

# $\alpha$ 1,3-Fucosyltransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human Fuc-TIX

Mika Kaneko<sup>a,b</sup>, Takashi Kudo<sup>a</sup>, Hiroko Iwasaki<sup>a</sup>, Yuzuru Ikehara<sup>a</sup>, Shoko Nishihara<sup>a</sup>, Satoshi Nakagawa<sup>c</sup>, Katsutoshi Sasaki<sup>c</sup>, Takashi Shiina<sup>d</sup>, Hidetoshi Inoko<sup>d</sup>, Naruya Saitou<sup>b</sup>, Hisashi Narimatsu<sup>a,\*</sup>

<sup>a</sup>Division of Cell Biology, Institute of Life Science, Soka University, 1-236 Tangi-cho, Hachioji-shi, Tokyo 192-8577, Japan

<sup>b</sup>Department of Genetics, School of Life Science, Graduate University for Advanced Studies, National Institute of Genetics, 1111 Yata, Mishima-shi, Shizuoka 411-8540, Japan

<sup>c</sup>Tokyo Research Laboratories, Kyowa Hakko Kogyo Company, Limited, 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, Japan

<sup>d</sup>Second Department of Molecular Life Science, School of Medicine, Tokai University, Isehara-shi, Kanagawa 259-1193, Japan

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**Abstract** The amino acid sequence of Fuc-TIX is very highly conserved between mouse and human. The number of non-synonymous nucleotide substitutions of the *Fuc-TIX* gene between human and mouse was strikingly low, and almost equivalent to that of the  $\alpha$ -actin gene. This indicates that Fuc-TIX is under a strong selective pressure of preservation during evolution. The human Fuc-TIX (hFuc-TIX) showed a unique characteristics, i.e. hFuc-TIX was not activated by  $Mn^{2+}$  and  $Co^{2+}$ , whereas hFuc-TIV and hFuc-TVI were activated by the cations. The hFuc-TIX transcripts were abundantly expressed in brain and stomach, and interestingly were detected in spleen and peripheral blood leukocytes.

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**Key words:** Glycosyltransferase; Fucosyltransferase;  $\alpha$ 1,3-Fucosyltransferase; Lewis x; CD15; SSEA-1

## 1. Introduction

The Lewis x ( $Le^x$ ) carbohydrate epitope, which is defined as  $Gal\beta 1,4(Fuc\alpha 1,3)GlcNAc$ , is synthesized by transferring a fucose (Fuc) to the *N*-acetylglucosamine (GlcNAc) residue of the type 2 chain,  $Gal\beta 1,4GlcNAc$ , with an  $\alpha$ 1,3-linkage. This fucose transfer is catalyzed by  $\alpha$ 1,3-fucosyltransferase ( $\alpha$ 1,3Fuc-T). So far, the human genes encoding five  $\alpha$ 1,3Fuc-Ts (Fuc-TIII, IV, V, VI and VII or FUT3, 4, 5, 6 and 7) have been cloned [1–6]. In a previous paper, we reported the molecular cloning of a new member of the  $\alpha$ 1,3Fuc-T family in the mouse and named the enzyme mouse Fuc-TIX (mFuc-TIX or mFUT9) [7].

CD15 is one of the differentiation markers of the cells, and the CD15 epitope has been determined as the  $Le^x$  carbohydrate structure. Several immunohistochemical studies have de-

tected CD15 antigens in certain neuronal cells and glial cells in the central nervous system (CNS) of humans and rodents [8–10]. The expression of CD15 antigens in the CNS is developmentally regulated, and considered to play a role in neuronal development. In our previous study, mFuc-TIX was identified as the most likely candidate for the enzyme synthesizing the  $Le^x$  structure (CD15 epitope) in the mouse CNS [7].

Some  $\alpha$ 1,3Fuc-Ts are known to be polymorphic in human. Three kinds of null alleles of the *Fuc-TIII* (*Le*) gene, named *le1*, *le2* and *le3* alleles, are widely distributed in the Japanese population [11–13]. Homozygotes of null *Fuc-TVI* alleles were also found in some populations [14]. In bovine, there is only one gene, named *futb*, corresponding to the *hFuc-TV-hFuc-TIII-hFuc-TVI* gene cluster [15]. In addition, the mouse gene orthologous to the *futb* gene seems to be a pseudogene [16]. In this sense, the *hFuc-TIII*, *hFuc-TV* and *hFuc-TVI* genes are regarded as non-essential for individual ontogeny, and therefore their sequences are not conserved among species.

Mice and humans possess functional *Fuc-TIV* and *Fuc-TVII* genes [3–6,16,17]. The distribution of Fuc-TVII is limited to specific tissues and cell populations such as leukocytes and high endothelial cells of the venule [18]. Fuc-TVII apparently participates in the synthesis of sialyl Lewis x ( $sLe^x$ ) epitopes in leukocytes: this epitope is required for leukocyte trafficking [18]. However, Fuc-TVII cannot synthesize the CD15 ( $Le^x$ ) epitope [4,5,19]. Fuc-TIV is ubiquitously expressed in various tissues, and probably participates in CD15 epitope synthesis in some tissues, but its biological function remains to be elucidated [16].

In this study, we cloned a human gene, named *hFuc-TIX*, orthologous to the *mFuc-TIX* gene, and analyzed its characteristics and tissue distribution. Interestingly, the nucleotide and amino acid sequences of hFuc-TIX were very highly conserved in comparison with those of mFuc-TIX [7]. This indicates that Fuc-TIX has been under a strong selective pressure during its evolution, suggesting that it is essential for ontogeny.

## 2. Materials and methods

### 2.1. cDNA cloning and sequencing of the *hFuc-TIX* gene

A cDNA library derived from a human gastric mucosa was constructed in our laboratory (Ikehara et al., unpublished). We screened the human stomach cDNA library with a mFuc-TIX cDNA probe

\*Corresponding author. Fax: (81) (426) 91-9315.  
E-mail: hisashi@scc1.t.soka.ac.jp

**Abbreviations:** Fuc-T, fucosyltransferase;  $Le^x$ , Lewis x; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; CNS, central nervous system;  $sLe^x$ , sialyl Lewis x; ORF, open reading frame; PA, pyridylamination; RT-PCR, reverse transcription-polymerase chain reaction;  $Le^y$ , Lewis y; LNT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; sialyl-LNT,  $\alpha$ 2,3-sialyl lacto-*N*-tetraose;  $Le^a$ , Lewis a; PBL, peripheral blood leukocytes

encompassing the full-length open reading frame (ORF), and obtained several clones encoding the *hFuc-TIX* gene. The inserts were subjected to nucleotide sequencing.

## 2.2. Calculation of the numbers of synonymous and non-synonymous nucleotide substitutions

To calculate the numbers of synonymous and non-synonymous nucleotide substitutions in *Fuc-T* genes between mouse (or other species) and human, we employed the ODEN program [20] of Supernig at the National Institute of Genetics, Japan.

## 2.3. Flow cytometric analysis of Namalwa cells stably expressing the *hFuc-TIX* gene and assaying of $\alpha 1,3$ Fuc-T activity

Namalwa cells stably expressing the *hFuc-TIX* gene were established, and subjected to flow cytometry analysis, using SSEA-1 (anti-Le<sup>x</sup>; Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA, USA) [21], AH6 (anti-Lewis y (Le<sup>y</sup>)) [22] and CSLEX-1 (anti-sLe<sup>x</sup>) [23] and assaying of  $\alpha 1,3$ Fuc-T activity using pyridylaminated (PA) oligosaccharides, as described previously [7].

## 2.4. Quantitative analysis of $\alpha 1,3$ Fuc-T transcripts in various human tissues using the competitive reverse transcription-polymerase chain reaction (competitive RT-PCR) method

The competitive RT-PCR methods for the measurement of the transcripts for the *hFuc-T III, IV, V, VI, VII* genes have been described in detail in our previous paper [24]. To quantify the *hFuc-TIX* transcripts by competitive RT-PCR, a competitor DNA plasmid (pBS-hFT9d) was prepared by deleting the 113-bp *Nhe*-*Nsi*I fragment from the standard DNA (pBS-hFT9s) which encodes the full-length ORF of the *hFuc-TIX* gene. A pair of PCR primers (forward primer, 5'-GGCAAGCATTGGGAGAATATGTCAAT-3' and reverse primer, 5'-ACCAACAGACTTATATTCTTGATGCC-3') amplified 398-bp and 285-bp fragments from pBS-hFT9s and pBS-hFT9d, respectively. cDNA samples were prepared from various human tissues by the method described previously [7,24]. The PCR conditions for the *hFuc-TIX* gene were as follows: a pre-PCR heating step at 95°C for 11 min, followed by 45 cycles of 1 min at 95°C, 1.5 min at 65°C, and 2 min at 72°C. To ascertain the efficiency of cDNA preparation from total RNAs, measurement of the  $\beta$ -actin transcripts in each sample was performed.

## 2.5. In situ hybridization and immunohistochemical analyses

Surgically resected stomach specimens for in situ hybridization were prepared as described previously [7]. The antisense and sense RNA probes, at the nucleotide positions 655–835 of *hFuc-TIX* cDNA, labeled with digoxigenin were synthesized using a Dig RNA Labeling Kit (Boehringer Mannheim) according to the supplier's manual. The hybridization and signal detection procedures were performed as described previously [7].

Immunohistochemistry was performed with monoclonal antibodies, PM-81 (anti-Le<sup>x</sup>) [25] and AH6 (anti-Le<sup>y</sup>), by the method described previously [26].

## 2.6. Construction of a phylogenetic tree of the $\alpha 1,3$ Fuc-T family

We used amino acid sequences for six human [1–6], three mouse [7,16,17], one rat [27], one bovine [15], one chicken [28], two zebrafish [29] and four *Caenorhabditis elegans* [30] genes. These 18 sequences were multiply aligned, and all positions with gaps were eliminated, resulting in the alignment of 356 amino acid residues. Clustal W [31] was used for multiple alignment and the neighbor-joining method [32] was used to construct a tree. We also performed a bootstrap analysis to gain a statistical measure of confidence in the phylogenetic tree. By bootstrap analysis, a total of 1000 trees were generated from the initial data set, and the percentage of trees containing a particular clade was measured.

## 3. Results

### 3.1. A putative amino acid sequence of *hFuc-TIX* deduced from the cDNA sequence which is highly conserved between mouse and human

The 3019-bp nucleotide sequence of the *hFuc-TIX* cDNA was registered by us in DDBJ/EMBL/GenBank with the ac-

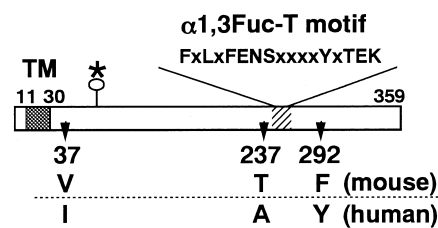


Fig. 1. A schematic diagram of the primary structure of *hFuc-TIX*. Three amino acid substitutions between *hFuc-TIX* and *mFuc-TIX* are presented at positions 37, 237 and 292. A putative transmembrane domain is indicated with a dark shaded box. The amino acid sequence and position of the  $\alpha 1,3$ Fuc-T motif conserved in all  $\alpha 1,3$ Fuc-Ts, including those of *Helicobacter pylori* [33], is presented. A possible N-glycosylation site is indicated with an asterisk.

cession number AB023021. The putative amino acid sequence predicted from the nucleotide sequence encodes a typical type II membrane protein, and is very similar to that of *mFuc-TIX*, i.e. only three amino acid residues, Val-37 to Ile, Thr-237 to Ala and Phe-292 to Tyr, differ among 359 amino acids (Fig. 1). The consensus amino acid sequence for the members of the  $\alpha 1,3$ Fuc-T family [33], FxL/VxFENS/TxxxxYxTEK, is conserved in the *hFuc-TIX* sequence.

### 3.2. Synonymous and non-synonymous substitutions of the members of the *Fuc-T* gene family

We calculated the numbers of synonymous ( $d_S$ ) and non-synonymous ( $d_N$ ) nucleotide substitutions between *mFuc-TIX* and *hFuc-TIX* genes to establish if there is some significant evolutionary pressure for maintaining the primary structure of the enzyme ( $d_S$ : number of substitutions per site which cause no amino acid replacement;  $d_N$ : number of substitutions per site which cause amino acid replacement) (Table 1). The other *Fuc-T* genes, *H (FUT1)*, *Fuc-TIII (FUT3)*, *Fuc-TIV (FUT4)*, *Fuc-TVII (FUT7)* and *FUT8* genes, were also analyzed to determine their  $d_S$  and  $d_N$  values (Table 1). The mouse *Fuc-TIII* and *FUT8* genes have not been cloned, therefore the bovine orthologue (*futb*) for *hFuc-TIII* gene and the porcine *FUT8* gene were compared with the respective human genes. We considered that it was essentially not a problem to use different species to calculate  $d_S$  and  $d_N$ , because they started to diverge more or less at the same time. The *Fuc-TIX* gene showed the lowest  $d_N$  value (0.005), followed by the *FUT8* gene (0.021), among the six members of the *Fuc-T* gene family. The difference in  $d_N$  values between the *Fuc-TIX* gene and the others was almost 40-fold. On the other hand, the  $d_S$

Table 1  
Synonymous ( $d_S$ ) and non-synonymous ( $d_N$ ) substitutions per site of  $\alpha 1,3$ Fuc-T genes

Gene	L <sup>a</sup>	$d_S$	$d_N$	Species pair
<i>H (FUT1)</i>	368	0.938	0.148	mouse vs. human
<i>Fuc-TIII (FUT3)</i>	366	0.516	0.218	bovine vs. human
<i>Fuc-TIV (FUT4)</i>	408	0.734	0.159	mouse vs. human
<i>Fuc-TVII (FUT7)</i>	343	0.950	0.111	mouse vs. human
<i>FUT8</i>	576	0.331	0.021	porcine vs. human
<i>Fuc-TIX (FUT9)</i>	360	0.527	0.005	mouse vs. human
<i>histone H4<sup>b</sup></i>	101	0.228	0.001	mouse vs. human
$\alpha$ -actin <sup>b</sup>	376	0.587	0.002	mouse vs. human

<sup>a</sup>Number of codons compared.

<sup>b</sup>Li et al. [40].

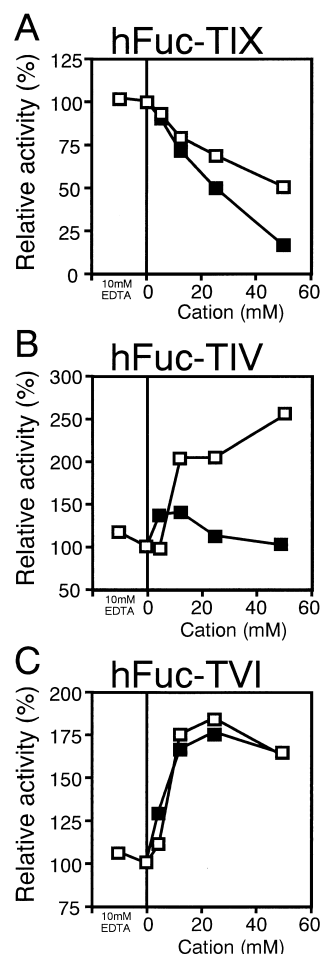


Fig. 2. Activation of hFuc-TIX, hFuc-TIV and hFuc-TVI by  $Mn^{2+}$  or  $Co^{2+}$ . The activities toward LNnT-PA were measured at various concentrations of  $Mn^{2+}$  ( $\square$ ) or  $Co^{2+}$  ( $\blacksquare$ ). The activity in the absence of the dications is presented as 100% activity.

values for *Fuc-TIX* gene (0.527) and the others (0.331–0.950) were similar.

### 3.3. Flow cytometry analysis of Namalwa cells transfected with the *hFuc-TIX* gene and measurement of $\alpha 1,3$ Fuc-T activity of *hFuc-TIX* towards oligosaccharides

Namalwa-hFT9 cells, stably expressing the *hFuc-TIX*

cDNA, were analyzed by flow cytometry. Namalwa-hFT9 cells showed a positive peak with SSEA-1 (anti- $Le^x$ ) and AH6 (anti- $Le^y$ ), but not with CSLEX-1 (anti- $Le^x$ ) (data not shown). In contrast, Namalwa-hFT4 cells (stable transformant with the *hFuc-TIV* gene) showed positive peaks with all three antibodies, SSEA-1, AH6 and CSLEX-1, although the positive peak with CSLEX-1 was very weak (data not shown).

The  $\alpha 1,3$ Fuc-T activities of hFuc-TIX were measured against PA oligosaccharides, i.e. lacto-*N*-neotetraose-PA (LNnT-PA), lacto-*N*-tetraose-PA (LNT-PA) and  $\alpha 2,3$ -sialyl lacto-*N*-tetraose-PA (sialyl-LNnT-PA). hFuc-TIX exhibited activity against LNnT-PA, resulting in the synthesis of  $Le^x$  epitope, but no activity against LNT-PA or sialyl-LNnT-PA, resulting in no synthesis of the Lewis a ( $Le^a$ ) or s $Le^x$  epitope, respectively (data not shown).

Thus, hFuc-TIX showed almost the same results of flow cytometry analysis and  $\alpha 1,3$ Fuc-T activities as mFuc-TIX [7].

### 3.4. Activation of $\alpha 1,3$ Fuc-Ts in the presence of $Mn^{2+}$ or $Co^{2+}$

Three human  $\alpha 1,3$ Fuc-Ts, i.e. hFuc-TIX, hFuc-TIV and hFuc-TVI, were examined for activity against LNnT-PA in the presence of  $Mn^{2+}$  or  $Co^{2+}$  (Fig. 2). Interestingly, hFuc-TIX was not activated at any concentration of  $Mn^{2+}$  or  $Co^{2+}$ , moreover it was markedly suppressed by  $Mn^{2+}$  and  $Co^{2+}$  (Fig. 2A). In contrast, hFuc-TIV was markedly activated in the presence of  $Mn^{2+}$ , but less so in that of  $Co^{2+}$  (Fig. 2B). hFuc-TVI was also markedly activated by both  $Mn^{2+}$  and  $Co^{2+}$  (Fig. 2C).

### 3.5. Tissue distribution and quantitative measurement of six human $\alpha 1,3$ Fuc-T transcripts

The amounts of six human  $\alpha 1,3$ Fuc-T transcripts expressed in various tissues were measured by competitive RT-PCR (Table 2). hFuc-TIII was abundantly expressed in gastrointestinal tissues, i.e. stomach, jejunum and colon, and faintly expressed in spleen, lung, kidney and cervix uteri. hFuc-TIII was not detected in brain, liver, adrenal cortex or peripheral blood leukocytes (PBL). hFuc-TIV was found to be ubiquitously expressed in all tissues examined and it was abundantly expressed in cervix uteri. hFuc-TV was not expressed in any tissues examined, except for spleen in which it was faintly expressed. The tissue distribution of hFuc-TVI was similar to that of hFuc-TIII, except that hFuc-TVI was expressed positively in liver whereas hFuc-TIII was not. hFuc-TVI was much more abundant in kidney than hFuc-TIII. hFuc-

Table 2

Quantitative analysis of transcripts of six  $\alpha 1,3$ Fuc-T genes in various human tissues by competitive RT-PCR

	Expression levels of $\alpha 1,3$ Fuc-Ts relative to the level of $\beta$ -actin (%)					
	<i>Fuc-TIII</i>	<i>Fuc-TIV</i>	<i>Fuc-TV</i>	<i>Fuc-TVI</i>	<i>Fuc-TVII</i>	<i>Fuc-TIX</i>
Forebrain	< 0.001	0.58	< 0.001	0.01	< 0.001	0.53
Stomach (antrum)	2.42	1.83	< 0.001	0.27	< 0.001	0.73
Stomach (corpus)	1.87	1.07	< 0.001	0.06	< 0.001	0.37
Jejunum	13.66	2.33	< 0.001	0.86	< 0.001	< 0.001
Colon	2.90	0.43	< 0.001	0.41	< 0.001	< 0.001
Liver	< 0.001	0.39	< 0.001	0.14	< 0.001	< 0.001
Spleen	0.02	0.25	0.02	0.004	0.13	0.004
Lung	0.01	0.01	< 0.001	< 0.001	< 0.001	< 0.001
Kidney	0.34	1.13	< 0.001	3.18	< 0.001	< 0.001
Adrenal cortex	< 0.001	0.35	< 0.001	< 0.001	< 0.001	< 0.001
Cervix uteri	0.12	5.14	< 0.001	0.07	< 0.001	< 0.001
PBL	< 0.001	0.29	< 0.001	< 0.001	0.15	0.01

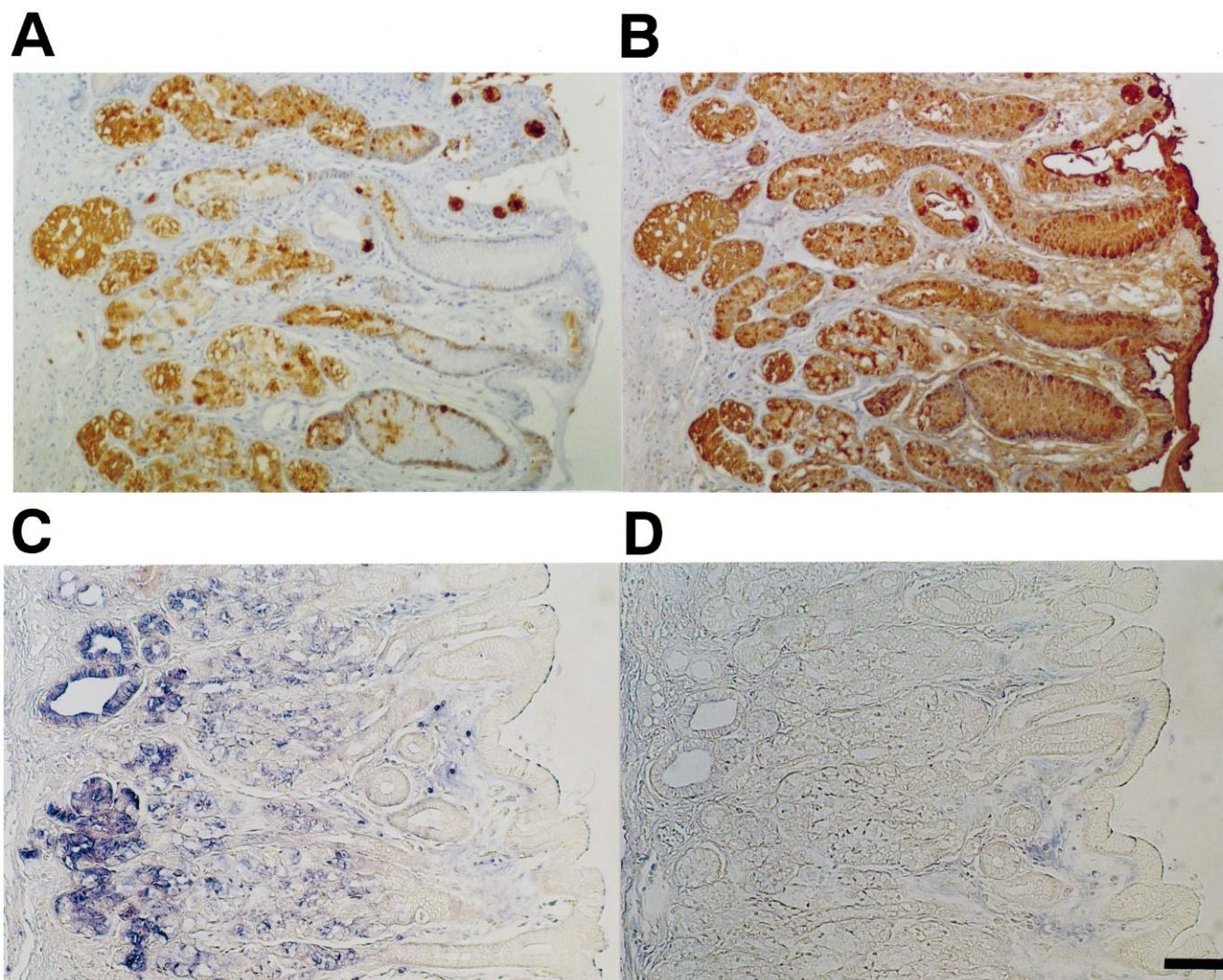


Fig. 3. Immunohistochemical staining with PM-81 (anti-Le<sup>x</sup>) and AH6 (anti-Le<sup>y</sup>) and in situ hybridization with the hFuc-TIX probe on the antrum portion of human gastric mucosa. Serial sections were immunohistochemically stained with PM-81 (A) and AH6 (B). C shows the positive signals detected with the antisense probe of segment 655–835, and D shows the negative signals with the sense probe of segment 655–835. The scale bar represents 10  $\mu$ m.

TVII was detected in spleen and PBL. hFuc-TIX was markedly expressed in forebrain and stomach, i.e. in both the antrum and corpus of the stomach, and, interestingly, was detected in spleen and PBL. hFuc-TIX was not expressed in kidney at all. In this regard, hFuc-TIX differs from mFuc-TIX which is abundantly expressed in mouse kidney [7].

### 3.6. Immunohistochemical analysis of Le<sup>x</sup> and Le<sup>y</sup> expression and in situ hybridization analysis of hFuc-TIX transcripts in the antrum portion of human gastric mucosa

A stomach specimen of a secretor individual, whose secretor (*Se*) genotype is *Se1/Se1* [26], was subjected to immunohistochemical analysis for Le<sup>x</sup> and Le<sup>y</sup> expression and in situ hybridization with the hFuc-TIX probe. As can be seen in Fig. 3A,B, the Le<sup>x</sup> antigens were mainly positive in the glandular compartments of the pyloric mucosa. In contrast, the Le<sup>y</sup> antigens were detected both in the glandular compartments and in the pit of the gastric mucosa. The hFuc-TIX transcripts were localized in the glandular compartments alone, being well co-localized with the Le<sup>x</sup> antigens (Fig. 3C).

### 3.7. A phylogenetic tree of the $\alpha$ 1,3Fuc-*T* gene family

We performed a FASTA search [34], and BLAST search [35] of the DDBJ/EMBL/GenBank International Nucleotide Sequence Database to identify the genes homologous to the hFuc-TIX gene. We used two specific search programs, tfastx and tblastn, which compare amino acid sequences with a DNA database. Mammalian, chicken, zebrafish, *C. elegans*, *Helicobacter pylori*, *Drosophila melanogaster*, and *Vibrio cholerae* genes were retrieved, while no members of the  $\alpha$ 1,2 or  $\alpha$ 1,6Fuc-*T* gene families were found to show homology with the hFuc-TIX gene.

The root was determined by assuming that *C. elegans* genes are located as outgroups (Fig. 4). The phylogenetic tree indicates that the Fuc-*T* genes evolved by independent gene duplication between vertebrates and *C. elegans*. There are four clusters in the vertebrate Fuc-*T* gene family, the Fuc-TIX gene subfamily being the first to diverge from the ancestral gene. Two zebrafish  $\alpha$ 1,3Fuc-Ts, zFT1 and zFT2, which are developmentally regulated in embryogenesis, form a cluster with mFuc-TIX and hFuc-TIX.



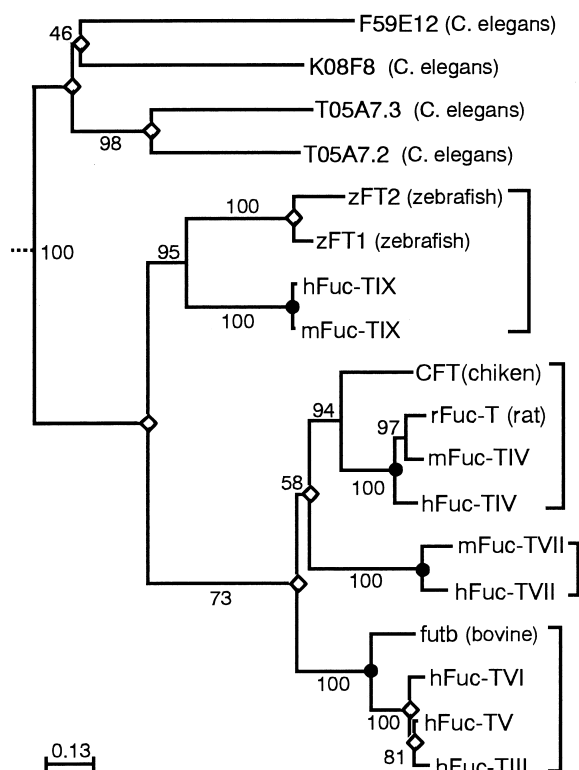


Fig. 4. Phylogenetic tree of  $\alpha 1,3$ Fuc-Ts. The branch lengths indicate the evolutionary distances between different genes. ◇ indicates the gene duplication and ● the speciation. Four cosmid clone names containing *C. elegans* genes are presented.

#### 4. Discussion

hFuc-TIX can synthesize the  $Le^x$  and  $Le^y$  epitopes, but not the  $Le^a$  or  $sLe^x$  epitopes, in contrast to hFuc-TIV's low, but apparently positive activity of  $sLe^x$  synthesis. These characteristics of hFuc-TIX were almost the same as those of mFuc-TIX. In the present study, we found that  $Mn^{2+}$  and  $Co^{2+}$  ions did not activate the hFuc-TIX activity at all.

Mollicone et al. reported that human brain homogenates exhibited activity for  $Le^x$  synthesis, but not for  $sLe^x$  synthesis [36,37]. The enzymes expressed in brain and leukocytes had similar levels of activity, i.e. both showed similar  $K_m$  values for GDP-fucose and H-type 2 compounds, the same optimum pH, and were resistant to *N*-ethylmaleimide. However, they differed in the enzyme activity when activated by dications. The enzyme(s) in leukocytes exhibited only 10–15% of the activity in the presence of  $Co^{2+}$  as compared to  $Mn^{2+}$ , whereas the activity of brain enzyme(s) did not differ in the presence of  $Mn^{2+}$  or  $Co^{2+}$  [36,37]. Foster et al. indicated that the enzyme purified from human neuroblastoma cells exhibited a level of activity in the presence of 10 mM  $Co^{2+}$  that was 75% of that in the presence of 10 mM  $Mn^{2+}$  [38]. The characteristics of hFuc-TIX determined in the present study were most similar to those of the enzyme(s) in adult brain [36]. However, there was some discrepancy between previous studies [36–38] and our study as to the characteristics of  $\alpha 1,3$ Fuc-T activities which can be explained as follows. As demonstrated in Table 2 in the present study, many tissues contain multiple  $\alpha 1,3$ Fuc-Ts. The previous studies measured the activities directed by mixed enzymes in tissue homogenates,

while we measured the activities of the recombinant enzymes. In tissue homogenates, there may still be unknown  $\alpha 1,3$ Fuc-Ts, the genes of which have not been cloned.

Cameron et al. [39] reported the tissue distribution of three hFuc-Ts, i.e. hFuc-TIII, V and VI, on Northern analysis and semi-quantitative RT-PCR. We employed the competitive RT-PCR method in the present study because of the following advantages. Competitive RT-PCR enabled us for the first time to compare all six  $\alpha 1,3$ Fuc-Ts in terms of the level of relative amounts of transcripts in various human tissues. The small amounts of transcripts, which could not be detected by Northern analysis, were quantitatively detected by this sensitive method. Fuc-TIV is believed to be a single enzyme capable of  $Le^x$  (CD15) synthesis in myeloid tissues. It is worth noting that hFuc-TIX was also expressed in spleen and PBL almost at the same level as hFuc-TIV. We will examine whether hFuc-TIX and hFuc-TIV are expressed in cells of different subpopulations in PBL or together in the same cells. hFuc-TIX was not expressed in human kidney at all, whereas mFuc-TIX was most abundantly expressed in mouse kidney as demonstrated in our previous study [7]. There may be species-specific and tissue-specific regulation of the *Fuc-TIX* gene. In this study, we detected both hFuc-TIX and hFuc-TIV expression almost at the same level in forebrain, while we did not detect mFuc-TIV in a previous study [7]. This discrepancy may be attributed to the different areas of brain examined, or to a species-specific regulation as in kidney.

The co-localization of hFuc-TIX transcripts with the  $Le^x$  antigens in the glandular compartments of the stomach strongly suggested that hFuc-TIX is involved in the synthesis of  $Le^x$  antigens in stomach. However, the absence of hFuc-TIX in the foveolar cells in the gastric pits indicated that some other  $\alpha 1,3$ Fuc-T(s) are involved in  $Le^y$  synthesis, because gastric mucosa expressed four kinds of  $\alpha 1,3$ Fuc-Ts, i.e. hFuc-TIII, IV, VI and IX, as shown in Table 2.

The position of Fuc-TIX in the phylogenetic tree indicates that the Fuc-TIX subfamily was the first to diverge in vertebrates. CFT1, a chicken homologue (probably orthologue) of Fuc-TIV, is clustered with the mammalian Fuc-TIV subfamily. This suggests that a chicken orthologue of Fuc-TIX may be found in the Fuc-TIX subfamily. It is of interest to know to what extent the Fuc-TIX sequence is evolutionarily conserved in non-mammal vertebrates. The numbers of non-synonymous substitutions ( $d_N$ ) of protein coding gene regions are known to vary considerably. Genes for conservative proteins such as histones and actins show very low  $d_N$  values (0.001 and 0.002, respectively, between mouse and human [40]), because selective constraints are strong. It should be emphasized that the  $d_N$  value of the *Fuc-TIX* gene is as low as those of *histone* and  *$\alpha$ -actin* genes. The very low  $d_N$  value of the *Fuc-TIX* gene clearly indicates a strong selective pressure for the preservation of the Fuc-TIX amino acid sequence during the mammalian evolution. Recently, two zebrafish  $\alpha 1,3$ Fuc-T genes, *zFT1* and *zFT2*, were found to be expressed in embryogenesis [29]. hFuc-TIX and mFuc-TIX form a cluster with *zFT1* and *zFT2* in the phylogenetic tree. Fuc-TIX may possibly be involved in mammalian embryogenesis, and play an essential role for ontogeny.

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