Expression Cloning and Characterization of a Novel Murine α1,3-Fucosyltransferase, mFuc-TIX, That Synthesizes the Lewis x (CD15) Epitope in Brain and Kidney*

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The 3-fucosyl-N-acetyllactosamine (Lewis x, CD15, SSEA-1) carbohydrate epitope is widely distributed in many tissues and is developmentally expressed in some rodent and human tissues, i.e. brain and lung, and mouse early embryo. In such tissues, the Lewis x epitope is considered to be involved in cell-cell interactions. We isolated a novel mouse α1,3-fucosyltransferase gene, named mFuc-TIX, from an adult mouse brain cDNA library using the expression cloning method. On flow cytometric analysis, Namalwa cells transfected stably with the mFuc-TIX gene showed a marked increase in Lewis x epitopes but not sialyl Lewis x epitopes. As seen experiments involving oligosaccharides as acceptor substrates, mFuc-TIX transfers a fucose to lacto-N-neotetraose but not to either α2,3-sialyl lacto-N-neotetraose or lacto-N-tetraose. The substrate specificity of mFuc-TIX was similar to that of mouse myeloid-type α1,3-fucosyltransferase (mFuc-TIV). The deduced amino acid sequence of mFuc-TIX, consisting of 359 residues, indicated a type II membrane protein and shows low degrees of homology to the previously cloned α1,3-fucosyltransferases, i.e. mFuc-TIV (48.4%), mouse Fuc-TII (39.1%), and human Fuc-TII (43.0%), at the amino acid sequence level. A phylogenetic tree of the α1,3-fucosyltransferases constructed by the neighbor-joining method showed that mFuc-TIX is quite distant from the other α1,3-fucosyltransferases. Thus, mFuc-TIX does not belong to any subfamilies of known α1,3-Fuc-Ts. The mFuc-TIX transcript was mainly detected in brain and kidney with the Northern blotting and competitive reverse transcription-polymerase chain reaction methods, whereas the mFuc-TIV transcript was not detected in brain with these methods. On in situ hybridization, the mFuc-TIX transcript was detected in neuronal cells but not in the glial cells including astrocytes. These results strongly indicated that mFuc-TIX participates in the Lewis x synthesis in neurons of the brain and may be developmentally regulated.

α1,3-Fucosyltransferase (α1,3Fuc-T) transfers a fucose (Fuc) from guanosine diphosphate-fucose (GDP-Fuc) to N-acetylgalactosamine (GlcNAc) of the type 2 chain, Galβ1,4GlcNAc-R, with an α1,3-linkage. The genes encoding α1,3Fuc-Ts form a family. Five human (1–8), two bovine (9), two mouse (10–12), one rat (13), and one chicken (14) α1,3Fuc-T gene have been cloned to date. Recently, two α1,3Fuc-T genes of Helicobacter pylori were cloned (15, 16). The enzymatic characteristics of all these α1,3Fuc-Ts from various species have been examined using recombinant enzymes directed by the genes. All five human α1,3Fuc-Ts, i.e. hFuc-TIII (hFUT3; Lewis enzyme), hFuc-TIV (hFUT4; myeloid-type α1,3Fuc-T), hFuc-TV (hFUT5; plasma-type α1,3Fuc-T), and hFuc-TVIII (hFUT6; plasma-type α1,3Fuc-T), can synthesize the sialyl Lewis x (sLeα), sialic acid α2Galβ1,4(Fucα1,3)GlcNAcβ-R, epitope, and four of them, i.e. hFuc-TIII, -TV, -TVIII, and -TVI, can synthesize the Lewis x (Leα). hFuc-TIV and -TVII are located at 11q21 and 9q34.3, respectively (7, 24). A bovine homologue, named the futb gene, corresponding to the hFuc-TII, -TV, or -TVI gene has been cloned (9). The futb gene, a single copy gene, is suggested to be an orthologous homologue of an ancestral gene, from which the present hFuc-TIV-hFuc-TIII-hFuc-TV gene cluster evolved. The two mouse genes, the mFuc-TIV and mFuc-TVII genes, homologues of the hFuc-TIV and -TVII genes, respectively, and the rat homologue, the rFuc-TV gene, corresponding to the hFuc-TIV gene, have been cloned, and their substrate specificities were determined to be similar to those of the human homologues (10–14). The mouse orthologous homologue corresponding to the futb gene, the ancestral gene for the hFuc-TV-hFuc-TIII-hFuc-TV gene cluster, seems to be a pseudogene (11, 25).

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1 The abbreviations used are: α1,3Fuc-T, α1,3-fucosyltransferase; mFuc-TIX, murine α1,3-fucosyltransferase IX; mFuc-TIV, murine α1,3-fucosyltransferase IV; Leα, Lewis x; sLeα, sialyl Lewis x; Fuc, fucose; Gal, galactose; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase(s); mAb, monoclonal antibody; ORF, open reading frame; MOPS, 3-[N-morpholino]propanesulfonic acid; PA, pyridylamidated; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; CNS, central nervous system; SSEA-1, stage-specific embryonal antigen-1.
Immunohistochemical and biochemical studies on man and rodents revealed the widespread distribution of Leα epitopes in many tissues, such as epithelial cells of intestinal tissues (26–28), myeloid cells (29–31), and certain neurons and glial cells in the central nervous system (CNS) (32–36). In addition, the developmentally regulated Leα expression has been well investigated immunohistochemically in some tissues, i.e., mouse early embryos (37–40), human lung (41), and human and rodent CNS (36, 42, 43). Stage-specific embryonic antigen-1 (SSEA-1), having the Leα epitope at its carbohydrate chain terminus, is highly expressed in the morulae of mouse embryos and decreases rapidly after compaction. The Leα epitope on SSEA-1 is considered to play a role as a cell-cell interaction molecule during development. The developmentally regulated expression of the Leα (CD15-reactive) epitope in the CNS has also attracted much attention since the Leα epitope may be involved in the neurodevelopmental process.

However, little is known as to which α1,3Fuc-T is responsible for the Leα synthesis in such native tissues. The α1,3Fuc-T partially purified from human neuroblastoma cells exhibited the ability of Leα and Leβ synthesis, but not that of αLeα synthesis, its substrate specificity being similar to that of hFuc-TIV, but quite different from those of the other four (44). Mollicone et al. (45) reported that the substrate specificity of α1,3Fuc-T expressed in the brain is very similar to that of hFuc-TIV, and the α1,3Fuc-T in the brain cannot be distinguished from hFuc-TIV by means of enzymatic characteristics. However, the transcript for the mFuc-TIV gene was not detected in mouse brain on Northern blot analysis (11), whereas it was abundantly expressed in other tissues, such as stomach, colon, spleen, and uterus, and at intermediate levels expressed in lung, testis, ovary, and small intestine (11). The above results raise a possibility that the Leα epitope in the CNS may be synthesized by unknown α1,3Fuc-T(s), of which genes have not been cloned yet.

In the present study, we isolated a novel α1,3Fuc-T gene from a mouse brain cDNA library by the expression cloning method and named it the mFuc-TIX mRNA the gene. The transcript for the mFuc-TIX gene, which was mainly expressed in brain and kidney, was apparently detected in neuronal cells but not in glial cells including astrocytes in the CNS on in situ hybridization in the present study. Previous immunohistochemical studies revealed the existence of the CD15 (Leα) epitope in both neuronal cells and astrocytes (32–36, 42, 43, 46–48). The present study strongly indicated that the novel mFuc-TIX is responsible for the Leα synthesis in the neurons but not in the glial cells in the CNS.

**EXPERIMENTAL PROCEDURES**

**Nomenclature for a New α1,3-Fucosyltransferase—To date, eight human fucosyltransferase (Fuc-T) genes have been cloned, i.e., two α1,2Fuc-Ts, five α1,3Fuc-Ts, and one α1,6Fuc-T. Costache and co-workers (25) named the eight Fuc-Ts, FUT1 to FUT8. FUT1 and FUT2 are α1,2Fuc-Ts corresponding to the H and S enzymes, respectively (49–52). FUT3, -4, -5, -6, and -7 are α1,3Fuc-Ts corresponding to Fuc-TIII, -IV, -V, -VI, and -VII, respectively, according to Lowe’s nomenclature (1, 2, 4, 5, 7, 8). FUT4 is an α1,6Fuc-T of which the gene was originally cloned by Uozumi et al. (53). The gene encoding a mouse homologue corresponding to the human FUT8 (54) has not been cloned yet. A novel α1,3Fuc-T, of which the gene was isolated from mouse brain tissue in the present study, will be referred to as murine Fuc-TIX (mFuc-TIX) according to the designation of Lowe and co-workers (1,2,4,5,7,54).

**Construction of a cDNA Library from Adult Mouse Brain and Ex-**

**pression Cloning of cDNA Encoding mFuc-TIX—Poly(A)+-enriched RNA was isolated with Oligotex<sup>TM</sup>40T<sub>Super</sub>-<sup>+</sup> (Roche, Tokyo, Japan) from an adult BALB/c mouse brain. Complementary DNAs were synthesized with oligo(dT) primers derived from poly(A)+-rich RNA using a Superscript Choice System for cDNA Synthesis (Life Technologies, Inc.). A cDNA library was constructed by inserting size-fractionated cDNAs (more than 1.5 kb) into an expression vector, pMAo (7, 55), using S/JI adaptors. We obtained about 1 × 10<sup>6</sup> independent clones as a cDNA library and extracted plasmid DNAs from the library.

The expression cloning method involving Namalwa cells (human Burkitt lymphoma cells) was described in detail in our previous papers (7, 55). Namalwa cells were transfected with cDNAs from the cDNA library using a Gene-Pulsor (Bio-Rad). The cells were exposed in the presence of geneticin (0.6 mg/ml) (Life Technologies, Inc.) for 2 weeks to obtain stable transformant cells. The Namalwa cells growing after the geneticin selection were stained with an anti-Leα monoclonal antibody (mAb) (PM-81; IgM), followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Cappel) and then cell sorting (FACSTAR Plus Epitope Coulter). The cells expressing the Leα epitope were enriched by four rounds of cell sorting. The plasmids were recovered from the Leα-positive cells by Hirt’s method (56).

For nucleotide sequencing of the DNA isolated, we excised the insert by KpnI and HindIII digestion, and then the insert was subcloned into a plBluescript SK(−) vector (pBS). Sequencing of the insert was performed by an ordinary method, i.e., the dyeon chain termination method using an ALP DNA sequencer (Amersham Pharmacia Biotech). The cDNA obtained was named murine Fuc-TIX (mFuc-TIX).

**Monoclonal Antibodies (mAbs) and Flow Cytometry Analysis—Na-**

malwa cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum. The pPM plasmid DNA containing the mFuc-TIX cDNA (pPM-mFT9) was transfected into Namalwa cells. After exposure to geneticin for 2 weeks, we established Namalwa cells which stably expressed the mFuc-TIX cDNA (Namalwa-mFT9 cells). In previous studies, we established a series of Namalwa cells stably expressing each of five different human α1,3Fuc-T genes, named Namalwa-hFT3, -hFT4, -hFT5, -hFT6, and -hFT7 cells (18, 57). In this study, we isolated the mFuc-TIV gene by the polymerase chain reaction (PCR) method, as described later, and inserted the mFuc-TIV gene into the pMAo vector, by which we established Namalwa cells stably expressing mFuc-TIV (Namalwa-mFT4 cells). These stable transformant cells were used as controls in the following experiments in comparison with Namalwa-mFT9 cells.

The mFuc-TIX and mFuc-TIV genes were subcloned into the pCDM8 vector for transient expression of the genes in COS-1 cells. The hFuc- TIV and hFuc-TII genes, which were subcloned into the pCDM8 vector in the previous study, were also transiently transfected into COS-1 cells. The COS-1 cells transfected transiently with the respective genes were subjected to flow cytometry analysis, and the cell homogenates were used for measurement of enzyme activity toward oligosaccharides.

For flow cytometry analysis, the cells were incubated with each of the mAbs, i.e., two anti-Leα mAbs, PM-81, which was a kind gift from Dr. D. Marcus, and 73–30 (IgM), which was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), an anti-Leα mAb, KM-95 (IgM), and an anti-Lewis a (Leα) mAb, 7LE (IgM), which were purchased from Seikagaku Kogyo, Co., Ltd., and an anti-Leα mAb, AH-6 (IgM), which was a kind gift from Otsuka Inc. (Tokushima, Japan). After incubation with the first antibody (10 μg/ml), the cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Cappel) and then subjected to flow cytometry analysis with an Epics Elite cell sorter (Coulter). The results were analyzed and expressed as flow cytometry histograms and dot plots for each antibody.

**Substrate Specificity of Fuc-TIX toward Oligosaccharides—Lacto-N-**

tetraose (LNT) and lacto-N-neotetraose (LNTn) were purchased from Oxford Glycosystems (United Kingdom) and pyridylaminated according to the method of Kondo et al. (58). Pyridylaminated LNnT (LNnT-Pa) was silylated with the recombiant β-galactoside α2,3-sialyltransferase from Namalwa cells (55), resulting in the production of pyridylaminated α2,3-sialyl lacto-N-neotetraose (α2,3-sialyl LNnT-Pa), and the product, α2,3-sialyl LNnT-Pa, was purified by high pressure liquid chromatography (Nihon Bunko, Tokyo, Japan).

Fifteen micrograms of pCDM8 DNA containing each gene was transfected into COS-1 cells in combination with 1 μg of the β-actin promoter-driven luciferase expression vector as an indicator of the transfection efficiency. Lysates of COS-1 cells transfected transiently with the respective genes were used for assaying the substrate specificities toward LNT-Pa, LNnT-Pa, and α2,3-sialyl LNnT-Pa. The cells were harvested after 48 h incubation and separated into 3 aliquots. Total RNA was extracted from 1 aliquot. The 2nd aliquot was subjected to the assaying of luciferase activity and fucosyltransferase activity and the 3rd aliquot to flow cytometry analysis by the same method as described above. The cells for the enzyme assay were rinsed with ice-cold 0.1 M HEPES (pH 7.4) and 2% Triton X-100 by brief sonication. Luciferase activity was measured as described previously (59). The luciferase activity was relatively constant from transfection to transfection with errors within ±20%. The level of the transcript of the transfected gene was determined by the competitive reverse transcription-PCR (competitive RT-PCR) method. The amount of the transcript expressed paral-
leaded the transfection efficiency monitored as the luciferase activity.

After determination of the protein concentrations with a Micro-BCA Protein Assay Reagent Kit (Pierce), the concentrations of cell lysates were adjusted based on the transfection efficiency, and the lysates were incubated in 50 mM cacodylate buffer (pH 6.8), 5 mM ATP (Sigma), 75 μM GDP-fucose (Sigma), 10 mM λ-fucose (Sigma), and 25 μM of the respective pyridylaminated acceptor substrate. After incubation at 37 °C for 2 h, enzyme reactions were terminated by boiling for 3 min and adding water. After centrifugation of the reaction mixture at 15,000 rpm for 5 min, 10 μl of the supernatant was subjected to high pressure liquid chromatography analysis on a TSK-gel ODS-80TM column (4.6 × 300 mm; Tosoh, Tokyo, Japan). The reaction products were eluted with 20 mM ammonium acetate buffer (pH 4.0) at the flow rate of 1.0 ml/min at 35 °C and monitored with a fluorescence spectrophotometer (JASCO FP-920, Tokyo, Japan). Parallel reactions were performed in the absence of GDP-Fuc to identify the products and to check substrate hydrolysis. The reaction products were identified by comparison of their retention times on high pressure liquid chromatography with those of pyridylaminated standard oligosaccharides, and their amounts were determined from their fluorescence intensities.

**PCR Cloning of the mFuc-TIV and Mice β-Actin (mβ-Actin) Genes**

The genes encoding the full-length open reading frames (ORF) of mFuc-TIV and mβ-actin were amplified by PCR, and the amplified fragments were subcloned into pBS. Complementary DNAs prepared from mRNAs of stomach tissues of BALB/c mice were used for the PCR amplification. The mFuc-TIV gene was amplified with a forward primer, 5'-TATCCG-AGTGCAGGCGAGGTTG-3', and a reverse primer, 5'-TATCCG- TGGAAACCGTGTCACAAG-3'. The mβ-actin gene was amplified with a forward primer, 5'-ACAGCTTCTTGGCATCTTTCG-3', and a reverse primer, 5'-GGTGAACGGCAACGTACAAAG-3'. Thus, the three mouse genes, i.e. the mFuc-TIV, mFuc-TIX, and mβ-actin genes, were subcloned into pBS, the resultant plasmids being named pBS-mFuc-T4d, pBS-mFuc-T9, and pBS-mβ-actin, respectively. These cloned DNA sequences were used as templates for construction of competitor DNAs for use with the competitive RT-PCR method.

**Quantitative Analysis of Glycosyltransferase Transcripts in Mouse Tissues Using the Competitive RT-PCR Method**—Competitor DNA plasmids carrying a small deletion within the respective full-length ORF cDNAs were constructed by appropriate restriction endonuclease digestion, as shown in Table I. For instance, a competitor DNA plasmid for the mFuc-TIV gene (pBS-mF4d) was prepared by deleting the 127-base pair Xenl-Bpu1102I fragment from pBS-mF4. Thus, pBS-mF9 was digested with XenI and Bpu1102I, followed by blunt-end ligation. The competitor plasmids for the mFuc-TIV and mβ-actin genes, pBS-mF4d and pBS-mβ-actin-d, respectively, were constructed by the same method using appropriate restriction enzymes, as shown in Table I.

Total cellular RNA was isolated from various tissues of BALB/c mice using the acid guanidinium thiocyanate/phenol/chloroform method (60). Six micrograms of the total RNA was treated with 5 units/ml DNase I (Life Technologies, Inc.) to remove residual genomic DNAs in the RNA preparation for 5 min at room temperature, followed by incubation for 15 min at 65 °C for inactivation of DNase I. Complementary DNAs were synthesized with an oligo(dT) primer from 6 μg of the DNase I-treated total RNA in a 20-μl total volume reaction mixture using a SUPER-SCRIPT™ Preamplification System for first strand cDNA synthesis (Life Technologies, Inc.). After cDNA synthesis, the reaction mixture was diluted 50-fold with H2O and then stored at −80 °C until use.

The competitive RT-PCR was performed with AmpliTaq Gold™ (Perkin-Elmer) in a total volume of 50 μl including 10 μl of standard plasmid DNA or sample cDNA, 10 μl of competitor DNA at the optimal concentration, which differed with the transcript, and 0.2 μM of each of the gene specific primer sets listed in Table I. The PCR buffer for the competitive RT-PCR comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 μM of each dNTP, and 0.001% (w/v) gelatin. PCR was performed with a pre-PCR heating step at 95 °C for 11 min, followed by the optimal number of PCR cycles of 1 min at 95 °C, 1 min at 65 °C, and 2 min at 72 °C. The optimal number of cycles and the optimal concentration of competitor DNA varied depending on the transcript, i.e. 38–42 cycles for mFuc-TIX, 38–42 for mFuc-TIV, and 22–26 for mβ-actin. A 10-μl aliquot after the competitive RT-PCR was electrophoresed in a 1% agarose gel, and the bands were visualized by ethidium bromide staining. The intensities of the bands of amplified fragments were quantified by scanning the positive pictures using an NIH Image system. To ascertain the efficiency of cDNA preparation from total RNAs, measurement of the β-actin transcript in each sample was performed using the competitive RT-PCR method as performed for the glycosyltransferase transcripts. Each value for the fucosyltransferase and β-actin transcripts was plotted on the respective standard curve to obtain the actual amount of the transcripts. The amount of β-actin varied depending on the tissue.

**Northern Blot Analysis of Mouse Tissues and Southern Blot Analysis of Mouse Genomic DNA**—For Northern blot analysis, 5 μg of poly(A)+-rich RNAs from mouse tissues was denatured in 50% (v/v) formamide containing 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA for 5 min at 65 °C and then electrophoresed on a 2% agarose gel. The RNA in the gel was subsequently transferred to a nitrocellulose filter (Optitran BA-85; Schleicher & Schuell, Germany) through capillary action in 20× SSC. The RNA on the filter was immobilized by baking at 80 °C for 1 h under a vacuum. Prehybridization and hybridization were performed in a buffer comprising 5× SSPE, 5× Denhardt’s solution, 1% SDS, and 0.2 mg/ml salmon sperm DNA (Amersham Pharmacia Biotech) at 65 °C. A 1.2-kb fragment encoding the full-length ORF of the mFuc-TIX gene was used as a hybridization probe. After washing with 0.1× SSC, 0.1% SDS buffer at 65 °C twice, the filter was exposed to an imaging plate, and the hybridized bands were detected with a BAS2000 Bio-Imaging Analyzer (Fuji, Tokyo, Japan).

For Southern blot analysis, genomic DNA from a mouse liver was prepared using a Qiagen Genomic-tip Kit and digested with each restriction endonuclease, EcoRI, BamHI, or HindIII, followed by electrophoresis in a 0.8% agarose gel. The methods for transference to the membrane, hybridization, and detection of hybridized signals were identical to those for Northern blot analysis.

**In Situ Hybridization Analysis**—For the construction of riboprobes for in situ hybridization analysis, we prepared two sets of each antisense or sense sequence. Two segments of the mFuc-TIX gene encompassing the nucleotide positions 506–683 and 693–855 were amplified by PCR and subcloned into plasmid vector pCR3 (Invitrogen). Antisense or sense riboprobes were generated using these plasmids as templates in SP6 or T7 RNA polymerase-directed in vitro transcription mixtures containing digoxigenin-labeled mix (Boehringer Mannheim).

The brain tissue of BALB/c mice was fixed in 4% paraformaldehyde/phosphate-buffered saline (−) (w/v) overnight and then immersed in paraffin. After deparaffinization and rehydration of paraffin sections, the sections were treated with 0.2 μM HCl for 20 min to inactivate endogenous alkaline phosphatase and then incubated with 4 μg/ml Protex K (Boehringer Mannheim) at 37 °C for 15 min. The sections were fixed in 4% paraformaldehyde/phosphate-buffered saline (−) (w/v) for 10 min and then immersed in 0.2% glycyne/phosphate-buffered saline (−) (w/v) for 10 min to avoid nonspecific staining. After dehydration of the sections, ribopores were hybridized on the sections in 50%
formamide, 10% dextran sulfate, 1× Denhardt's solution, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.6 M NaCl, and 200 μg/ml yeast tRNA at 50 °C for 16 h. They were then washed with 2× SSC at room temperature for 15 min twice and 0.1× SSC at 50 °C for 30 min. The hybridized signals were reacted with an anti-digoxigenin antibody and then detected with a 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Boehringer Mannheim).

Phylogenetic Tree of α1,3-Fucosyltransferase Genes—Amino acid sequence data for the cloned α1,3Fuc-Ts were obtained from the GenBank™ database. A phylogenetic tree was constructed using the neighbor-joining method (61).

RESULTS
Expression Cloning of a cDNA Encoding a Novel Le Syndrhea—Although the previous studies implied the Le determinant was present in neurons and astrocytes in the CNS, the α1,3Fuc-T involved in the Le synthesis in brain tissues remains to be identified. To isolate novel α1,3Fuc-T genes expressed in the brain, we employed the expression cloning method using a mouse brain cDNA library and the anti-Le mAb, PM-81. Namalwa cells transfected with cDNAs constructed in the expression vector, pAMo, were selected by cell sorting to obtain Le-positive cells by the same method as employed in our previous study (7). Six kinds of cDNA clones were recovered from the Le-positive Namalwa cells after four rounds of sorting with PM-81. Each of the six cDNAs was independently transfected into Namalwa cells, and one of the six cDNAs was found to direct the Le expression on the cell surface of Namalwa cells. The single cDNA clone that directed the Le expression was subjected to nucleotide sequencing.

A Putative Amino Acid Sequence of mFuc-TIX Deduced from the cDNA Sequence and Homology to Other α1,3Fuc-Ts—The cDNA rescued from the Le-positive Namalwa cells was subcloned into pBS for sequencing. cDNA sequencing analysis revealed that the cDNA contains an ORF encoding a protein of 359 amino acids with a calculated molecular mass of 42,040 daltons (Fig. 1A). The position of the AUG start codon was determined according to the Kozak consensus sequence (62). A hydrophoby profile determined by the Kyte and Doolittle method indicated that the ORF encodes a typical type II membrane protein with a cytoplasmic tail of a 10-amino acid peptide, a transmembrane domain of a 20-amino acid N-terminal hydrophobic portion, and a large catalytic portion of a 329-amino acid peptide (Fig. 1B). The topology of the protein encoded by the cDNA is consistent with that of mammalian glycosyltransferases which have been cloned previously.

The homologies of the amino acid sequence encoded by the cDNA with those of the other α1,3Fuc-Ts are summarized in Table II. Although none of the α1,3Fuc-Ts, which have been cloned to date, show a high degree of homology to the amino acid sequence encoded by the cDNA, there was a significant homology indicating that the cDNA encodes a new member of the α1,3Fuc-T family. Thus, the novel α1,3Fuc-T isolated from mouse brain tissue in the present study was named mFuc-TIX. Among the α1,3Fuc-Ts, CPT1 (chicken Fuc-TIV) and mFuc-TV showed the highest homology, 50.5 and 48.4%, respectively, to mFuc-TIX at the amino acid sequence level; however, these homologies are not high enough for cross-hybridization with nucleic acid probes. The consensus amino acid sequence for the members of the α1,3Fuc-T family, YYFX(L/V)XPEN(ST)-XXXXXYTEK, which was recently found on comparison of the mammalian α1,3Fuc-Ts and the α1,3Fuc-Ts of H. pylori (15, 16), is conserved in the mFuc-TIX sequence (Fig. 1A), except that the first tyrosine in the consensus sequence is replaced by cysteine in mFuc-TIX.

A phylogenetic tree of the α1,3Fuc-T family including mFuc-TIX was constructed by the neighbor-joining method (61) based on the amino acid sequences (Fig. 2). Regarding the α1,3Fuc-Ts other than mFuc-TIX, we obtained the same tree as established by Oulmouden et al. (9), in which they proposed the futh gene is an orthologous homologue of an ancestral gene, from which originated the present hFuc-TV-hFuc-THI-hFuc-TVII cluster. The position of mFuc-TIX in the tree is quite distant from those of the known α1,3Fuc-Ts. Thus, mFuc-TIX does not belong to any of the subfamilies of α1,3Fuc-Ts, the genes of which have been cloned.
were determined to exhibit very weak activity as to sLea expression, whereas hFuc-TIV and mFuc-TIV were consistent with those of previous studies by us (18) and others (5, 11), in which hFuc-TVI was determined to exhibit a very strong activity as to Lea synthesis, whereas mFuc-TIX and mFuc-TIV were determined to exhibit very weak activity as to sLea synthesis. Thus, hFuc-TIVA and mFuc-TIX were determined to obtain the relative activities of the four enzymes.

Results indicated that the mFuc-TIX gene can synthesize Lea and Lea' epitopes, but not sLea, and thus it is categorized as a member of the α1,3Fuc-T family. Namalwa cells are not suitable host cells for detection of Lea and sLea epitopes, because they do not express these epitopes when transfected with the hFuc-TIII gene (7). Therefore, we could not determine whether or not the mFuc-TIX gene exhibits the ability of mFuc-TIX on flow cytometry analysis involving Namalwa cells.

COS-1 cells are known to be appropriate host cells having the intracellular acceptor substrates of the precursor structures for both type 1- and type 2-Lewis antigens (11, 63). The hFuc-TIII cDNA was introduced into COS-1 cells as a positive control for these epitopes when transfected with the hFuc-TIII gene (7).

Therefore, we could not determine whether or not the mFuc-TIX gene was expressed in Namalwa cells. The distribution pattern of mFuc-TIX transcripts was consistent with the result of Gersten et al. (11), i.e., they detected mFuc-TIV transcripts in various mouse tissues on Northern blot analysis. mFuc-TIV transcripts were abundantly detected in stomach, colon, and epididymis and at intermediate levels in uterus, ovary, testis, spleen, lung, and kidney in this study and by others (11). mFuc-TIV was not expressed in brain, thymus, liver, or heart, even though it could be detected with this sensitive method, competitive RT-PCR. In contrast, mFuc-TIX transcripts were highly expressed in brain and much more abundantly in kidney. Substantial amounts of mFuc-TIX transcripts were detected in some other tissues, such as stomach, colon, uterus, and epididymis. With this sensitive method, we did not detect any mFuc-TIX transcripts in thymus, liver, spleen, ovary, lung, heart, testis, or small intestine. The tissue distribution of mFuc-TIX transcripts was determined to be quite different from that of mFuc-TIV transcripts.

Northern blot analysis demonstrated that the mFuc-TIX transcripts were detectable in brain and kidney as two positive bands, corresponding to approximately 5.5- and 3.0-kbs (Fig. 5). No bands were detected for the other 13 tissues of an adult mouse on Northern blot analysis (data not shown). The results of Northern blot analysis corresponded to those of the competitive RT-PCR.

**Tissue Distribution and Quantitative Measurement of mFuc-TIX Transcripts**—The amounts of the transcripts for the mFuc-TIX gene were determined in a variety of mouse tissues by competitive RT-PCR. The expression levels of mFuc-TIX transcripts were also measured as controls. As summarized in Fig. 4, the distribution pattern of mFuc-TIX transcripts was consistent with the result of Gersten et al. (11), i.e., they detected mFuc-TIV transcripts in various mouse tissues on Northern blot analysis. mFuc-TIV transcripts were abundantly detected in stomach, colon, and epididymis and at intermediate levels in uterus, ovary, testis, spleen, lung, and kidney in this study and by others (11). mFuc-TIV was not expressed in brain, thymus, liver, or heart, even though it could be detected with this sensitive method, competitive RT-PCR. In contrast, mFuc-TIX transcripts were highly expressed in brain and much more abundantly in kidney. Substantial amounts of mFuc-TIX transcripts were detected in some other tissues, such as stomach, colon, uterus, and epididymis. With this sensitive method, we did not detect any mFuc-TIX transcripts in thymus, liver, spleen, ovary, lung, heart, testis, or small intestine. The tissue distribution of mFuc-TIX transcripts was determined to be quite different from that of mFuc-TIV transcripts.

**Measurement of α1,3Fuc-T Activity toward Oligosaccharides**—The acceptor specificity of mFuc-TIX expressed in COS-1 cells toward pyridylaminated oligosaccharide substrates was analyzed. The activities of mFuc-TIV, hFuc-TIII, and hFuc-TVI expressed transiently in COS-1 cells were also determined to obtain the relative activities of the four enzymes. Lysates of COS-1 cells expressing mFuc-TIX exhibited significant α1,3Fuc-T activity toward LNnT-PA compared with those of mock-transfected COS-1 cells but exhibited no activity toward either LNT-PA or α2,3-sialyl-LNnT-PA (Table III). The relative activities of mFuc-TIX as to Lea synthesis toward LNnT-PA in comparison with those of mFuc-TIV and hFuc-TVI were 1.62 and 1.31, respectively. mFuc-TIX was demonstrated to encode an α1,3Fuc-T, the substrate specificity of which is similar to that of Fuc-TIV.

**Flow Cytometry Analysis**—The mFuc-TIX gene in the pAMo vector was named pAMo-mFT9. We transfected Namalwa cells with the purified pAMo-mFT9 DNA and obtained a stable transformant, which was named Namalwa-mFT9 cells. On flow cytometry analysis, Namalwa-mFT9 cells showed positive profiles for both the Lea and Lea' epitopes (Fig. 3A). The intensities of the Lea' and Lea'-positive peaks of Namalwa-mFT9 cells were almost equal to those of Namalwa-mFT4 and Namalwa-hFT6 cells. Regarding the sLea expression in Fig. 3A, mock-transfected Namalwa cells (Namalwa-Mo cells) showed a positive peak stained with KM-93 (anti-sLea). Namalwa-hFT6 cells apparently showed a sLea'-positive peak with stronger intensity than that in the case of Namalwa-Mo cells, whereas the intensities of the sLea'-positive peaks of Namalwa-mFT9 and Namalwa-mFT4 cells did not differ from Namalwa-Mo cells.

The results for Namalwa-hFT6 and Namalwa-mFT4 cells are consistent with those of previous studies by us (18) and others (5, 11), in which hFuc-TVI was determined to exhibit a very strong activity as to sLea synthesis, whereas hFuc-TIV and mFuc-TIV were determined to exhibit very weak activity as to sLea synthesis in the cells. The staining profile of Namalwa-mFT9 cells was similar to that of Namalwa-mFT4 cells but different from that of Namalwa-hFT6 cells in terms of sLea expression. These results indicated that mFuc-TIX can synthesize Lea and Lea', but not sLea, and thus it is categorized as a member of the α1,3Fuc-T family.

Namalwa cells are not suitable host cells for detection of Lea and sLea epitopes, because they do not express these epitopes when transfected with the hFuc-TIII gene (7). Therefore, we could not determine whether or not the mFuc-TIX gene was expressed in Namalwa cells. The distribution pattern of mFuc-TIX transcripts was consistent with the result of Gersten et al. (11), i.e., they detected mFuc-TIV transcripts in various mouse tissues on Northern blot analysis. mFuc-TIV transcripts were abundantly detected in stomach, colon, and epididymis and at intermediate levels in uterus, ovary, testis, spleen, lung, and kidney in this study and by others (11). mFuc-TIV was not expressed in brain, thymus, liver, or heart, even though it could be detected with this sensitive method, competitive RT-PCR. In contrast, mFuc-TIX transcripts were highly expressed in brain and much more abundantly in kidney. Substantial amounts of mFuc-TIX transcripts were detected in some other tissues, such as stomach, colon, uterus, and epididymis. With this sensitive method, we did not detect any mFuc-TIX transcripts in thymus, liver, spleen, ovary, lung, heart, testis, or small intestine. The tissue distribution of mFuc-TIX transcripts was determined to be quite different from that of mFuc-TIV transcripts.

**TABLE II**

| Species | Gene | DNA (ORF) | Amino acids | Homology (AA) |
|---------|------|-----------|-------------|--------------|
| Human   | Fuc-TIII | 1086     | 361         | 43.0         |
|         | Fuc-TIV  | 1218     | 405         | 47.5         |
|         | Fuc-TV   | 1125     | 374         | 43.0         |
|         | Fuc-TVII  | 1080    | 359         | 42.6         |
|         | Fuc-TVIII | 1029    | 342         | 42.1         |
| Mouse   | Fuc-TIV   | 1302     | 433         | 48.4         |
|         | Fuc-TVII  | 1170     | 389         | 39.1         |
|         | Fuc-TIX   | 1080     | 359         | 42.1         |
| Bovine  | futh     | 1098     | 365         | 41.9         |
| Rat     | Fuc-TIV   | 1302     | 433         | 48.4         |
| Chick   | CFTI     | 1071     | 356         | 50.5         |
| Helicobacter | fucT1 | 1035     | 345         | 28.3         |
|         | fucT2     | 999      | 333         | 28.4         |

**FIG. 2. A phylogenetic tree of α1,3-fucosyltransferases constructed by the neighbor-joining method (61).** A phylogenetic tree was constructed based on the amino acid sequences. The branch lengths indicate the evolutionary distances between different sequences.
The expression of Lewis antigens on the surface of Namalwa-mFT9, -mFT4, or -hFT6 cells transfected stably with the mFuc-TIX, mFuc-TIV, or hFuc-TVI gene, respectively, and COS-1 cells transfected with the mFuc-TIX or hFuc-TIII gene was analyzed by flow cytometry. The shaded peak in each panel represents the results for mock-transfected Namalwa or COS-1 cells stained with the respective antibody. The panels in A show the three stable transformants of Namalwa cells that were stained with PM-81 (anti-Lex), 73-30 (anti-Le\(^\alpha\)), KM-93 (anti-sLe\(^x\)), and AH-6 (anti-Le\(^\beta\)). The panels in B show the 7LE (anti-Le\(^\alpha\)) staining of COS-1 cells that were transiently transfected with the mFuc-TIX or hFuc-TIII gene.

**DISCUSSION**

The synthesis of the Le\(^\alpha\) epitope is terminated on the addition of Fuc to the GlcNAc residue of the type 2 chain, Gal\(\beta\)1,4GlcNAc-R, with an α,1,3-linkage. The expression of Le\(^\alpha\) antigens on the O- and N-glycans of glycoproteins and glycolipids has been found in a variety of human and rodent tissues. On *in vitro* assaying to determine the substrate specificities of the α1,3Fuc-Ts using the recombinant enzymes, four of them, Fuc-TIII, -IV, -V and -VI, were demonstrated to be able to synthesize the Le\(^\alpha\) epitope. In the present study, we found and cloned a novel α1,3Fuc-T, mFuc-TIX, that can synthesize the Le\(^\alpha\) epitope. mFuc-TIX was demonstrated to have the following characteristics. 1) mFuc-TIX is quite distant from the previously cloned α1,3Fuc-Ts in the phylogenetic tree. 2) Its substrate specificities are similar to, but clearly distinct from, those of Fuc-TIV, *i.e.*, Fuc-TIV is able to synthesize the sLe\(^x\) epitope at low efficiency, whereas Fuc-TIX exhibits no ability to make the sLe\(^x\) epitope. 3) The GC content of the nucleotide sequence of mFuc-TIX cDNA is very low (41.9%), whereas those of the other α1,3Fuc-Ts are GC-rich, *i.e.*, mFuc-TIV (63.7%), mFuc-TII (60.1%), and hFuc-TIII (63.1%). This indicated that the chromosomal localization of the mFuc-TIX gene is distinct from those of the others. 4) The tissue distribution of the mFuc-TIX transcript strongly indicated that this gene is responsible for the Le\(^\alpha\) expression in the mouse CNS.

A previous immunohistochemical study revealed the detailed distribution of the Le\(^\alpha\) epitope within an adult mouse brain. In *in situ* hybridization was performed using two antisense probes derived from the ORF sequence of the mFuc-TIX gene to localize the mFuc-TIX transcript in adult mouse brain tissue. Two sense probes corresponding to the respective antisense probes were used for *in situ* hybridization as negative controls. Fig. 7A shows that the antisense probe apparently gave positive signals of the mFuc-TIX transcript in the cell bodies of neurons in the hippocampal region but not in any glial cells. Fig. 7B shows the negative staining on *in situ* hybridization with the sense probe as a negative control.

**Figure 3. Flow cytometry analysis of Namalwa-mFT9, -mFT4, and -hFT6 cells and COS-1 cells transfected with the mFuc-TIX or hFuc-TIII gene.**

**Table III**

Incorporation of fucose into pyridylaminated oligosaccharides by cell extracts of COS-1 transfectants with four α1,3fucosyltransferase genes

| Substrate | mFuc-TIX | mFuc-TIV | hFuc-TIII | hFuc-TVI |
|-----------|---------|---------|---------|---------|
| LNT       | 0       | 0       | 123.3   | 0       |
| LNaT      | 83.8    | 51.6    | 32.3    | 63.7    |
| α2,3-Sialyl LNaT | 0.4   | 16.4    | 100     |         |

\(^a\) The levels of each of the four fucosyltransferase activities were normalized as to the amounts of transcripts determined by competitive RT-PCR.

**Alignment of the Amino Acid Sequences Conserved in the α1,3Fuc-Ts of Various Species—**Fig. 8 shows a comparison of the putative peptide sequences of representative α1,3Fuc-Ts of four species, *i.e.*, mouse, human, bovine, and chicken. As indicated by asterisks, 80 amino acid residues are conserved in all sequences. Among them, four cysteine residues are common to all sequences. Cys\(^\text{143}\) of hFuc-TIII, an N-ethylmaleimide-sensitive site (64), is replaced by threonine in mFuc-TIX, as in mFuc-TIV and mFuc-TVII. The length of the stem region of mFuc-TIX is similar to those of the other α1,3Fuc-Ts, except for that of mFuc-TIV, which is longer by an extra 33 amino acids.
two phases during fetal CNS development in mouse. The CD15 epitope appears initially at E10 of mouse embryos in the ventricular surface of the hippocampal primordium and in the early hippocampal marginal zone. The CD15 expression in the second phase appears in astrocytes around birth, and the positive staining of astrocytes persists in the adult CNS. In later postnatal life, the anti-CD15 antibody stained the astrocytes more predominantly than the neurons in the hippocampal region (36). In an adult human brain, the astrocytes are more strongly stained with the anti-CD15 antibody than the neurons (43). The present in situ hybridization experiment demonstrated that mFuc-TIX is responsible for the expression of the Le\(^x\) epitope in the neurons in the mouse CNS, as reflected by

![Quantitative analysis of transcripts of mFuc-TIX, mFuc-TIV, and mβ-actin in various mouse tissues by competitive RT-PCR.](image)

![Northern blot analysis](image)

![Genomic Southern blot analysis](image)
the positive signals in the hippocampal region. However, we did not observe positive signals of the mFuc-TIX transcript in astrocytes on in situ hybridization. This could lead to the interesting interpretation that α1,3Fuc-Ts responsible for the Le\(^a\) synthesis in the CNS are differentially utilized in neurons and astrocytes.

CD15 is one of the differentiation markers of cells in the CNS useful for the pathological diagnosis of brain tumors. The expression of the CD15-reactive Le\(^a\) epitope has been immunohistochemically examined in a variety of brain tumor tissues (46). A human Fuc-TIX (hFuc-TIX) gene corresponding to the mFuc-TIX gene will be a useful tool for determining whether or not the Le\(^a\) (CD15) epitope expression in human brain tumor tissues is regulated through expression of the hFuc-TIX gene.

There have been some studies on the expression levels of each α1,3Fuc-T transcript in various tissues. Gersten et al. (11) described in detail the tissue distribution of the mFuc-TIV transcript in various mouse tissues observed on Northern analysis and in situ hybridization. The mFuc-TIV transcript is most abundant in stomach and colon, and substantial amounts are also present in the lung, testis, uterus, and small intestine, but it was not detected in brain on Northern analysis. The distribution of the mFuc-TIV transcript in the cells of these tissues was also confirmed by in situ hybridization (11). The expression levels of the mFuc-TIV transcript in various mouse tissues examined in the present study as controls, which were determined by competitive RT-PCR, were well consistent with the results of Gersten et al. (11). Expression of three human α1,3Fuc-Ts, i.e., hFuc-TIII, hFuc-TV, and hFuc-TVI, in some human normal tissues has been examined by Northern analysis and RT-PCR (65). In studies comparing the expression levels of each α1,3Fuc-T in normal and cancer tissues, human normal colon epithelial cells expressed the hFuc-TIII transcript at the highest level, followed by the levels of expression of hFuc-TIV and hFuc-TVI (66), and human normal lung tissues also expressed the hFuc-TIII transcript most abundantly, followed by the hFuc-TIV and hFuc-TVI transcripts (data not shown). However, there has been no study as to which α1,3Fuc-T is expressed in the human CNS and is responsible for the Le\(^a\) and sLe\(^a\) expression in the CNS.

The tissue distribution of the mFuc-TIX transcript determined in the present study is quite distinct from those of the other α1,3Fuc-Ts. Among the mouse tissues examined in the present study, kidney showed the highest level of Fuc-TIX transcript expression, of which the level was much higher than that of the mFuc-TIV transcript. We also detected the mFuc-TIX transcript in stomach, colon, and uterus, where the levels were comparable to those of the mFuc-TIV transcript. Considering the relative activity of Le\(^a\) synthesis determined in the present study, both enzymes, i.e., mFuc-TIV and mFuc-TIX, may participate redundantly in the Le\(^a\) synthesis in the tissue, in which both are expressed. In the future, experiments involving in situ hybridization with two probes of mFuc-TIV and mFuc-TIX will reveal whether they are co-localized in the same cells or differentially expressed in cells of the same tissues.

The peptide sequence, YX\(\text{F}(L/V)XFEN(S/T)X\)XXYXTEK,

![Fig. 7. In situ hybridization analysis of mFuc-TIX transcripts in mouse brain. In situ hybridization was performed using two antisense probes encompassing segments 506–663 and 693–855, as described under “Experimental Procedures.” Both antisense probes gave the same results. A, shows the positive signals detected with the antisense probe of segment 693–855, and B shows the negative signals with the sense probe of segment 693–855.](image-url)
Novel α,1,3-Fucosyltransferase Expressed in Brain and Kidney

Multiple conserved peptide sequences for the α,1,3Fuc-T family, which are conserved in all α,1,3Fuc-Ts from bacteria to mammals, have been proposed to be essential for α,1,3Fuc-Ts of higher eukaryotes. In this sense, the mFuc-TIX peptide sequence, not being so highly homologous to those of the above five α,1,3Fuc-Ts, is very informative as to the discovery of conserved peptide sequences for the α,1,3Fuc-Ts of higher eukaryotes. On comparison of the mFuc-TIX protein sequence with those of the other mammalian and chicken α,1,3Fuc-Ts (Fig. 8), we found several peptide sequences which are conserved in all α,1,3Fuc-Ts. Four cysteine residues are conserved in all α,1,3Fuc-Ts, which suggested that they are essential for maintenance of the tertiary structures of the α,1,3Fuc-Ts.

Many immunohistochemical studies on CD15 (Lea) expression in the CNS have suggested a biological role of the Lea epitope in the CNS (32–36, 42, 43, 46–48). In the future, mFuc-TIX will be a very useful tool for investigating whether the Lea epitope plays an important role in neuronal development or not.

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Fig. 8. Peptide sequence alignment of mFuc-TIX with other α,1,3Fuc-Ts. The aligned sequences are of three mouse α,1,3Fuc-Ts, mFuc-TIX, mFuc-TIV, and mFuc-TVII, one human α,1,3Fuc-T, hFuc-TI, one bovine α,1,3Fuc-T, fubt, and one chicken α,1,3Fuc-T, CPT-I. The putative transmembrane domains are underlined. Amino acid residues conserved in all sequences are indicated by asterisks. The amino acid residues conserved in the consensus peptide sequence of α,1,3Fuc-Ts from bacteria to mammals are boxed. An arrowhead indicates the Cys residues in hFuc-TIII which is sensitive to iodoacetamide.
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