ALLHEESP	<13>	FNHT	ATFSRSDYPLTTQ	<91>	YKF	HLAFENGLCPDYITEK	LWR-PLHVGSPVYVG	266	7	93	
FUT10/FUT11																				
D.pseudoobsc. A	43	E	LLW	WTH	<32>	FYGT	FL	<12>	HS	WALLHEESP	<13>	FNHT	STFSRSDYPLTTQ	<91>	YKF	MIATENAVCDYITEK	FWR-PLVGVPIPIYVG	268	56	44
D.pseudoobsc. C	43	E	LLW	WTH	<32>	FYGT	FL	<12>	HS	WALLHEESP	<13>	FNHT	STFSRSDYPLTTQ	<91>	YKF	MIATENAVCDYITEK	FWR-PLVGVPIPIYVG	268	56	44
D.pseudoobsc. B	43	E	LLW	WTH	<32>	FYGT	FL	<12>	HS	WALLHEESP	<13>	FNHT	STFSRSDYPLTTQ	<91>	YKF	MIATENAVCDYITEK	FWR-PLVGVPIPIYVG	268	52	48
D.persimilis	43	E	LLW	WTH	<32>	FYGT	FL	<12>	HS	WALLHEESP	<13>	FNHT	STFSRSDYPLTTQ	<91>	YKF	MIATENAVCDYITEK	FWR-PLVGVPIPIYVG	268	52	48
D.melanogaster	42	E	LLW	WTH	<32>	FYGS	NI	<12>	QW	ALLHEESP	<13>	FHHT	STFSRYSNPLTTQ	<92>	YKF	MIATENAACPDYITEK	FWR-PLIMGVPIPIYVG	268	43	57
D.simulans	42	E	LLW	WTH	<32>	FYGS	NI	<12>	QW	ALLHEESP	<13>	FHHT	STFSRYSNPLTTQ	<92>	YKF	MIATENAACPDYITEK	FWR-PLIMGVPIPIYVG	268	47	53
D.yakuba	42	E	LLW	WTH	<32>	FYGS	NI	<12>	QW	ALLHEESP	<13>	FHHT	STFSRYSNPLTTQ	<91>	YKF	MIATENAACPDYITEK	FWR-PLIMGVPIPIYVG	267	47	53
D.erecta	42	E	LLW	WTH	<32>	FYGS	NI	<12>	QW	ALLHEESP	<13>	FHHT	STFSRYSNPLTTQ	<92>	YKF	MIATENAACPDYITEK	FWR-PLIMGVPIPIYVG	268	41	59
FUT3-7 and FUT9																				
FUT7_HUMAN	49	IVLW	WHPF	<36>	FHHR	EL	<15>	QF	WVWMSMES	<11>	FNW	LSYRSDSDIFPYG	<67>	YRFL	SPFNSQHRDYITEK	FWRNALVAGTVPVVLG	256	54	46	
FUT9_HUMAN	66	IVLW	WHPF	<34>	IHHRI	D	<15>	QF	WVWMSMES	<11>	FNLT	LYRSDSDIFPYG	<67>	C	KFLSPFNSQHRDYITEK	NAFLAGSVPVVLG	271	48	52	
FUT6_HUMAN	64	IVLW	WHPF	<35>	VHHR	EV	<16>	QF	WVWMSMES	<11>	FNLT	SYRSDSDIFPYG	<70>	YRFL	SPFNSQHRDYITEK	LWRALNAEAVWVVLG	274	46	54	
FUT5_HUMAN	78	IVLW	WHPF	<35>	VHHR	EV	<16>	QF	WVWMSMES	<11>	FNLT	SYRSDSDIFPYG	<70>	YRFL	SPFNSQHRDYITEK	LWRNALNAEAVWVVLG	288	46	54	
FUT3_HUMAN	65	IVLW	WHPF	<35>	VHHR	EV	<16>	QF	WVWMSMES	<11>	FNLT	SYRSDSDIFPYG	<70>	YRFL	SPFNSQHRDYITEK	LWRNALNAEAVWVVLG	275	42	58	
FUT4_HUMAN	66	IVLW	WHPF	<38>	FHHR	EL	<47>	QF	WVWMSMES	<12>	FNW	LSYRSDSDIFPYG	<75>	YRFL	SPFNSQHRDYITEK	LWRNALNAEAVWVVLG	316	36	64	

FIGURE 1. ClustalW sequence alignment of the conserved peptide motifs I–V of α 1,3-fucosyltransferases. Positions conserved (>50%) in the classical monoexonic α 1,3-fucosyltransferases (FUT3–FUT7 and FUT9) are highlighted in pink. Positions conserved in the FUT10 and FUT11 families are yellow. Positions conserved in FUT10 are indicated in pale blue, and positions conserved in FUT11 are shown in orange. In each line, the sequence positions of the first amino acid of motif I and the last amino acid of motif V are represented at the beginning and at the end of each sequence, respectively. The number of intermotif amino acids is given between the less than and greater than symbols. The sizes of intermotif distances and the motifs IV and V are well preserved in all families. Motifs I and III are less well preserved, and motif II is strikingly different between the classical monoexonic α 1,3-fucosyltransferase families and the two new FUT10 and FUT11 vertebrate families. The last two columns correspond to the percentage of FUT10- and FUT11-specific amino acids in the total alignment of the 206 G-block-selected positions, in decreasing order from 100 to 0% for FUT10 and the complementary increasing order from 0 to 100% for FUT11. This simple calculation ascribes each of the sequences of the FUT10–FUT11 branch to either one or the other of these two families. The insect enzymes and the classical monoexonic α 1,3-fucosyltransferases reveal intermediate percentages of FUT10- and FUT11-specific positions and could not be ascribed to any of them. The FUT10 and FUT11 families ascribed by this method were fully confirmed by the phylogeny shown in Fig. 2.

to either of FUT10 or FUT11. This simple method, based on the similarities between sequences is complementary to the more sophisticated phylogeny calculations that are mainly based on the differences between sequences. Phylogeny was carried out with PhyloWin (42) (available on the World Wide Web) using BIONJ, Poisson correction, and 500 bootstrap replicates (43).

RESULTS

Conserved Peptide Motifs of FUT10 and FUT11—The hydrophobic cluster analysis (44, 45) of the α 1,3-fucosyltransferase family (FUT3–FUT7 and FUT9) first revealed two conserved peptide motifs originally called I and II. Then a third conserved motif of six amino acids was called the acceptor motif ((I/V/F)HH(R/W)(D/E)(I/V/L)), because a single amino acid change (Trp¹¹¹ → Arg) was able to transform the type 1 α 1,4-fucosylation of FUT3 (leading to the Le^a epitope) into the type 2 α 1,3-fucosylation (leading to the Le^x epitope) (46, 47). A fourth conserved motif, located on the NH₂-terminal side of the sequence, before the acceptor motif, was later called motif III (12, 47), giving the following order for these four conserved motifs: III, acceptor motif, I and II. Finally, while writing the present paper, we found a fifth conserved motif located between the acceptor motif and motif I. In order to avoid confusion, we have here renamed these five peptide motifs from I to V, according to their order in the protein sequence (Fig. 1).

As expected, the two main conserved motifs (IV and V in Fig. 1) are present in all of the α 1,3-fucosyltransferases, including the FUT10 and FUT11 isoforms. These two motifs contribute to recognition and binding of the donor substrate GDP-Fuc (12, 46), whereas the first three motifs occur toward the NH₂ end of the sequence, in a region that has been shown (by domain swapping experiments) to be involved in the recognition of the acceptor substrate (48). In the middle of this region, the acceptor motif II is present in FUT3–FUT7 and FUT9, but we did not find it in either the FUT10 or FUT11 sequences. However, a different conserved peptide motif (FYGTDF) was present in the equivalent positions of FUT10 and FUT11 (Fig. 1). The classical acceptor motif II shares only one negatively charged amino acid at position 5 (Asp or Glu) with this new acceptor motif II of FUT10 and FUT11 (Fig. 1). The motifs I and III, flanking the acceptor motif II that is involved in acceptor recognition contain six conserved amino acids specific of FUT10 and FUT11.

The intermotif distances between the motifs I, II, III, IV, and V are relatively well preserved in all of the α 1,3-fucosyltransferase families, including FUT10 and FUT11 (Fig. 1). This plus the very good conservation of motifs IV and V and the less good but significant conservation of motifs I and III suggests that all of the α 1,3-fucosyltransferases derive from a single ancestral gene.

Overall, 24 amino acid positions within the five conserved peptide motifs were specific to both FUT10 and FUT11 and

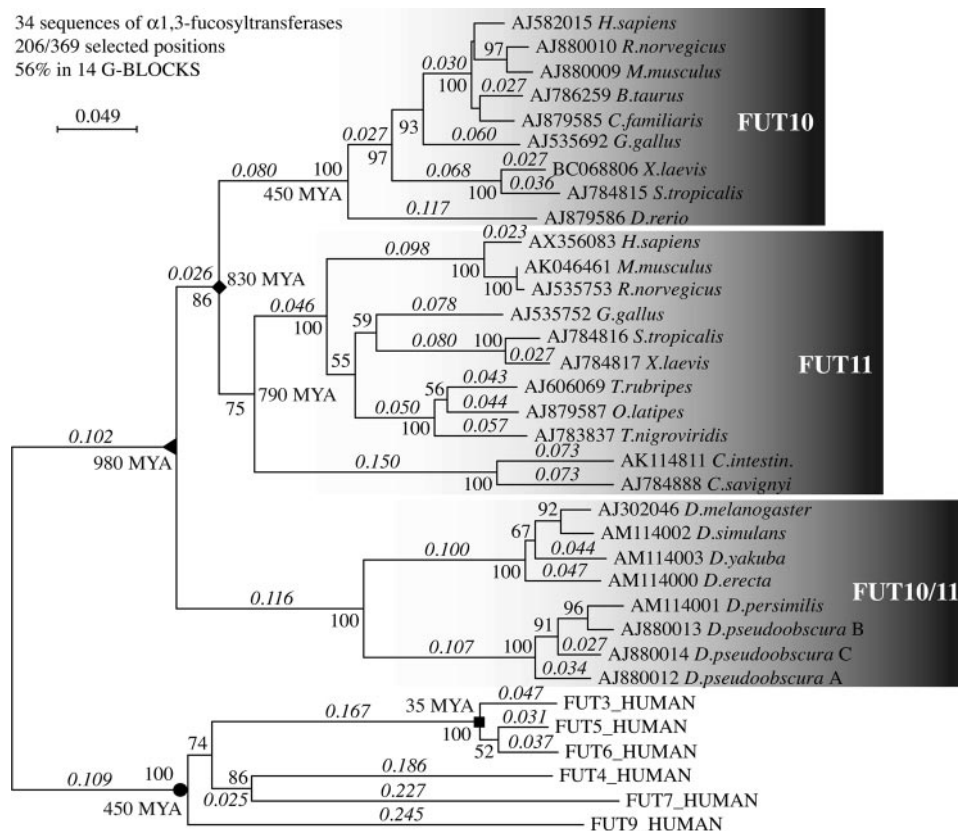


FIGURE 2. **Neighbor-joining phylogenetic tree of α 1,3-fucosyltransferase families.** The 34 selected sequences comprise all of the FUT10- and FUT11-like sequences plus the human FUT3–FUT7 and FUT9 α 1,3-fucosyltransferases. The *solid circle* illustrates the expected position of the retrotransposition event in the FUT3–FUT7 and FUT9 branch. The *solid square* illustrates the first duplication at the origin of the present FUT3, FUT5, and FUT6 genes. The *solid triangle* illustrates the duplication event that separated the FUT10/11 arthropod branch from an ancestor of the FUT10 and FUT11 families. The *solid diamond* illustrates the duplication at the origin of the present FUT10 and FUT11 vertebrate families. The robustness of the tree branches was tested by bootstrap calculations from 500 sets of data, and values higher than 50% are reported at the left of each node. Values in *italic type* are genetic distances, and the scale bar represents the number of substitutions/site for a unit of branch length.

absent from the classical monoexonic α 1,3-fucosyltransferases (Fig. 1). In addition, the existence of other FUT10- and FUT11-specific amino acids in the remaining positions of the 14 G-blocks (41), comprising the 206 informative amino acids selected for phylogeny (not shown), allowed us to clearly ascribe all of the vertebrate FUT10 and FUT11 enzymes to one or the other of these two families, because they contain more than 80% of conserved positions specific for either one or the other family (Fig. 1, *last two columns*). Alternatively, the presence of equivalent proportions of specific positions of FUT10 and FUT11 ($50 \pm 9\%$) in the insect FUT10/11 enzymes suggests that they are at the same genetic distance from either FUT10 or FUT11 and therefore can be orthologous to the common ancestor that preceded the duplication event at the origin of the FUT10 and FUT11 families. The same concept applies to the classical monoexonic α 1,3-fucosyltransferases (FUT3–FUT7 and FUT9) (11) that are also at the same genetic distance, as evaluated by the percentage of specific positions ($50 \pm 14\%$) from either the FUT10 or FUT11 family. They have almost no FUT10- or FUT11-specific conserved positions within the five peptide motifs (Fig. 1) and only a small number among the 206 positions selected for phylogeny.

In addition to the above mentioned FUT3–FUT11 sequences, other α 1,3/4-fucosyltransferases have been characterized in invertebrates (core insect) and in plants (core plant and Le^a) (not shown). They all had the large donor-related conserved motifs IV and V, but none had significant numbers of FUT10- or FUT11-specific positions. Furthermore, the sequences of motifs I, II, and III of the core invertebrate (28, 49–51), the core plant (52, 53), and the Le^a plant enzymes (51, 54) are more closely related to the FUT3–FUT7 and FUT9 than to the FUT10 or FUT11 families. Nevertheless, they could not be ascribed to any of the FUT3–FUT7 and FUT9 families of α 1,3/4-fucosyltransferases, suggesting that they are at similar genetic distances from each of the FUT3, FUT4, FUT5, FUT6, FUT7, and FUT9 enzymes. Therefore, they might be orthologous to a common ancestor present before the duplication events at the origin of the paralogous FUT3–FUT7 and FUT9 genes.

Phylogeny of FUT10 and FUT11—The phylogenetic tree of Fig. 2 contains the FUT10 and FUT11 sequences on the first branch and the human FUT3–FUT7 and FUT9 (11) on the second branch. The root is probably between these two

branches, since the classical monoexonic α 1,3-fucosyltransferases appear as an out-group clearly distinct from FUT10 and FUT11. The enzymes of the FUT3–FUT7 and FUT9 branch started to appear early in the vertebrate lineage (about 450 MYA), since they were not found among invertebrates, but they are present in fishes, amphibians, birds, and mammals (1, 19, 55). They are all monoexonic with the exception of FUT7, whose cds is assembled from two exons (56), whereas all of the genes coding for the enzymes of the FUT10–FUT11 branch (Fig. 2) plus all of the other invertebrate and plant α 1,3-fucosyltransferases are polyexonic (1). This suggests that a retrotransposition event of an ancestral rearranged gene occurred in the FUT3–FUT7 and FUT9 branch, before the duplications at the origin of these six paralogous genes (represented by a *solid circle* in Fig. 2), which is at the origin of the intron loss of the sequences of this branch (57).

Another reference point can be dated at about 35 MYA (47) in this same branch (*solid square*), since only Old World anthropoid apes and humans have the three Lewis genes (FUT3, FUT5, and FUT6) (58), whereas all of the New World monkeys and lower mammals have a single gene (FUT3/5/6), orthologous to the ancestor present before the duplications at the origin of FUT3, FUT5, and FUT6 genes.

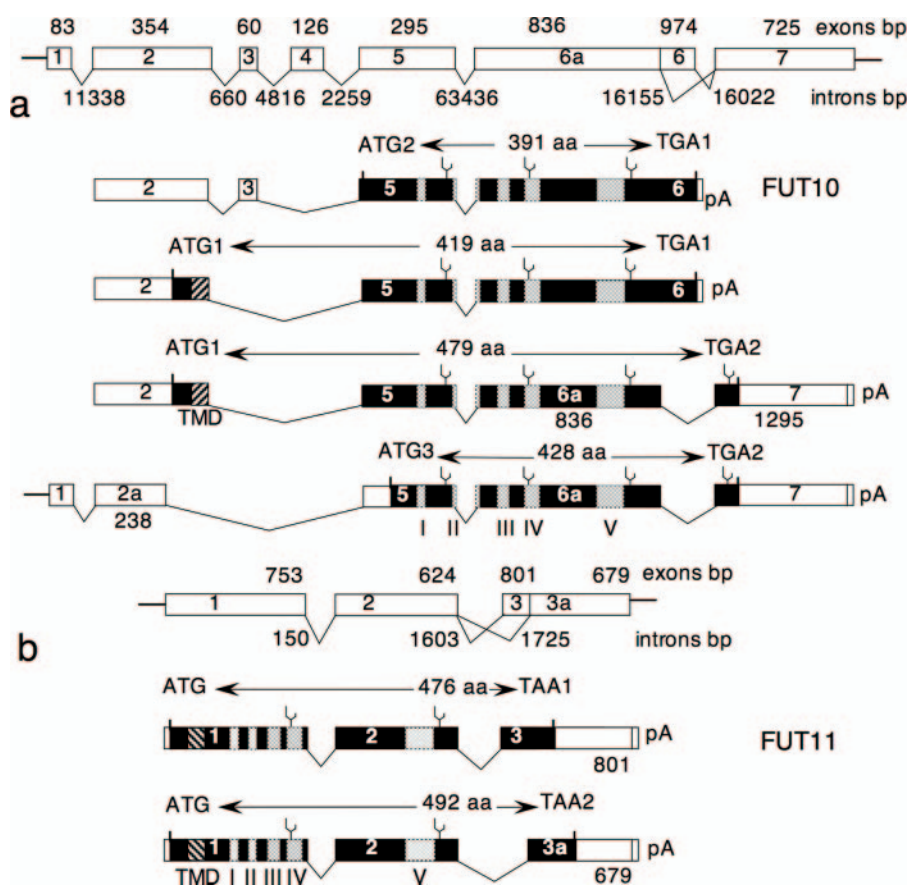


FIGURE 3. Genomic organization of *H. sapiens* FUT10 and FUT11 genes and their splice variants. *a*, scheme of the *FUT10* gene, spanning 102 kb on the chromosome band 8p11.23 (61). Exons 1–7 are represented by open rectangles, and introns are shown with V-shaped lines. The embryo splice variant FUT10-391 starts with the ATG2 and terminates with the TGA1, predicting a soluble protein of 391 amino acids with three putative N-glycosylation sites (Y-shaped lines). An alternative splicing event skipping exon 3 generates the embryo FUT10-419 transcript, initiating with the ATG1 and ending with the same TGA1 as FUT10-391. After translation, the predicted protein of 419 amino acids has a TMD and the same N-glycosylation sites as FUT10-391. The adult brain splice variant FUT10-479 starts with ATG1 and terminates with TGA2. It predicts a protein of 479 amino acids with four N-glycosylation sites. The *in silico* predicted 428-amino acid protein (22) differs from our adult FUT10-479 on its amino side, because it has a different start codon (ATG3). *b*, scheme of the *FUT11* gene spanning 4 kb on the chromosome band 10q22.2 (61). A differential splice generates the FUT11-476 and FUT11-492 variants, which start with the same ATG and end with different stop codons, TAA1 for FUT11-476 and TAA2 for FUT11-492. cds exons are represented by solid rectangles, and the cds-flanking exons are shown by empty rectangles. The hatched box represents the TMD, and the α 1,3-fucosyltransferase conserved peptide motifs are represented by gray boxes, labeled I, II, III, IV, and V. The predicted N-glycosylation sites are represented with Y-shaped lines, and the poly(A) sequences are labeled pA.

In the FUT10–FUT11 branch, a first duplication (solid triangle in Fig. 2) was at the origin of the separation of the insect FUT10/11 group from the ancestor of the two vertebrate FUT10 and FUT11 families, and a second duplication (solid diamond) generated the FUT10 and FUT11 families, which are found in vertebrates and urochordates, since two ciona sequences (*C. intestinalis* and *C. savignyi*) (Fig. 2) have 81 and 85% of the FUT11-specific positions (Fig. 1).

The duplication node at the origin of the FUT10 and FUT11 families is flanked by the separation of arthropods 980 MYA (59) and the separation of urochordates 790 MYA (60), suggesting that it occurred about 830 MYA (Fig. 2). It is interesting to note that the insect FUT10/11 are the only sequences of the FUT10 and FUT11 families that lack the last two amino acids (DF) of the acceptor substrate motif II (Fig. 1). Therefore, these insect FUT10/11 sequences, which are the most ancient pro-

teins of the FUT10–FUT11 branch (Fig. 2), might have different acceptor specificity.

Identification of the Embryo and Adult Transcript Splice Variants of FUT10—The presence of the *FUT10* splice variants was first verified in our human 50-day embryo cDNA library using the double PCR method reported under “Experimental Procedures.” A broad PCR product of ~1400 bp was amplified with the primers F10-8s and F10-12as (Table 2). After cloning in the PCR3.1 mammalian vector, we selected 20 clones for sequencing and found two new *FUT10* splice variants of 1373 and 1433 bp, reported in Fig. 3a as FUT10-391 and FUT10-419, respectively.

The embryo cDNA variant of 1433 bp (FUT10-391) has an open reading frame (ORF) of 1176 bp, including exons 2, 3, 5, and 6 and skipping exons 4 and 7 (Fig. 3a). After translation, a soluble protein of 391 amino acids without TMD is expected. The insertion of exon 3 of 60 bp at position 177 of the cDNA induces the use of the ATG2 start codon (position 239 of the cDNA) and the stop codon TGA1. The predicted protein has three putative N-glycosylation sites, located at positions 82, 140, and 290 (Fig. 3a).

The second splice variant of 1373 bp (FUT10-419) has an ORF of 1354 bp, including exons 2, 5, and 6. It skips exons 3, 4, and 7 and uses the ATG1 (position 95 of the cDNA) and the same TGA1 stop codon as the FUT10-391. Post-translational topology suggests a type II trans-membrane protein of 419 amino acids with a short TMD of 16 amino acids and an NH₂-terminal ER membrane retention signal (VRIQ), similar to that of the yeast ALG7 enzyme, also localized in the ER. The FUT10-419 has the same three N-glycosylation sites of the soluble isoform FUT10-391 (Fig. 3a). Repetitive PCRs in the different cDNA pools could only amplify these two variants in the embryo library and not in the adult cDNA brain library.

Using the conditions expected to amplify the *in silico* defined human sequence FUT10-428 (AJ431184 (22)), we were unable to amplify the corresponding transcript from either embryo or adult cDNA libraries, but we did amplify a new splice variant reported as FUT10-479 from the adult library. The complete cDNA of 2312 bp was composed of exons 2, 5, 6a, and 7. We made a double PCR using first primers F10-1s and F10-as2 and then primers F10-8s and F10-as2, generating a fragment of

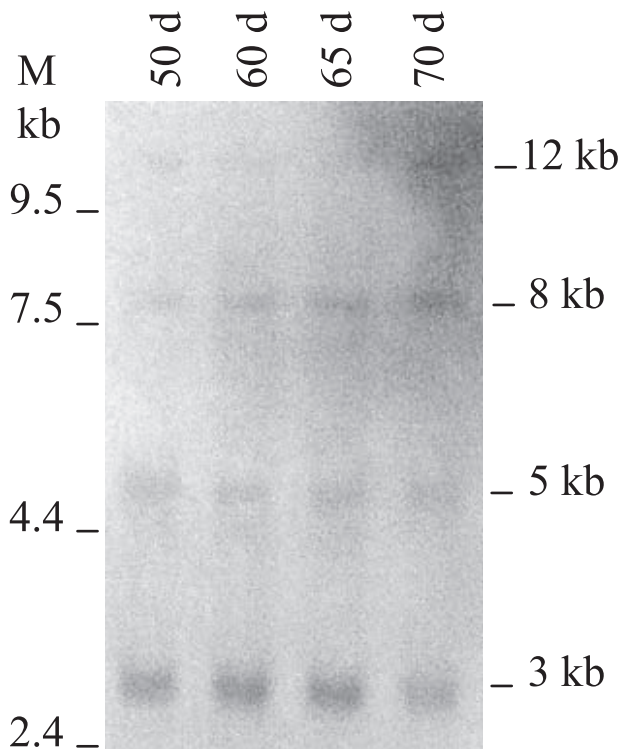


FIGURE 4. Northern blot with 4 μ g/lane of poly(A)⁺ extracted from 50–70-day-old whole human embryos. The blot was hybridized with the *cds*-FUT10 probe of 350 bp obtained by PCR with F10-8s and F10-4 as primers. The same profile with four mRNA bands of 3, 5, 8, and 12 kb was detected in the four embryos of 50, 60, 65, and 70 days (*d*). Positions of RNA markers (kb) are shown on the *left*, and estimated sizes of FUT10 transcripts are indicated on the *right*.

1866 bp, from the adult brain cDNA library. After cloning this fragment in the mammalian PCR3.1 vector, it has an ORF of 1440 bp, using the ATG1 and a new stop codon TGA2 (Fig. 3*a*). The FUT10-479 has the same TMD and NH₂-terminal ER membrane retention signal found in the FUT10-419 splice variant plus a new ER membrane retention signal (LVFK) in the COOH terminus. It has three putative *N*-glycosylation sites identical to those of the other two FUT10 variants plus a fourth one located at position 466 (Fig. 3*a*). The corresponding transcript was never amplified from our embryo cDNA library even after repetitive PCR. Compared with the published FUT10-428 sequence, our three FUT10 variants have two amino acid substitutions, Leu to Phe at position 31 and Leu to Asp at position 340 of the FUT10-391, and use different start codons (Fig. 3*a*).

Tissue Distribution of the Human FUT10 mRNA Transcripts—The size and tissue distribution of the human FUT10 variants were determined by Northern blot analysis with the specific FUT10 *cds* probe. The poly(A)⁺ RNA from entire 50–70-day-old embryos show the same transcript profile, with four FUT10 mRNA bands, ranging from 3 to 12 kb (Fig. 4). The 3-kb transcript is a broad band always more abundant than the 5, 8, or 12 kb bands. They are expressed in all of the embryos with similar intensity, but we have no information about their tissue distribution, because they correspond to mRNA from entire embryos.

The fetal and adult tissue distribution profiles were studied with commercial Northern blots. Table 3 summarizes the relative intensity evaluation of the bands of the embryo, fetus, and

TABLE 3

Comparisons of embryo, fetal, and adult expression of FUT10 transcripts after Northern blot analysis

The presence of the transcript bands is shown with the symbols + + +, + +, +, and +/–, corresponding to the relative intensities of the bands. Absence of transcripts is noted with the negative symbol (–).

	FUT10 transcript bands			
	3 kb	5 kb	8 kb	12 kb
Embryos				
50–70 days	+++	++	++	+
Fetal tissues				
Skin, 20 weeks	+++	++	+	+/-
Small intestine, 28 weeks	+++	++	+	+/-
Liver, 24 weeks	++	++	++	+/-
Kidney, 28, 32 weeks	++	+	+/-	–
Lung, 37 weeks	+	+	–	++
Muscle, 28 weeks	+	+	–	+/-
Heart, 12, 13, 26, 37 weeks	+	–	–	–
Brain, 20 weeks	+/-	–	–	–
Adult tissues				
Lung	+++	++	++	+
Jejunum	+++	++	+/-	–
Colon	+++	++	–	–
Rectum	+++	++	–	–
Stomach	++	+	–	–
Ileum	+	+/-	–	–
Placenta	+	+/-	–	–
Gall bladder	+++	–	–	–
Kidney	++	–	–	–
Uterus	++	–	–	–
Brain	+	–	–	–
Spleen	–	–	–	–
Heart	–	–	–	–
Muscle	–	–	–	–
Liver	–	–	–	–
Pancreas	–	–	–	–

adult tissues. In all fetal tissues, the FUT10 profile with four bands is found in skin, small intestine, and liver with an intensity similar to that seen in the embryos. In kidney, we found a profile with three weak bands of 3, 5, and 8 kb. Lung and muscle expressed weakly the 3, 5, and 12 kb bands. Heart and brain show only the 3 kb band. The adult tissues also revealed these transcripts but with different patterns of expression. In the lung, the four transcripts were similar to the embryo. Tissues of the digestive tract (jejunum, colon, rectum, and stomach) expressed the 3- and 5-kb transcripts strongly. A similar but weaker pattern was observed with ileum and placenta. The gall bladder reveals strongly expression of the 3 kb band only. This same transcript is also expressed with moderate intensity in kidney and uterus and weakly in brain. Adult spleen, heart, muscle, liver, and pancreas did not express any of these FUT10 transcripts (Table 3).

Identification of the Transcript Splice Variants of FUT11—The two cDNA splice variants of FUT11 were integrated in the PCR3.1 vector, sequenced, and named FUT11-476 and FUT11-492 (Fig. 3*b*). The ORF sizes of the selected clones are 1431 and 1479 bp, respectively. A differential splicing event occurred in exon 3, giving the FUT11-476 with the entire exon 3 and the FUT11-492 when it utilizes the exon 3a, starting at position 123 and skipping 15% of this exon. This induces a change of ORF and generates a new stop codon (TAA2). The use of two distinct stop codons predict two proteins with a short TMD of 16 amino acids. The FUT11-476 has no ER membrane retention signal, but the FUT11-492 variant has a KKXX-like motif (KRQH) in its COOH terminus that could retain it in the ER. These pro-

Expression of Two New FUT Families, FUT10 and FUT11

teins have only two of the four potential *N*-glycosylation sites found in FUT10 (Fig. 3b).

Genomic Organization of the FUT10 and FUT11 Genes—The Blast search of the EMBL data bank retrieved AC091144, located on the chromosome band 8p11.23 (61) that contains the complete *FUT10* genomic organization spanning 102 kb. The splice intron/exon boundaries followed the AG/GT rule (62) (Table 4). We also found a microsatellite sequence with a 17-GT repeat localized on the 3'-side of exon 4 (about 500 bp downstream; not shown). The gene organization of *FUT11* (Fig. 3b and Table 4) is included in the EMBL genomic sequence AC022400 located on the chromosome band 10q22.2 (61), and it spans ~4 kb.

Fucosyltransferase Activity Profiles of the Recombinant Protein Variants—COS7 cells were transiently transfected for 18–24 h with the empty PCR3.1 vector, the three FUT10-cDNA constructs (FUT10-391, FUT10-419, and FUT10-479), the two FUT11 constructs (FUT11-476 and FUT11-492), two

classical monoexonic α 1,3-fucosyltransferases (FUT3 and FUT4), and the α 1,6-fucosyltransferase (FUT8) (Table 5).

The natural biantennary oligosaccharide substrate, BGA-biotin, is a well known and very good acceptor for the FUT8 α 1,6-fucosyltransferase (1550 pmol/h/mg), because it is the specific substrate of FUT8, and the assay conditions used were optimal for this enzyme (30). These conditions without MnCl₂ are also appropriate for the three FUT10 isoforms (FUT10-391, FUT10-419, and FUT10-479), giving respective activities of 70, 120, and 105 pmol/h/mg. The FUT11-476 displayed an activity of 93 pmol/h/mg, and a very weak activity of 17 pmol/h/mg for the FUT11-492 isoform was noted. The FUT10 and FUT11 activities are at least 10 times lower than that noted for the FUT8 enzyme but are higher than the activities of the two α 1,3-fucosyltransferases, FUT3 and FUT4 (30 and 55 pmol/h/mg, respectively).

The FUT8, FUT10, and FUT11 activities toward the acceptor 0989-BM were increased by about 50% compared with those observed using BGA-biotin. The 0989-BM acceptor possesses the same *N*-glycan structure present in the BGA-biotin, but the sugar structure is connected to the biotin via a linking arm NHCOCH₂NHCO(CH₂)₅ instead of the natural asparagine (Table 1). We found activities of 2480 pmol/h/mg for FUT8; 105, 175, and 160 pmol/h/mg for FUT10-391, FUT10-419, and FUT10-479, respectively; and finally an activity of 139 pmol/h/mg for FUT11-476, and again a weak activity of 25 pmol/h/mg for the FUT11-492 isoform was observed. By contrast, the FUT3 and FUT4 enzymes revealed similar activities toward both acceptors.

When the 0989-BM acceptor was compared with 0988-BM (0989-BM is elongated at the nonreducing end by a galactose residue linked β 1,4- onto each of the terminal nonreducing GlcNAc residues; see Table 1), FUT8 activity was reduced by 50% toward the latter acceptor (1350 pmol/h/mg; see Table 5). This reduction reaches 75% for the FUT10 enzymes with 31, 40, and 37 pmol/h/mg for FUT10-391, FUT10-419, and FUT10-479, respectively, and 80% for the FUT11-476 enzyme with 24

TABLE 4

Genomic organization of human FUT10 and FUT11 genes

Exons		Introns		Intron-exon boundaries ^c
name	size bp	name	size bp	
<i>FUT10^a</i>				
1	83	1-2	11338	GTCTTCCCGCGCCCC..CGCGAG/ gt gagtgc
2	354	2-3	660	tg ttgcag /AATTTT..CTCCAG/ gt accacag
3	60	3-4	4816	ttaaac ag /GTGAAA..TAGAAG/ gt gagttt
4	126	4-5	2259	cttttt tg /GCCTTG..AAAGAG/ gt aacact
5	295	5-6	63436	tctttt ag /GTACATG..TCTATG/ gt aagcag
6	974	6-7	16022	tctcct ag /GTACTG..GCTTTGCAGGAAGGT
6a	836	6a-7	16155	tctcct ag /GTACTG..GAAAAG/ gt taatac
7	725			tctcgc ag /GGCTTA..GTTTCAAGT+polyA
<i>FUT11^b</i>				
1	753			GGGGTCGGTGCTGGC..ATCCCG/ gt gagtga
2	624	1-2	150	cggtct ag /gtAGAC..TGACAG/ gt aagagt
3	801	2-3	1603	tttcac ag /CAGTCT..GTCAAA+polyA
3a	679	2-3a	1725	Tatttt ag /TTGGAA..GTCAAA+polyA

^a The *FUT10* sequence is in the genomic sequence AC091144 of 185,872 bp, and it spans 101,065 bp.

^b The *FUT11* sequence is included in the genomic sequence AC022400 of 183,797 bp, and it spans around 4000 bp.

^c Exon sequences are in uppercase, intron sequences in lowercase, and donor/acceptor splice sites in boldface type.

TABLE 5

α 1,3- and α 1,6-fucosyltransferase activities (pmol/h/mg) in homogenates of transiently transfected COS7 cells

COS7 cells were transiently transfected with the FUT10, FUT11, FUT3, FUT4, and FUT8 constructs and the vector PCR3.1 alone for 18–24 h. Twenty-five μ g of total protein extracts were used to assay α 1,3- and α 1,6-fucosyltransferase activities, using the acceptors on the left side column and 4 h of incubation at 37 °C. Activities are expressed in pmol/h/mg, and the S.D. values were calculated in 10 independent assays. When no S.D. is shown, the activities are the means of three independent experiments.

Constructs	α 3-FT activity								α 6-FT activity (FUT8)
	PCR3.1	FUT10-391	FUT10-419	FUT10-479	FUT11-476	FUT11-492	FUT3	FUT4	
				pmol/h/mg					pmol/h/mg
BGA-biotin	0	70 \pm 7	120 \pm 9	105 \pm 9	93 \pm 10	17 \pm 5	30 \pm 5	55 \pm 10	1550 \pm 70
0989-BM	0	105	175	160	139	25	40	50	2480
0988-BM	0	31	40	37	24	5	ND ^a	ND	1350
0987-BM	0	<1	1	<1	1	<1	ND	ND	12
0990-BM	0	1	2	2	1	0	ND	ND	50
H-type-2-Ogr	0	4 \pm 2	7 \pm 2	5 \pm 2	2	1	63 \pm 9	748 ^b \pm 15	7 \pm 3
LacNAc-type-2-Ogr	0	<1	2 \pm 1	2 \pm 1	<1	<1	9 \pm 2	120 ^c \pm 10	2 \pm 1
H-type-1-Ogr	0	4	2	4	ND	ND	675 ^d \pm 20	<0.1	ND
LacNAc-type-1-Ogr	0	2	4	4	ND	ND	ND	ND	ND
Chitobiose-BM	0	0	0	0	ND	ND	0	0	0
OP-392-Ogr	0	0	ND	0	ND	ND	0	ND	14
OP-395C-Ogr	0	0	ND	0	ND	ND	0	ND	0
SHT-2156-Ogr	0	3	2	0	ND	ND	0	ND	0

^a ND, not determined.

^b In the presence of 20 mM MnCl₂, the activity for the acceptor is 1115 pmol/h/mg.

^c In the presence of 20 mM MnCl₂, the activity for the acceptor is 230 pmol/h/mg.

^d In the presence of 20 mM MnCl₂, the activity for the acceptor is 1280 pmol/h/mg.

TABLE 6

 α 1,3- and α 1,6-fucosyltransferase activities of FUT10 and FUT8 constructs using biantennary modified acceptors

Constructs	α 1,3-FT activity ^a			α 1,6-FT activity ^b (FUT8)
	FUT10-391	FUT10-419	FUT10-479	
		dpm/reaction		dpm/reaction
0989-BM	23,400 (100)	42,000 (100)	32,000 (100)	95,000 (100)
0988-BM	4480 (19)	8400 (20)	6400 (20)	20,550 (22)
7OS-ManNAc-BM	2130 (9)	3500 (8)	2650 (8)	9775 (10)
7OS-amino-alditol-GlcNAcBM	200 (0,9)	50 (0,1)	120 (0,4)	2450 (3)
Fuc α 1,6-0989-BM	11,750 (40)	17,760 (42)	15,000 (47)	1650 (2)

^a Activities are expressed in dpm/reaction and as the percentage of activity (%) of the 0989-BM acceptor.

pmol/h/mg and negative for FUT11-492, suggesting that these enzymes are more affected by the terminal Gal than FUT8. The other two *N*-glycan acceptors, 0987-BM and 0990-BM, are not substrates for FUT10 and FUT11 (0–2 pmol/h/mg), and they are poor acceptors for the FUT8 α 1,6-fucosyltransferase (12 pmol/h/mg with 0987-BM and 50 pmol/h/mg with 0990-BM; see Table 5).

In the absence of MnCl₂, H-type-2-Ogr still remains the best acceptor for the α 1,3-fucosyltransferase FUT4 (748 pmol/h/mg); a poor acceptor for FUT3 (63 pmol/h/mg); and a poorer acceptor for the FUT10, FUT11, and FUT8 enzymes (range of 1–7 pmol/h/mg). The type 2 lactosamine (LacNAc-type-2-Ogr) is a good substrate for FUT4 (120 pmol/h/mg) and weak for FUT3 (9 pmol/h/mg), and we considered FUT10, FUT11, and FUT8 to be inactive toward this acceptor. H-type-1-Ogr is a good acceptor for the FUT3 enzyme only (675 pmol/h/mg). The other substrates tested are not acceptors for any of the enzymes, with the exception of OP-392-Ogr, which gives a weak activity with FUT8 (14 pmol/h/mg; Table 5).

The transfer activity profiles of the FUT10 and FUT11 isoforms are clearly distinguishable from those of the classical monoexonic α 1,3-fucosyltransferases, and this difference increases in the presence of 20 mM MnCl₂, which is a cofactor for FUT3 and FUT4, but not for FUT10, FUT11, or FUT8 (see Footnotes *b–d*, Table 5). In the presence of MnCl₂, the activity obtained with short lactosaminyl acceptors is doubled for FUT3 and FUT4, reaching 1115 pmol/h/mg for FUT4 with H-type-2-Ogr and 230 pmol/h/mg with LacNAc-type-2-Ogr. For FUT3, the transfer onto H-type-1-Ogr increased to 1280 pmol/h/mg. By contrast, with FUT10 and FUT11, the presence of MnCl₂ reduces by 80% the transfer onto *N*-glycan acceptors (0989-BM or BGA-biotin) and by 50% the activity of FUT8. From these results, we conclude that FUT10 and FUT11 function well without MnCl₂, use biantennary *N*-glycan acceptors, and cannot use short linear acceptors.

As for the natural BGA-biotin acceptors, two types of fucosylation site are possible for these enzymes on the 0989-BM acceptor: either or both of the GlcNAc residues linked β 1,2 onto the trimannosyl core, and the innermost GlcNAc of the chitobiose unit. For this reason, we prepared new acceptors modified at the innermost GlcNAc of the chitobiose (7OS-ManNAc-BM and 7OS-amino-alditol-GlcNAc-BM) (Table 1). Data presented in Table 6 indicate that these modified acceptors gave very low rates of transfer with the FUT10 and FUT8 enzymes when compared with the native 0989-BM acceptor. The 2-NAc epimerization of the innermost GlcNAc (7OS-ManNAc-BM) inhibited acceptor potential drastically, show-

ing only 8–10% of that observed for the parent structure. The acceptor potential of 7OS-amino-alditol-GlcNAc-BM was even lower, reaching only 0.1–0.9% for the three FUT10 variants and around 3% for FUT8, compared with that of the non-modified acceptor 0989-BM (Table 6).

A comparison of the acceptor potential of 0989-BM with its fucosylated counterpart (Fuc α 1,6-0989-BM) revealed an approximately 50% reduction for the FUT10 variant when the acceptor was fucosylated. The FUT8 α 1,6-fucosyltransferase cannot use this fucosylated acceptor, and the apparent transfer rate was reduced to 2%. Therefore, this acceptor permits discrimination between FUT10- and FUT8-mediated fucosylations (Table 6).

Under the same assay conditions and after 18 or 48 h of transfection, we tested the fucosyltransferase activities expressed in the FUT10-transfected cell supernatants, and no detectable activity was found using the best acceptor for these enzymes (0989-BM) (not shown).

In order to locate more precisely the fucosylation site used by FUT10-419, the ¹⁴C-fucosylated BGA-biotin reaction products generated by this enzyme were analyzed by TLC before and after NAG digestion. The undigested material yielded a predominant species designated Fuc-BGA-biotin (Fig. 5) as well as three minor slower migrating components. After NAG digestion (16 h), Fuc-BGA-biotin is converted into a component whose migration position is compatible with Fuc-BGA-biotin having lost its two terminal GlcNAc residues (see Fig. 5, –2 GlcNAc). In order to confirm that the product obtained lost both terminal GlcNAc residues, we performed shorter (6-h) NAG incubations. Under these conditions, in addition to the above mentioned digestion product (Fig. 5, –2 GlcNAc), a second component, whose migration position is compatible with a product having lost only one GlcNAc, was observed (not shown). The ensemble of these results indicates that the FUT10 enzyme predominantly fucosylates the innermost chitobiose core GlcNAc.

Conalbumin-Glycopeptide Fucosylation—Conalbumin glycopeptides carry natural nonsialylated, nongalactosylated biantennary glycoasparagine *N*-glycans, used as acceptors for FUT10-419, FUT8, and FUT3 α -L-fucosyltransferases. Data presented in Table 7, demonstrates that the FUT10-419 enzyme is able to transfer α -L-fucose onto native conalbumin (2450 dpm) or directly onto conalbumin glycopeptides (10,500 dpm), suggesting that FUT10 fucosylation is more efficient using conalbumin glycopeptides as acceptors. This transfer is of the same order of magnitude as that obtained with the FUT8 enzyme, which transfers 2800 dpm onto native glycoprotein

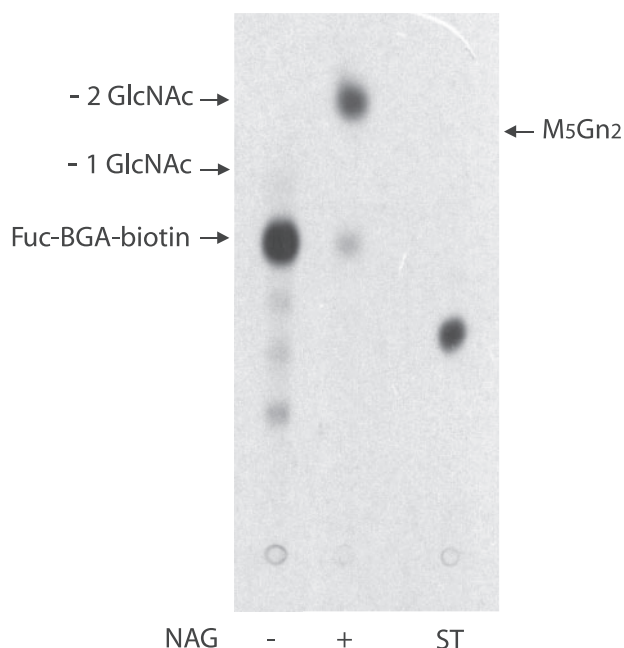


FIGURE 5. Thin layer chromatography of the BGA-biotin acceptor after FUT10-419 fucosylation and incubation in either the presence or the absence of human placenta NAG. The NAG-digested and nondigested Fuc-BGA-biotin products were resolved on TLC alongside the standard (ST) biantennary-free oligosaccharide OS-¹⁴C-galactosylated *N*-glycan (Gal₂GlcNAc₂Man₃GlcNAc₂). The nondigested Fuc-BGA-biotin product (–) has a main strong spot and three very faint spots. After NAG digestion (+) the main spot migrates faster, at the position expected for the product that has lost the two terminal nonreducing GlcNAc residues linked β1,2 to the trimanosyl core of the glycosylparagine *N*-glycan. The expected migration positions of Fuc-BGA-biotin products having lost one (–1 GlcNAc) or two GlcNAc (–2 GlcNAc) and the marker Man₃GlcNAc₂ (M₅Gn₂) are also indicated.

TABLE 7

α1,3- and α1,6-fucosyltransferase activities of FUT3, FUT10-419, and FUT8 constructs using conalbumin glycopeptide acceptors

Constructs ^a	α1,3-FT	α1,3-FT	α1,6-FT
FUT3 control	200		
FUT3 ^b	950		
FUT3	350		
FUT10-419 control		200	
FUT10-419 ^b		2450	
FUT10-419		10,500	
FUT8 control			400
FUT8 ^b			2800
FUT8			9000

^a COS7 cells were transiently transfected with the indicated constructs. Cell homogenates were incubated for 16 h at 37 °C with pronase-treated chicken egg white conalbumin glycopeptides as acceptor. The reaction mixtures were then subjected to Biogel P2 size exclusion chromatography as described under "Experimental Procedures." Specific fucosyl glycopeptides activities are expressed as the dpm recovered from the pooled fractions 9–15 (glycopeptides) of the Biogel P2 columns.

^b The fucose transfer was performed on native conalbumin glycoprotein, and then the glycoprotein was digested with pronase to obtain radioactive fucosylated glycopeptides. After Pronase digestion, the radioactive products were quantitated after Biogel P2 chromatography as described above.

and 9000 dpm on the corresponding conalbumin glycopeptides. Under these conditions, whatever the acceptor, FUT3 fucosylated weakly compared with FUT8 and FUT10-419 (Table 7).

Kinetic Studies of FUT10—Apparent K_m values were calculated for GDP-Fuc (range of concentration 3–420 μ M) in the presence of 60 μ M BGA-biotin and with optimal conditions for the FUT10 enzyme (Table 8). The FUT10-391 and the FUT10-479 variants had similar apparent affinities (K_m) for GDP-Fuc

TABLE 8

Kinetic parameters of the fucosyltransferase activities for the donor GDP-Fuc and the natural desialylated-degalactosylated biantennary glycosylparagine acceptor substrate linked to biotin (BGA-biotin)

Substrate	FUT10-391		FUT10-479	
	K_m	V_{max}	K_m	V_{max}
	μ M	pmol/h/mg	μ M	pmol/h/mg
GDP-Fuc	13	285	15	146
BGA-biotin	154	119	150	59

(between 13 and 15 μ M). Under these conditions, the maximum velocity obtained for the soluble protein FUT10-391 (V_{max} = 285) is twice that of the ER-resident enzyme FUT10-479 (V_{max} = 146) (Table 8). Apparent K_m values were also calculated for the natural BGA-biotin acceptor (3–310 μ M) at a saturating concentration of GDP-Fuc (125 μ M). The soluble (FUT10-391) and the ER-resident (FUT10-479) enzymes have higher apparent K_m values for this acceptor than those observed for the GDP-Fuc donor, but the K_m values for the acceptor are similar for the two variants (154 and 150 μ M). The V_{max} values for the acceptor were lower than those observed for GDP-Fuc but with the same difference between the soluble FUT10-391 (V_{max} = 119) and the ER-resident FUT10-479 enzyme (V_{max} = 59) (Table 8).

Subcellular Localization of the GFP-tagged Fusion Proteins—The three-tagged FUT10 isoforms were expressed into COS7 cells and visualized by immunofluorescence after 12, 18, 24, and 48 h of transfection. At 12 h, the soluble FUT10-391-GFP enzyme (green) did not co-localize with the ER marker calnexin (red) (Fig. 6, *a–c*), nor with the Lamp-1 lysosomal protein marker (not shown). By contrast, FUT10-391 did fully co-localize with giantin (red) in the Golgi apparatus (Fig. 6, *d–f*), as do the classical monoexonic α1,3-fucosyltransferases. Eighteen hours after transfection, the majority of this soluble protein still co-localized with giantin (Fig. 6, *g–i*), but some had migrated outside the Golgi apparatus and appeared to be dissociated from giantin. Twenty-four hours after transfection, it increased its migration outside the Golgi apparatus (Fig. 6, *j–l*), and more than 20% of the FUT10-391-GFP-transfected cells revealed a partition of the GFP fusion protein between the Golgi apparatus and cytoplasmic vacuolated vesicles. At 48 h, all of the FUT10-391-GFP labeling is completely dissociated from giantin (Fig. 6, *m–o*) and calnexin (Fig. 6, *p–r*). Strikingly, at 48 h, the staining with Lamp-1 antibody (Fig. 6, *s–u*) shows full co-localization of FUT10-391-GFP with this lysosome marker. These results demonstrate that the subcellular localization of the soluble FUT10-391-GFP changes with time. It is only transiently present in the Golgi before appearing in the lysosomal compartment associated with Lamp-1.

The two fusion proteins FUT10-419-GFP and FUT10-479-GFP appear to co-localize with the ER marker calnexin (Fig. 7, *a–c*) at all times of transfection, from 12 to 48 h. Lamp-1 labeling was dissociated from both FUT10-419-GFP (not shown) and FUT10-479 (Fig. 7, *j–l*), and giantin was also dissociated from both FUT10-419-GFP (Fig. 7, *d–f*) and FUT10-479-GFP (Fig. 7, *g–i*). By contrast, transfection with the FUT8-GFP enzyme illustrates co-localization of FUT8 with the Golgi apparatus marker (Fig. 7, *m–o*), as reported for all of the classical α1,3-fucosyltransferases.

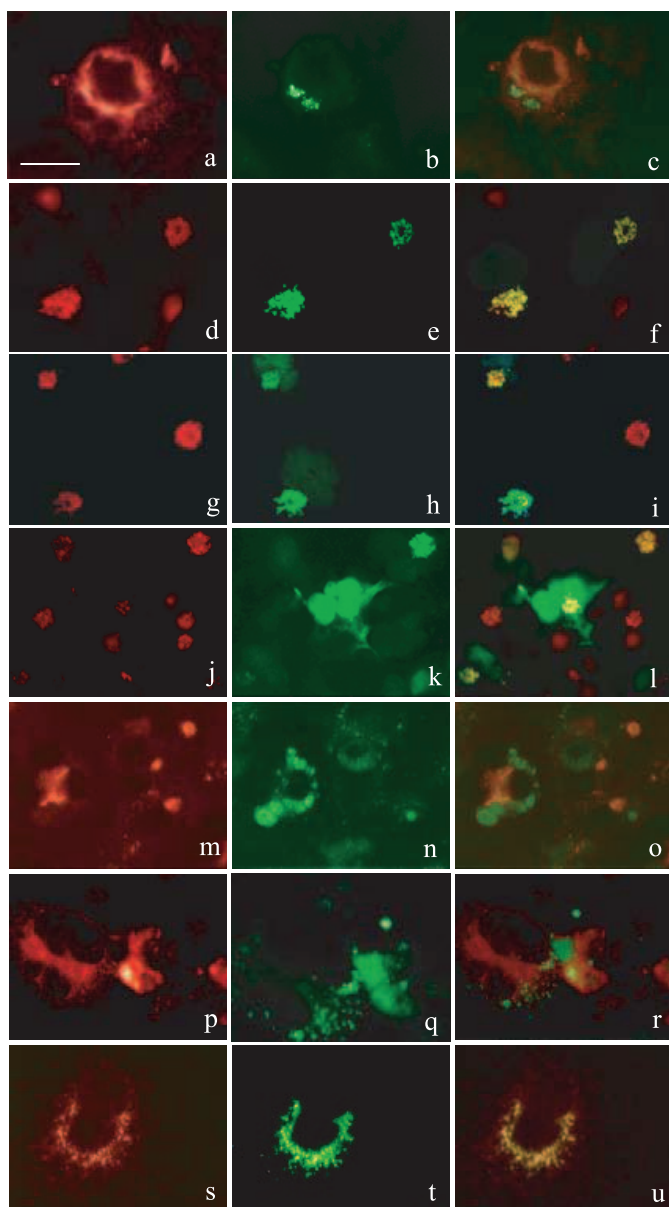


FIGURE 6. Immunofluorescence localization of FUT10-391-GFP-tagged recombinant protein (green) in COS7 cells after 12, 18, 24, and 48 h of transfection. Three subcellular markers are labeled in red: giantin for the Golgi, calnexin for the ER, and Lamp-1 for the lysosome. In each horizontal series of three pictures, the left image is the red staining of these subcellular cell markers alone, the central image is the green staining of the recombinant FUT10-391-GFP tagged protein alone, and the right image is the superimposition of the two color stains. The first two lines (a–f) correspond to 12 h of transfection; the third line (g–i) to 18 h of transfection; the fourth line (j–l) to 24 h of transfection; and the fifth, sixth, and seventh lines (m–u) to 48 h of transfection. The first line illustrates independent staining of calnexin and FUT10-391-GFP (a–c), and the second line shows the full co-localization of giantin and FUT10-391-GFP (d–f) at 12 h. The third (g–i) and the fourth (j–l) lines illustrate progressive partial dissociation of giantin and FUT10-391-GFP at 18 and 24 h, respectively. At 48 h, the fifth line (m–o) illustrates the full dissociation of giantin and FUT10-391-GFP, the sixth line (p–r) illustrates the independence of calnexin and FUT10-391-GFP stains, and finally, the seventh line (s–u) illustrates full co-localization of Lamp-1 and FUT10-391-GFP stains. Scale bar, 10 μ m in all panels.

DISCUSSION

The human FUT10 and FUT11 share the two main α 1,3-fucosyltransferase motifs that constitute a signature for the α 1,3-fucosyltransferases (23), and these motifs are not present

in the FUT8 α 1,6-fucosyltransferase (16). The phylogeny study suggests that the ancestor of FUT10 and FUT11 appeared about 980 MYA and that these two families emerged about 830 MYA. Therefore, they are more ancient than the classical monoexonic α 1,3-fucosyltransferase families that emerged in the vertebrate lineage about 450 MYA.

In the present study, we report the characterization of three new active splice variants of the *FUT10* gene, distinct from the published soluble FUT10-428 variant that has a truncated amino terminus with a different start codon. The main differences between these enzymes and the classical monoexonic α 1,3-fucosyltransferases are in the acceptor substrate-related area, located toward the NH_2 -end of the protein, suggesting that the FUT10 enzymes may have different acceptor substrate requirements for their activity. In good agreement with this idea, our results show that FUT10 activity profiles are clearly distinguishable from those of the classical monoexonic α 1,3-fucosyltransferases, since they branch α -L-fucose onto biantennary *N*-glycan structures in the absence of MnCl_2 . By contrast, the classical monoexonic α 1,3-fucosyltransferases need MnCl_2 as cofactor and prefer short linear lactosamine-related acceptors.

The elongation at the nonreducing end of the biantennary *N*-glycan 0989-BM acceptor, with galactose residues (0988-BM) or with galactose and *N*-acetylneuraminic acid residues (0987-BM), or its shortening, by removal of the terminal nonreducing GlcNAc residues (0990-BM), reduces or inhibits the enzymatic transfer of FUT10 and FUT11. These results illustrate that, in the acceptor panel used for this work, the best structure for these novel enzymes is the biantennary *N*-glycan substrate (0989-BM), which theoretically permits the fucosylation in the α 1,3-orientation, onto the terminal GlcNAc residue linked β 1,2 to the trimannosyl core or onto the innermost GlcNAc residue of the chitobiose unit. To check where the variants of the FUT10 enzymes linked the fucose, we made two analogs of the 0989-BM acceptor that abolish the possibility of fucosylation onto the innermost GlcNAc. These modifications reduced drastically the transfer of the FUT10 variants, suggesting that FUT10 preferentially fucosylates the innermost GlcNAc of the chitobiose. Furthermore, the absence of FUT10 α 1,3-fucosylation onto the peripheral nonreducing GlcNAc residues was confirmed when both of these GlcNAc residues were removed from the FUT10 ^{14}C -fucosylated product (Fuc-BGA-biotin) with NAG glycosidase. This experiment is in good agreement with the fact that FUT10 is able to fucosylate in α 1,3 the innermost GlcNAc of the biantennary *N*-glycan structure.

The acceptor previously fucosylated in α 1,6 onto the core chitobiose permits discrimination between the FUT10- and FUT8-mediated fucosylations. This Fuc α 1,6-0989-BM substrate keeps a substantial fucosyltransferase activity for FUT10 enzymes, although the presence of the α 1,6-fucose reduces the FUT10 transfer by 50%, suggesting a steric constraint for the FUT10 enzymes. This confirms that the α 1,3-fucosylation mechanism of human FUT10 is different from the activity of the *D. melanogaster* core Fuc-TA that is, on the contrary, favored by previous core α 1,6-fucosylation (26). Under the same conditions, the FUT8 enzyme does not work at all, because the α 1,6-fucosylation position is already occupied. In

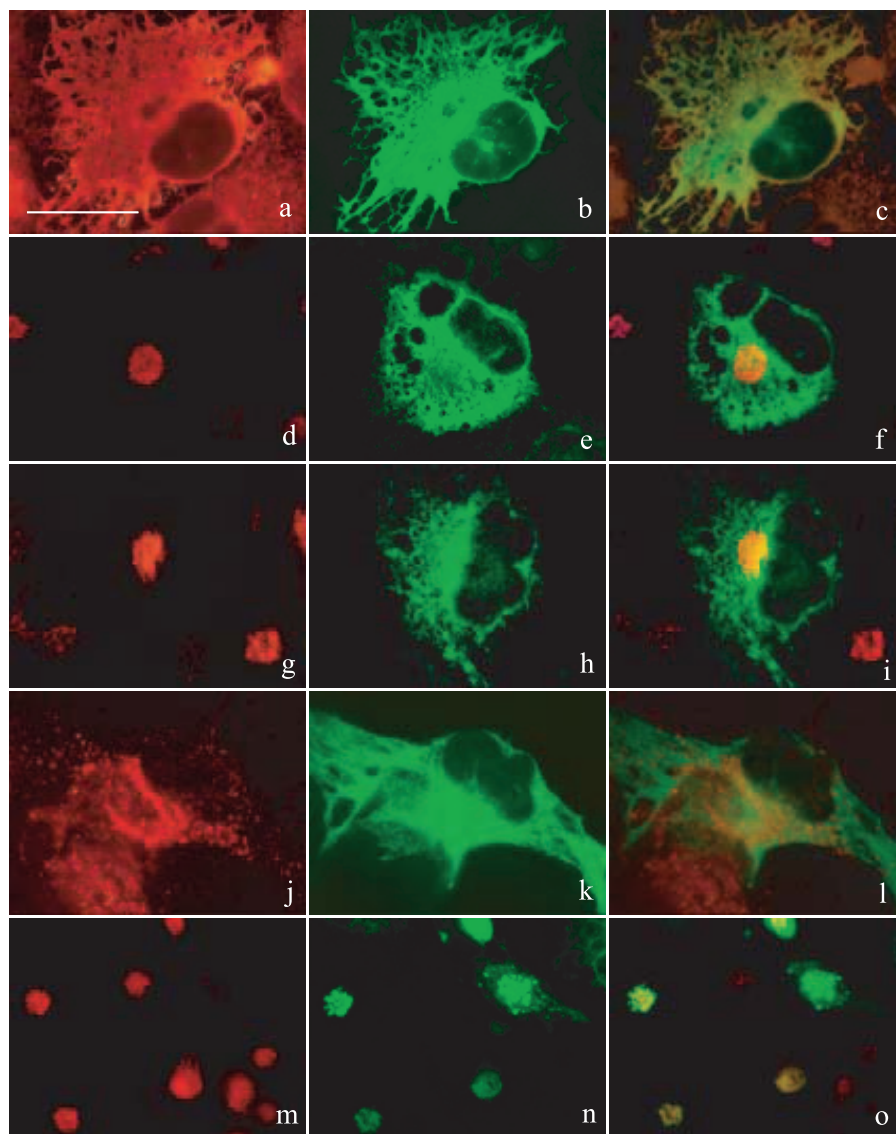


FIGURE 7. Immunofluorescence localization of FUT10-419, FUT10-479, and FUT8 GFP-tagged recombinant proteins in COS7 cells after 48 h of transfection. As in Fig. 6, the images on the left represent the staining of the subcellular markers alone (red), the central images are the recombinant fucosyltransferase-GFP tagged enzymes stain alone (green), and the right images are the superimposition of the two color stains. The first line (a–c) illustrates full co-localization of the FUT10-419-GFP isoform and calnexin in the ER. The second line (d–f) illustrates independent stains for the same FUT10-419-GFP protein and giantin. The third line (g–i) illustrates independent stains for FUT10-479-GFP and giantin. The fourth line (j–l) shows independent stains of FUT10-479-GFP and Lamp-1 in the lysosome. Finally, the fifth line (m–o) illustrates full co-localization of FUT8-GFP α 1,6-fucosyltransferase with giantin in the Golgi apparatus. Scale bar, 10 μ m in all panels.

fact, this is a good control for the α 1,3-fucosylation activity of FUT10. In addition, the FUT10 fucosyltransferase is able to transfer fucose onto native conalbumin glycoprotein or onto its derivative glycopeptides. This FUT10 glycoprotein fucosyltransferase activity profile is similar to the one obtained for the FUT8 enzyme but distinct from the classical α 3-fucosyltransferase profile.

In our conditions, the linear chitobiose-BM disaccharide acceptor is not an acceptor for either FUT10 or FUT11 nor for the classical monoexonic α 1,3-fucosyltransferases. Although some fucose transfer onto linear acceptors, such as chitobiose, chitotriose, or chitotetraose, has been described for classical α 1,3-fucosyltransferases, long incubations of 4 days are required. The structures of the final products were well charac-

terized by NMR and mass spectrometry and revealed a mixture of chitobiose products fucosylated in α 1,3- and α 1,6-orientations and chitotriose and chitotetraose products with only weak α 1,3-fucosylation (63). In good agreement with these results, we also detect a very weak α -L-fucosyltransferase activity onto the innermost GlcNAc of BGA-biotin acceptor and onto the chicken egg white conalbumin glycoprotein and their derived glycopeptides with FUT3 and FUT4.

Considering the enzyme activity only, classical monoexonic α 1,3/4-fucosyltransferases prefer short lactosaminyl acceptors, whereas the polyexonic FUT10 enzymes prefer biantennary *N*-glycans linked to glycopeptides or to biotin aglycone, illustrating that linear short acceptors cannot be used to follow the FUT10 and FUT11 transfer of α -L-fucose. This may explain some of the previously reported negative results obtained with these enzymes (24)⁵ and the fact that all of the FUT10 fucosyltransferase sequences already reported have differences in motif II implicated in the acceptor substrate recognition.

Regarding the subcellular localization of the FUT10 isoforms, the FUT10-391 variant is a soluble protein showing no TMD and no ER membrane retention signal. Immunofluorescent staining confirms that this protein is transiently co-localized with the Golgi marker giantin during the first 24 h and then migrates to the lysosome, to be probably later degraded in this com-

partment. In our hands, this protein never co-localized with the ER marker calnexin. Alternatively, the ER-resident FUT10-419 and FUT10-479 variants have a type II transmembrane topology and an ER membrane retention signal at their NH₂ terminus. FUT10-479 has, in addition, a second ER membrane retention signal at its COOH terminus. Immunofluorescent staining confirmed that the recombinant FUT10-419 and FUT10-479 proteins stably reside in the ER, since they are always co-localized with calnexin. This is the first time that active α 1,3-fucosyltransferases have been found in the ER. Only the soluble protein-*O*-fucosyltransferase 1 enzyme has previously been shown to be retained, in the ER lumen via a COOH-terminal KDEL-like motif (64). It is not clear why FUT10 and protein-*O*-fucosyltransferase proteins are located in the ER, because no

GDP-Fuc transporter has been characterized in this compartment. However, the addition of *O*-fucose to epidermal growth factor-like repeats has been demonstrated in the ER, suggesting the existence of a novel GDP-Fuc transporter in this compartment (64) or a retrograde flux of GDP-Fuc from the Golgi apparatus. Protein-*O*-fucosyltransferase 1, FUT10-419, and FUT10-479 are active enzymes *in vitro* and perhaps can have other additional functions *in vivo*, such as the reported chaperone role of protein-*O*-fucosyltransferase 1, which promotes the correct folding of the epidermal growth factor repeat of the Notch receptor (3).

We hypothesize that the α 1,3-fucosylation onto the chitobiose unit of biantennary *N*-glycans can occur in the ER of embryo cells (FUT10-419) or in the ER of adult cells (FUT10-479) and transiently in the Golgi apparatus of embryo cells for the soluble variant FUT10-391. However, in the adult, this core α 1,3-fucosylation has to be a transient intracellular signal, because secreted outside or expressed at the surface of the cell, the core α 1,3-fucose on glycoproteins becomes a strong immunogen. It has been shown to be a major cause of allergic reactions, induced by insect and plant allergens containing this α 1,3-core fucose epitope (25, 65, 66). Because this type of core α 1,3-fucosylation is not a regular signal found in mammalian glycoproteins, expressed only in certain cell compartments, it could help to recognize and select the incorrectly glycosylated proteins that escape from the regular quality control mechanism, reorienting them to lysosomes or other compartments able to degrade aberrant glycoproteins.

Finally, enzyme activity was also detected with FUT11, in particular with FUT11-476. The results suggest a substrate acceptor pattern similar to FUT10 variants, but this enzyme activity needs further characterization.

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