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Molecular Cloning and Characterization of Two Zebrafish α(1,3)Fucosyltransferase Genes Developmentally Regulated in Embryogenesis[1]

Norihiko Kageyama, Shunji Natsuka, and Sumihiro Hase[2]

Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043

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Some α(1,3)fucosylated oligosaccharides serve as counter receptors to lectin-like adhesion proteins or are expressed with temporal precision during embryogenesis, and α(1,3)-fucosyltransferase is a key enzyme in the production of these oligosaccharides. Two α(1,3)-fucosyltransferase genes, designated zFT1 and zFT2, were cloned from zebrafish. Sequence comparisons with other genes indicated that zFT1 and zFT2 share about 30% amino acid sequence identity with human α(1,3)fucosyltransferases. Although the α(1,3)fucosyltransferases cloned so far can be classified into three types—myeloid, Lewis, and leukocyte—by virtue of their amino acid sequences, phylogenetic analysis indicated that neither zFT1 nor zFT2 belongs to any of these categories. The expression of zFT1 or zFT2 in mammalian cells induces α(1,3)fucosyltransferase activity to synthesize the Lewis x structure from pyridylaminated lacto-*N*-neotetraose; however, lacto-*N*-tetraose does not serve as a substrate. Reverse transcriptase-polymerase chain reaction analysis revealed that zFT1 is transcribed during a restricted period before hatching, whereas the mRNA for zFT2 was detected only after hatching.

Key words: embryo, fucosyltransferase, gene cloning, Lewis x, zebrafish.

The coordinated and temporally precise expression of cell-surface oligosaccharides has been observed during embryogenesis. The stage-specific embryonic antigen SSEA-1 is one such oligosaccharide (*1*). Although its expression pattern has been studied in detail (*2, 3*), the molecular mechanism regulating its expression remains poorly understood. The epitope of SSEA-1 is identical to the Lewis x structure, comprising *N*-acetyllactosamine and α-1,3-linked fucose residues (*4*). Hence, to elucidate the molecular mechanism controlling the expression of the fucosylated oligosaccharide, it is important to identify an α(1,3)fucosyltransferase, a key enzyme in SSEA-1 biosynthesis.

The genes for five human α(1,3)fucosyltransferases have been cloned and characterized (*5-13*), but none has been shown to synthesize SSEA-1/Lewis x during embryogenesis. Three of the genes, Fuc-TIII, Fuc-TV, and Fuc-TVI, constitute a sub-family of α(1,3)fucosyltransferases, termed the Lewis-type, by reason of the striking similarity of their primary structures. The other two, Fuc-TIV and Fuc-TVII, have been identified as myeloid- and leukocyte-type enzymes, respectively. Using the gene knock-out technique, Lowe's group has clearly demonstrated that Fuc-TVII in particular plays an essential role in synthesizing the oligosaccharides concerned in leukocyte homing (*14*). This strategy should also be effective in elucidating the roles of fucosyloligosaccharides in embryogenesis. For this, the cloning of a fucosyloligosaccharide-synthesizing α(1,3)fucosyltransferase gene(s) is important.

We thus attempted to isolate an α(1,3)fucosyltransferase gene(s) from zebrafish (*Danio rerio*) with the aim of clarifying the molecular mechanism of biosynthesis and the functions of α(1,3)fucosylated oligosaccharides during embryogenesis. Here, we report the cloning and characterization of two zebrafish α(1,3)fucosyltransferase genes, zFT1 and zFT2, that are expressed during the embryonic period and were found to be capable of synthesizing the Lewis x structure.

EXPERIMENTAL PROCEDURES

Standard PA-Oligosaccharides—Lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) were purchased from Seikagaku Kogyo (Tokyo), and pyridylaminated as described previously (*16*). PA-lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), PA-Lacto-*N*-fucopentaose II {Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-PA}, and PA-Lacto-*N*-fucopentaose III {Galβ1-4(Fucα1-

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[2] To whom correspondence should be addressed. Tel: +81-6-6850-5380, Fax: +81-6-6850-5383, E-mail: sumihase@chem.sci.osaka-u.ac.jp

Abbreviations: PA-, pyridylamino; RT, reverse transcriptase; PCR, polymerase chain reaction; SSEA-1, stage specific embryonic antigen-1.

3)GlcNAcβ1-3Galβ1-4Glc-PA} were purchased from Takara Biomedicals (Kyoto).

Zebrafish—Zebrafish were cared for and their embryos handled as described previously (*15*).

PCR Cloning of a Zebrafish Gene Fragment Homologous to Mammalian α(1,3)Fucosyltransferases—The PCR method was employed to isolate a zebrafish gene fragment homologous to mammalian α(1,3)fucosyltransferases. The sense primer, 5′-gcgcctcgagGA(C/T)TCNGA(C/T)(A/G)TCTT(C/T)(A/G)(C/T)(A/G)CC(C/T)TA(C/T)GG-3′, and antisense primer, 5′-gcgcctcgagGAA(A/G)TCGTCCACGTG(G/T)A(C/T)(A/G)AAGGC-3′, were constructed from consensus sequences of known α(1,3)fucosyltransferases. The PCR product (0.4 kbp) from zebrafish genomic DNA was digested with *Xho*I and subcloned into the *Sal*I site of the plasmid vector pTZ19R (Pharmacia). The sequence of the PCR product was analyzed by a genetic analyzer (ABI Prism 310; Perkin-Elmer).

Screening of a Zebrafish Genomic DNA Library—A zebrafish genomic DNA library was purchased from Stratagene (La Jolla, CA). Approximately 1.0×10^6 recombinant phages were screened by plaque hybridization. The zebrafish gene fragment homologous to mammalian α(1,3)fucosyltransferases isolated by PCR was utilized as a probe after labeling with digoxygenin using a DIG labeling kit (Boehringer Mannheim). Positive plaques were detected with a DIG detection kit (Boehringer Mannheim). The nine independent clones obtained from the library were characterized by restriction endonuclease digestion, Southern blotting, and sequencing. They included two genes homologous to mammalian α(1,3)fucosyltransferase genes. One of them, which hybridized strongly to the probe, was termed zFT1 and subcloned into plasmid vector pTZ19R to make pTZ19R-zFT1. The other, which hybridized weakly, was named zFT2 and similarly subcloned to make plasmid pTZ19R-zFT2.

In Vitro α(1,3)Fucosyltransferase Assay—A 1.2-kbp *Bam*HI-*Eco*RI fragment that included the open reading frame of zFT1 was obtained by PCR using pTZ19R-zFT1 as a template, two oligonucleotides (5′-gcgcggatccTCTTCCTTGCACAGTGATGG-3′ and 5′-gcgcgaattcCATTAACCCCAGAACCACCC-3′) as PCR primers, and pfu DNA polymerase (Stratagene). The 1.2-kbp zFT1 fragment was inserted into the mammalian expression vector pcDNA1 (Invitrogen), and the resultant plasmid was designated pcDNA1-zFT1. A plasmid named pcDNA1-zFT2 that included the open reading frame of zFT2 was obtained in the same manner. As positive controls, gene fragments containing open reading frames of hFuc-TIII and hFuc-TIV were prepared by PCR on the basis of sequences reported by Lowe *et al.* (*5, 6*), and inserted into the expression vector pcDNA1. Plasmids with or without the fucosyltransferase genes were transfected into COS 7 cells (ATCC No. CRL-1651) by the DEAE-dextran method. Transfected cells were harvested 72 h after the addition of DEAE-dextran-DNA complex, and cell extracts containing 1% Triton X-100 were prepared as described previously (*5*). The α(1,3)fucosyltransferase assay was performed at 37°C for 4 h in a total volume of 30 μl consisting of 0.1 M sodium cacodylate (pH 6.8), 5 mM ATP, 10 mM L-fucose, 25 mM $MnCl_2$, 75 μM GDP-fucose, 40 μM pyridylaminated sugar as an acceptor substrate, and 15 μl of cell extract. The reactions were stopped by heating at 100°C for 5 min, and the reaction products were analyzed by reversed phase HPLC on a Cosmosil 5C18P column (0.46×15 cm) with a fluorescence detector (*16*). The column was eluted with 0.05% 1-butanol in 0.1 M ammonium acetate, pH 6.0, at a flow rate of 1.5 ml/min. Size-fractionation HPLC was performed on a Shodex NH2-P column (0.46×5 cm) eluted with buffer containing 80% acetonitrile and 0.3% ammonium acetate (pH 7.0) at a flow rate of 0.8 ml/min. The products were identified by comparison with standard PA-oligosaccharides.

RT-PCR Analysis—Total RNAs from embryos at selected developmental stages were isolated using a QuickPrep total RNA extraction kit (Pharmacia). First strand cDNA was synthesized from 1 μg of total RNA by AMV reverse transcriptase with random primers using a 1st strand synthesis kit for RT-PCR (Boehringer Mannheim). The cDNA of zFT1 or zFT2 was amplified by PCR with the following primers: 5′-CGCCAAATCCTGCACCGGCTACTGC-3′ (248–272) and 5′-ATTGAAGTGCCCTCCATAGGCCTCC-3′ (765–741) for the zFT1 fragment; 5′-CCAAATCCAGCTAAAGGGGATCAAG-3′ (268–292) and 5′-AGGAAGTCATTTTGATTGCAATGGG-3′ (539–515) for the zFT2 fragment. The zebrafish *max* gene (*17, 18*), which is constitutively expressed during embryogenesis was used as a PCR quantitative control. The *max* primers were 5′-GCCGAAGAATGAGCGACAAC-3′ and 5′-CTGCTGTGTGTGTGTGGTTTTTC-3′. The PCR products were analyzed by electrophoresis in 2.5% agarose gels.

Alignment of Amino Acid Sequences and Construction of a Phylogenetic Tree for Vertebrate α(1,3)Fucosyltransferases Including zFT1 and zFT2—The amino acid sequences of vertebrate fucosyltransferases were obtained from the GenBank/EMBL database. Neighbor-joining analysis (*19*) of the distance matrix calculated from amino acid sequence alignment provided the phylogenetic tree. The bootstrap probabilities for clusters were examined. The resampling procedure was repeated one thousand times.

RESULTS

Molecular Cloning of Zebrafish α(1,3)Fucosyltransferase Genes—PCR was applied to isolate a zebrafish gene(s) homologous to mammalian α(1,3)fucosyltransferase genes. A 400-bp zebrafish gene fragment with a sequence significantly homologous to mammalian α(1,3)fucosyltransferase genes was obtained (data not shown). To isolate a gene(s) containing a full open reading frame, a zebrafish genomic DNA library was screened using the PCR product as a probe. Nine independent positive clones (#1–#9, Fig. 1) were obtained, and the results of restriction enzyme digestion and Southern blot analysis indicated that two genes homologous to mammalian α(1,3)fucosyltransferase genes were included in them. One hybridized strongly with the probe and the other weakly. The former, which included the nucleotide sequence coincident with the PCR product (Fig. 2A), was named zFT1. The latter was termed zFT2. The distance between the two genes on the chromosome was only 10.5 kbp and they were arranged in a tandem orientation (Fig. 1). The open reading frames of the two genes were predicted and identified by referring to Kozak's consensus rules for translation initiation sites (*20*) and by comparison with cloned mammalian α(1,3)fucosyltransferases (Figs. 2A and 3A). Since two suitable initiation

codons were present in zFT2, the upstream ATG of the zFT2 gene was temporarily assumed to be the initiation codon. The open reading frames of zFT1 and zFT2 were predicted to be polypeptides of 391 and 392 amino acids, respectively. Hydropathy analysis (*21*) of the predicted amino acid sequences indicated them to be type II trans-

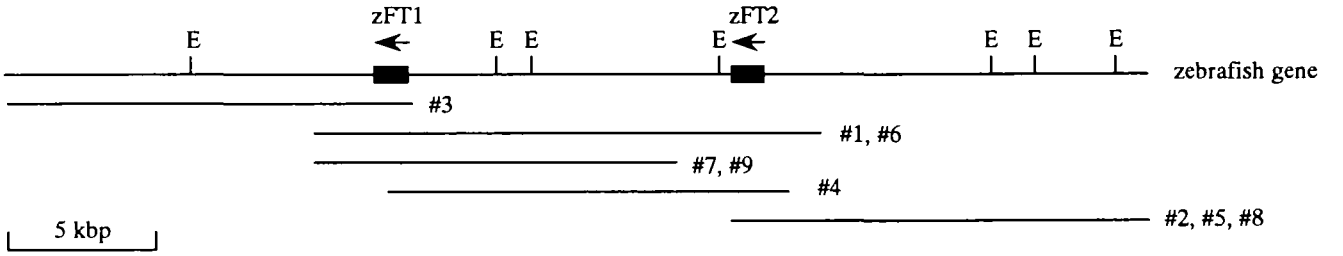

Fig. 1. **Map of zebrafish α(1,3)fucosyltransferase genes.** The shorter horizontal lines indicate the locations of the genes from the cloned phages. The positions marked E are the *Eco*RI restriction sites. The positions and orientations of the zFT1 and zFT2 open reading frames are shown by thick bars and arrows, respectively.

A

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                                                                -176 acaaccccaaatcagatatagagatgggacagtatggacgcgtcaaatattttaa
    attaatcacatgcgttaacacagtaatttgacagccctgatatatatatatgtatatatatatagacaatttttattaatttgacaatattttatttttcccttcttccatgcaacagtt

  1 ATGGATAAAATAACGCTCACACCTCCATCCAAAGCCGCTCAGAAGGTAATCATTGCCACATTCATGTTGATATCTTTTGTATGTATATTTTTTGTCTACTACAATCCAAACACAACCTTT 120
  1 M  D  K  I  T  L  T  P  P  S  K  A  A  Q  K  V  I  I  A  T  F  M  L  I  S  F  V  C  I  F  F  V  Y  Y  W  P  N  T  T  F  40

121 TTCAAATTCCCTGCTGTGGACGTCCATACAAACTGCAGCTGCACAGAAACTTGCCTCGATGTTTTGAAGAAGCAGAACTACAAATGCACTATAAAAAACGCCTCAGACAATCCATCGACA 240
 41 F  K  F  P  A  V  D  V  H  T  N  C  S  C  T  E  T  C  L  D  V  L  K  K  Q  N  Y  K  C  T  I  K  N  A  S  D  N  P  S  T  80

241 ACACCTGCGCCAAATCCTGCACCGGCTACTGCTAAAAAAGACGATCAAGAAACCATCCTGTTGATCTGGGTGTATCCTTTCGGAGCACGTTTTGACTTGGGAGTGTGTGGTTCGCAGTTC 360
 81 T  P  A  P  N  P  A  P  A  T  A  K  K  D  D  Q  E  T  I  L  L  I  W  V  Y  P  F  G  A  R  F  D  L  G  V  C  G  S  Q  F  120

361 AACATCCACGGCTGTCATTTAACAGACGACAGAAGTATGATCCAGAAAGCGCATGGAGTTATGTTTCACCACAGAGATCTGAGCGCGGATTTGCCGCAACCCCCTCGACCAGCTTTCCAA 480
121 N  I  H  G  C  H  L  T  D  D  R  S  M  I  Q  K  A  H  G  V  M  F  H  H  R  D  L  S  A  D  L  P  Q  P  P  R  P  A  F  Q  160

481 AAGTGGATATGGTGGAATATGGAGTCTCCGAGTAATTCCCATCCAAACAGCTTGATAAATGATGGGTTTAATCTGACGTCAAGTTTCCGCAAGGATTCAGATATTCCAGTGCCTTACGGT 600
161 K  W  I  W  W  N  M  E  S  P  S  N  S  H  P  N  S  L  I  N  D  G  F  N  L  T  S  S  F  R  K  D  S  D  I  P  V  P  Y  G  200

601 CGACTCATCGATGCCACCGACGAACAGAAAAACTTCACCATCCCCAAAAAGGATAAACTAGTTTGCTGGATAGTAAGTAACTTTCAAGAACACCACAAACGTTCACAGTACTATAATGAG 720
201 R  L  I  D  A  T  D  E  Q  K  N  F  T  I  P  K  K  D  K  L  V  C  W  I  V  S  N  F  Q  E  H  H  K  R  S  Q  Y  Y  N  E  240

721 TTGGTGAAACACATTAAAGTGGAGGCCTATGGAGGGCACTTCAATAACCGGGTTAATGATGACGATTATGGAAATGTGGTATCCAGTTGTAAATTCTACCTTTCCTTTGAGAACTCCATT 840
241 L  V  K  H  I  K  V  E  A  Y  G  G  H  F  N  N  R  V  N  D  D  D  Y  G  N  V  V  S  S  C  K  F  Y  L  S  F  E  N  S  I  280

841 CACAGAGACTATTTCACTGAGAAGCTGTTCAATCCTTTAGCGCTCGGTACGGTTCCTGTTGTACTTGGCCCTTCCAGAGATAACTATGAGGAGTTTATACCAAGAGACGCCTTCATCCAT 960
281 H  R  D  Y  F  T  E  K  L  F  N  P  L  A  L  G  T  V  P  V  V  L  G  P  S  R  D  N  Y  E  E  F  I  P  R  D  A  F  I  H  320

961 GTGGATGATTTCCCAACTCCCAAGGAACTGGCGGATCACCTTAAATCCTTGGACCAAAACGAGGAACAGTACAGACAGTATTTCAACTGGAGAAAACATTTTATTTCAATGAGTTCATCA 1080
321 V  D  D  F  P  T  P  K  E  L  A  D  H  L  K  S  L  D  Q  N  E  E  Q  Y  R  Q  Y  F  N  W  R  K  H  F  I  S  M  S  S  S  360

1081 TTTGGGCTTGAACATGCCTGTAGGACTTGTGATTATCTTAAAAGGAATAAGCACTATATAGCTATTAAAGATCTGAAAGGCTGGTTCTGGGGTTAA 1176
 361 F  G  L  E  H  A  C  R  T  C  D  Y  L  K  R  W  K  H  Y  I  A  I  K  D  L  K  G  W  F  W  G     391

    tgggattgttcacccattaatgaatattctgtaattaattattcaggtcatacctaactcttaagaccctcgttcgtcttcagaaaacaaatgaagatattttagatgaaatcctggagc
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B

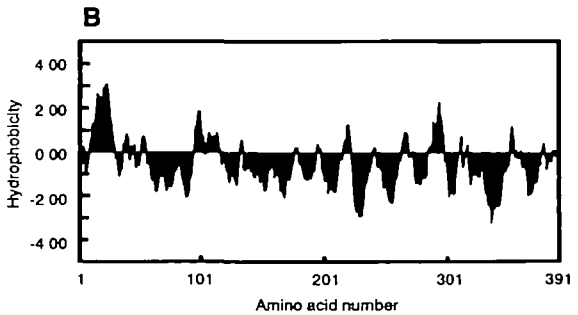

Fig. 2. **Nucleotide and deduced amino acid sequences of the zFT1 gene.** A: Nucleotide and deduced amino acid sequences of zFT1. The adenine residue of the putative initiation codon is assigned as residue 1 of the zFT1 nucleotide sequence. The methionine residue corresponding to this putative initiation codon is assigned as residue 1 of the deduced amino acid sequence. The amino acid sequence of the predicted transmembrane domain is double-underlined and consensus sequences for potential *N*-glycosylation sites are single-underlined. The DNA sequence corresponding to the PCR product is underlined B: Hydropathy plot of the predicted zFT1 polypeptide. The plot was calculated by the method of Kyte and Doolittle (*21*).

A

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                                -331 tatgagaaacgtctttaagctgccatcgccaaaaagccttgtattaaatagtattaaatacatttcaaatacagtagttcaggactttctt
    cttgtgtcattccattgatattaaacaacatgtttttctgatttatttgttttgtatcgtttttatgtttgggtttttaccaaaatctggttcaactccatgtcaacagctccttggaa
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  1 ATGGATAAAATAACAGTGATGGATAAAATAAAGCTCACAGCTCCGTCAAAATCCACTCAGAAACTAATCCTTGTCACATTCATGTTAATATCTTTAGTATGTATATTCTATGTCTACTAC 120
  1 M  D  K  I  T  V  M  D  K  I  K  L  T  A  P  S  K  S  T  Q  K  L  I  L  V  T  F  M  L  I  S  L  V  C  I  F  Y  V  Y  Y  40

121 AATCCAAACACAACCTTTTTCAAATACCCTACTATGGGCATCCACGCAAACAGCAGCTGCTCAGAAACTTGCCTCCATGTGCTGAAAATGCAGAACTATGAATGCATTATGAAAAACGCC 240
 41 N  P  N  T  T  F  F  K  Y  P  T  M  G  I  H  A  N  S  S  C  S  E  T  C  L  H  V  L  K  M  Q  N  Y  E  C  I  M  K  N  A  80

241 TCCTATAATGCGTTGCCAACACCAGCGCCAAATCCAGCTAAAGGGGATCAAGACATCATCGTGTTGATCTGGATGGCTCCTTTCAGAGTACCTTTTGACCTGAAAGATTGTGGTTTGGAG 360
 81 S  Y  N  A  L  P  T  P  A  P  N  P  A  K  G  D  Q  D  I  I  V  L  I  W  M  A  P  F  R  V  P  F  D  L  K  D  C  G  L  E  120

361 TATAACATCCACGGCTGTCAATTAACATACGACAGAAGTCTGATCCAGAAAGCGCATGGAGTTATGTTTCACCACAGAGATATGAGCACAGATTTTCCGCAACCCCCGCGACCAGATTTC 480
121 Y  N  I  H  G  C  Q  L  T  Y  D  R  S  L  I  Q  K  A  H  G  V  M  F  H  H  R  D  M  S  T  D  F  P  Q  P  P  R  P  D  F  160

481 CAAAAGTGGATATGGTGGAATATGGAGTCTCCGACCCATTGCAATCAAAATGACTTCCTAAATGATCGGTTTAATGTGACGTCAAGTTACAACAGGGATTCAGATATTCCAGTGCCTTAC 600
161 Q  K  W  I  W  W  N  M  E  S  P  T  H  C  N  Q  N  D  F  L  N  D  R  F  N  V  T  S  S  Y  N  R  D  S  D  I  P  V  P  Y  200

601 GGTCGACTCGTCGATGCCACAGAGGAACAGAAAAAGTTCACCATCCCCAAAAAGGATAAACTAGTTTGCTGGATAGTAAGTAACTTTCAAGGGCACCACAAGCGCACACATTTCTTCTAC 720
201 G  R  L  V  D  A  T  E  E  Q  K  K  F  T  I  P  K  K  D  K  L  V  C  W  I  V  S  N  F  Q  G  H  H  K  R  T  H  F  F  Y  240

721 GAGTTTGCGAAACACATTAATGTGAGCACCTATGGAGGGCACTTCAACAATCGAATTAATGGTGACGATTATGGAAATGTCGTATCCAGTTGTAAATTCTACCTGTCCTTTGAGAACTCC 840
241 E  F  A  K  H  I  N  V  S  T  Y  G  G  H  F  N  N  R  I  N  G  D  D  Y  G  N  V  V  S  S  C  K  F  Y  L  S  F  E  N  S  280

841 ATTCACAGAGACTATTTCACTGAGAAGCTGTTCAATCCTTTAGCACTCGGTACAGTTCCTGTCGTAATTGGCCCGCCCAGATATAACTATGAAGAGTTCATACCAAGAGACGCCTTCATC 960
281 I  H  R  D  Y  F  T  E  K  L  F  N  P  L  A  L  G  T  V  P  V  V  I  G  P  P  R  Y  N  Y  E  E  F  I  P  R  D  A  F  I  320

961 CATGTGGATGATTTCCCAACTCCCAAGGAACTGGCGGATCACCTTATATCCTTGGACCAAAACGAGGAACAGTACAAACAGTTTTCAACTGGAGGAAACTTTATGTTTCAAAGAGTACA 1080
321 H  V  D  D  F  P  T  P  K  E  L  A  D  H  L  I  S  L  D  Q  N  E  E  Q  Y  K  Q  F  F  N  W  R  K  L  Y  V  S  K  S  T  360

1081 TCGTTTGGGCTTGAACATTCCTGTAGGATTTGTGATTATCTTAAAAGGAATAAGCACTATATAGCCGTTACGGATCTGAAAGGGTGGTTCTGGGGTTAA 1179
 361 S  F  G  L  E  H  S  C  R  I  C  D  Y  L  K  R  N  K  H  Y  I  A  V  T  D  L  K  G  W  F  W  G     392

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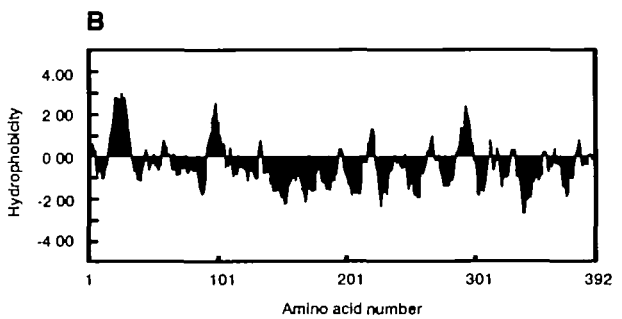

Fig. 3. **Nucleotide and deduced amino acid sequences of the zFT2 gene.** A: Nucleotide and deduced amino acid sequences of zFT2. The adenine residue of the putative initiation codon is assigned as residue 1 of the zFT2 nucleotide sequence. The methionine residue corresponding to this putative initiation codon is assigned as residue 1 of the deduced amino acid sequence. The predicted transmembrane domain is double-underlined, and consensus sequences for potential *N*-glycosylation sites are single-underlined. B: Hydropathy plot of the predicted zFT2 polypeptide. The plot was calculated by the method of Kyte and Doolittle (*21*).

membrane proteins, which are characteristic of most of glycosyltransferases (Figs. 2B and 3B). Sequence comparison showed that zFT1 shares 34.3% amino acid sequence identity with hFuc-TIII, 29.6% identity with hFuc-TIV, and 32.9% identity with hFuc-TVII, while zFT2 shows 28.7% amino acid sequence identity with hFuc-TIII, 27.1% identity with hFuc-TIV, and 29.5% identity with hFuc-TVII. zFT1 and zFT2 show relatively high sequence similarity—up to 75%—with each other (Fig. 4). Recently, a new mouse α(1,3)fucosyltransferase gene, Fuc-TIX, was reported (*22*). This gene shows a slightly higher sequence identity to zFT1 and zFT2—39.3 and 37.5% respectively—than other mammalian α(1,3)fucosyltransferase genes.

Enzyme Activities of zFT1 and zFT2 Products—To confirm that the sequences of the zebrafish genes homologous to mammalian α(1,3)fucosyltransferase genes code α(1,3)fucosyltransferases, the zFT1 and zFT2 gene products were assayed by expression in mammalian cells using PA-oligosaccharides as acceptor substrates. The enzyme activities of zFT1 and zFT2 catalyze the synthesis of the Lewis x structure using PA-lacto-*N*-neotetraose as a substrate are shown in Fig. 5. The reaction products were confirmed by reversed phase and size-fractionation HPLCs. In this assay, both zFT1 and zFT2 had α(1,3)fucosyltransferase activity and could effectively catalyze the synthesis of the Lewis x structure. On the other hand, when PA-lacto-*N*-tetraose was used as a substrate, neither zFT1 nor zFT2 could catalyze the synthesis of the Lewis a structure (data not shown).

Phylogenetic Tree of Vertebrate α(1,3)Fucosyltransfer-

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zFT1   1     MDKIT------LTPPSKAAQKVIIATFMLISFVCIFFVYYNPNTTFFKFP
zFT2   1     *****VMDKIK**A***ST**L**V******L****Y**********Y*

zFT1   45    AVDVHTNCSCTETCLDVLKKQNYKCTIKNASDNPSTTPAPNPAPATAKKD
zFT2   51    TMGI*A*S**S****H***M***E*IM****Y*ALP*******-----*G

zFT1   95    DQETILLIWVYPFGARFDLGVCGSQFNIHGCHLTDDRSMIQKAHGVMFHH
zFT2   96    **DI*V***MA**RVP***KD**LEY*****Q**Y***L**********

zFT1   145   RDLSADLPQPPRPAFQKWIWWNMESPSNSHPNSLINDGFNLTSSFRKDSD
zFT2   146   **M*T*F******D***********THCNQ*DFL**R**V***YNR***

zFT1   195   IPVPYGRLIDATDEQKNFTIPKKDKLVCWIVSNFQEHHKRSQYYNELVKH
zFT2   196   ********V***E***K****************G****THFFY*FA**

zFT1   245   IKVEAYGGHFNNRVNDDDYGNVVSSCKFYLSFENSIHRDYFTEKLFNPLA
zFT2   246   *N*ST********I*G**********************************

zFT1   295   LGTVPVVLGPSRDNYEEFIPRDAFIHVDDFPTPKELADHLKSLDQNEEQY
zFT2   296   *******I**P*Y*************************I*********

zFT1   345   KQFFNWRKLYVSKSTSFGLEHSCRICDYLKRNKHYIAVTDLKGWFWG
zFT2   346   R*Y*****HFI*M*S******A**T***********IK********
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Fig. 4. **Amino acid homology between zFT1 and zFT2.** The deduced amino acid sequences are indicated by the one letter notation. Amino acid residues in zFT2 identical to those in zFT1 are shown by asterisks. Gaps are indicated by dashes.

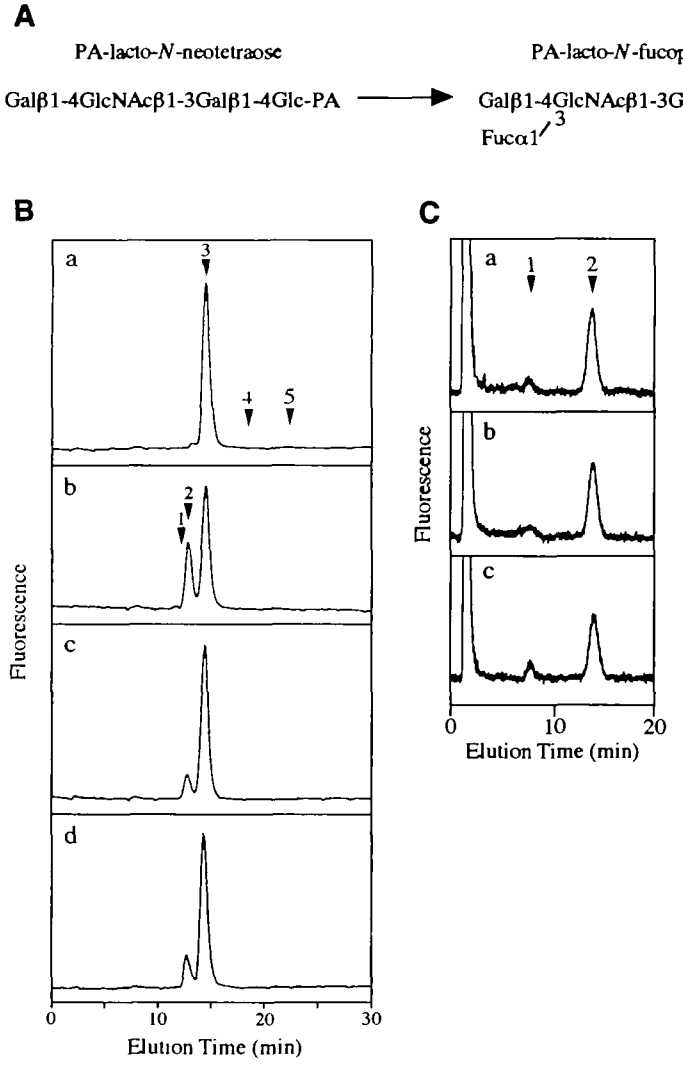

Fig. 5. **Lewis x-synthesizing activity of zFT1 and zFT2.** A: Scheme of the assay reaction. B: Reversed phase HPLC profiles of the reaction mixtures of zFT1 and zFT2 using PA-lacto-*N*-neotetraose as a substrate. Assays were performed as described in "EXPERIMENTAL PROCEDURES." Mock (a), zFT1 (b), zFT2 (c), and hFuc-TIV (d) transfected cells were used for the assay. Arrowheads 1 to 5 indicate the positions of standard PA-oligosaccharides, PA-lacto-*N*-fucopentaose II, PA-lacto-*N*-fucopentaose III, PA-lacto-*N*-neotetraose, PA-lacto-*N*-tetraose, and PA-lacto-*N*-fucopentaose I, respectively. C: Size-fractionation HPLC profiles of the reaction products. The peaks eluting at the same position as PA-lacto-*N*-fucopentaose III on reversed phase HPLC were collected and re-analyzed by size-fractionation HPLC. The products were from the assays of hFuc-TIV (a), zFT1 (b), zFT2 (c). Arrowheads 1 and 2 indicate the positions of PA-lacto-*N*-neotetraose and PA-lacto-*N*-fucopentaose III, respectively. Peaks in the pass through fractions originated from the elution buffer for reversed phase HPLC.

ases Including zFT1 and zFT2—We predicted the evolutional distances of zFT1 and zFT2 by comparing them with the mammalian α(1,3)fucosyltransferase genes cloned and characterized so far. We then drew up a phylogenetic tree for vertebrate α(1,3)fucosyltransferases, including zFT1 and zFT2, on the basis of their amino acid sequence alignment (Fig. 6). The genes cFT1 (*23*), hFuc-TIV (*6-8*), mFuc-TIV (*24*), and rFuc-TIV (*25*) were categorized as myeloid-type, hFuc-TVII (*12, 13*) and mFuc-TVII (*26*) as leukocyte-type, and Futb (*27*), hFuc-TVI (*10, 11*), hFuc-TIII (*5*), and hFuc-TV (*9*) as Lewis-type. The zebrafish genes zFT1 and zFT2 were placed on the same branch with mFuc-TIX (*22*).

RT-PCR Analysis of zFT1 and zFT2 Expression Patterns during Zebrafish Embryogenesis—To determine whether the genes are expressed during embryogenesis, embryos at each developmental stage were analyzed by RT-PCR. As shown in Fig. 7, zFT1 mRNA was definitely detected 15 h after fertilization, increased up to 18 h, and then rapidly disappeared. The mRNA was scarcely detected during the period from 48 h after fertilization, which is

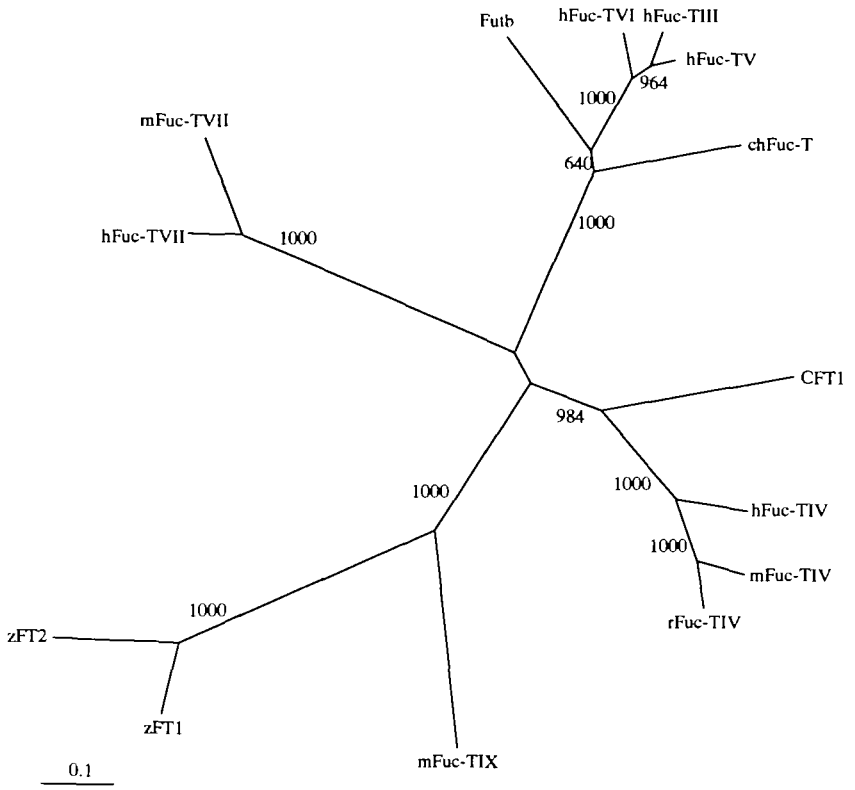

Fig. 6. **Phylogenetic tree of the vertebrate α(1,3)fucosyltransferase family.** The phylogenetic tree was drawn on the basis of the predicted amino acid sequences of vertebrate α(1,3)fucosyltransferases as described in "EXPERIMENTAL PROCEDURES." Bootstrap probabilities for clusters are shown as percentages. The bar below the tree represents the number of amino acid substitutions.

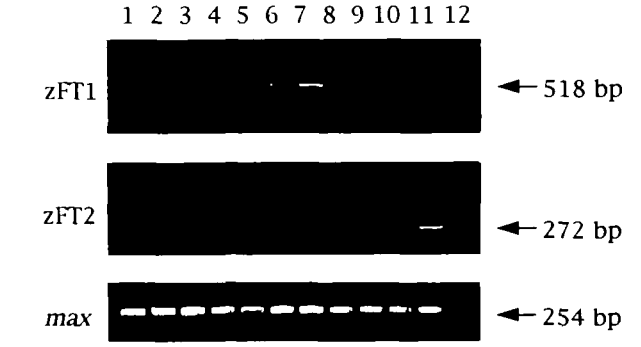

Fig. 7. **mRNA expression of zFT1 and zFT2 during embryogenesis.** Each cDNA was synthesized from total RNA isolated from selected developmental stages. RT-PCR analysis was performed as described in "EXPERIMENTAL PROCEDURES." The relative consistency of the *max* signals throughout embryogenesis indicates that approximately equivalent amounts of cDNAs were assayed in the PCR analyses of zFT1 and zFT2. The lanes represent the RT-PCR product from mRNA at the following times after fertilization: lane 1, 2 h; lane 2, 6 h; lane 3, 8 h; lane 4, 10 h; lane 5 , 12 h; lane 6, 15 h; lane 7, 18 h; lane 8, 24 h; lane 9, 36 h; lane 10, 48 h; lane 11, 72 h; lane 12, adult zebrafish.

the hatching time of the zebrafish embryo. In contrast, no mRNA for zFT2 was detected until 48 h after fertilization, and the amount was only evident 72 h after fertilization. Since the *max* gene transcript was detected in almost the same amounts through out the period of development used in this experiment, the changes in the amounts of zFT1 and zFT2 expressed are considered to be reliable.

DISCUSSION

Two zebrafish α(1,3)fucosyltransferase genes, zFT1 and zFT2, were cloned in order to identify the α(1,3)fucosyltransferase catalyzing the biosynthesis of fucosyloligosaccharides during development. Sequence comparison revealed that the two zebrafish genes share about 30% amino acid identity with human α(1,3)fucosyltransferase genes, and that they share over 70% amino acid identity with each other. zFT1 and zFT2 are present on the same chromosome and arranged in a tandem orientation; the distance between them is only 10.5 kbp. This resembles the case of the human α(1,3)fucosyltransferase genes hFuc-TIII and hFuc-TVI, which are about 13 kbp apart and are considered to have arisen by gene duplication (*28*). Interestingly, zFT1 and zFT2 show relatively high homology to the mouse Fuc-TIX gene which was cloned recently from a brain cDNA library (*22*).

When zFT1 or zFT2 was transfected into mammalian cells, their gene products could synthesize the Lewis x structure from pyridylaminated type II tetrasaccharide (PA-lacto-*N*-neotetraose), but neither zFT1 nor zFT2 synthesized the Lewis a structure from pyridylaminated type I tetrasaccharide (PA-lacto-*N*-tetraose). These results indicate that zFT1 and zFT2 encode α(1,3)fucosyltransferases. Only one enzyme with α(1,4)fucosylation activity can synthesize the Lewis a structure, Fuc-TIII, and is known to be a Lewis blood group enzyme (*5*). A murine counterpart of this gene was found not to have a functional structure (*24*), and to be a pseudogene in the mouse genome. A bovine enzyme, Futb, which is an orthologous homologue of a putative ancestor gene of the human Lewis-

type enzyme family comprising Fuc-TIII, Fuc-TV, Fuc-TVI, also does not have α(1,4)fucosyltransferase activity in spite of its high sequence similarity with hFuc-TIII (*27*). Therefore, it seems that α(1,4)fucosylation activity is exceptional in vertebrates.

RT-PCR analysis indicated that the mRNAs for both zFT1 and zFT2 are expressed specifically during embryogenesis, but at different stages during embryonic development. Furthermore, the results of *in vitro* enzyme assays showed that both gene products have enzyme activities capable of synthesizing the Lewis x structure, which is an epitope of SSEA-1. These findings point to the possibility that zFT1 and zFT2 are responsible for SSEA-1 biosynthesis during embryogenesis. However, since zFT1 and zFT2 are separately transcribed before or after hatching, respectively, they may play different roles in zebrafish embryogenesis. Further study is needed to determine the relation of zFT1 and zFT2 to SSEA-1, for instance, *in situ* hybridization of zFTs and an investigation of SSEA-1 histochemistry in the zebrafish embryo.

By comparing the zebrafish and mammalian α(1,3)fucosyltransferases, we were able to draw a phylogenetic tree of vertebrate α(1,3)fucosyltransferase genes including zFT1 and zFT2. The tree shows that the zFTs comprise a novel class of α(1,3)fucosyltransferase genes since they are on a different branch from "authentic" categories, *i.e.*, the Lewis-, myeloid-, or leukocyte-types. The chicken enzyme apparently diverged from mammalian enzymes before the bovine enzyme diverged from human enzymes, but the bovine and chicken enzymes belong to the Lewis- and myeloid-types, respectively. In contrast with the bovine and chicken enzymes, the zebrafish enzymes branched off at the first node to form a new category separate from the other groups. It is not yet clear how this new class of α(1,3)fucosyltransferase genes should be categorized. One possibility is that the zFTs may comprise a class of embryonic α(1,3)fucosyltransferases, because their expression is limited to restricted periods during embryogenesis. Future work aimed at locating a human or mouse gene(s) orthologous to zFT1 or zFT2 will be important to ascertain whether this is the case.

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