Analysis of brain-specific UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases in zebrafish

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(*Received September 12, 2005*
Revised December 19, 2005)

Abstract

A UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase (GalNAc-transferase), which catalyzes the initial step of mucin-type oligosaccharide biosynthesis, consists of a large gene family, with 15 isozyme genes cloned to date. These isozymes have specific, although partially overlapped, substrate specificity and are expressed in a temporally and spatially distinct manner. The author investigated the biological roles of the brain-specific GalNAc-transferases in model organisms. The author isolated a novel human cDNA clone from brain, which is homologous to GalNAc-T9, by homology-based PCR. Nucleotide sequence analysis of the cDNA showed that it contained structural features characteristic of the GalNAc-transferase family. The clone was, therefore, designate a putative GalNAc-transferase (pt-GalNAc-T). It was also found that human *pt-GalNAc-T* was identical to the gene *WBSCR17*, which is reported to be in the critical chromosome region of patients with Williams-Beuren Syndrome, a neurodevelopmental disorder, and to be predominantly expressed in brain and heart. In order to investigate the expression of pt-GalNAc-T in brain in more detail, Northern blot analysis was carried out. The 5.0-kb mRNA was most abundantly expressed in cerebral cortex with somewhat less amount in cerebellum. The author expressed soluble recombinant pt-GalNAc-T in insect cells to investigate biochemical roles of this molecule. Recombinant human pt-GalNAc-T, however, did not glycosylate several peptides derived from mammalian mucins. The brain-specific expression of pt-GalNAc-T may suggest that its has a narrow substrate specificity and is involved in O-glycosylation of proteins in the brain.

The author, then, performed the knock-down studies of brain-specific GalNAc-transferases from zebrafish to investigate their biological function. I cloned orthologue genes of brain-specific isozymes from zebrafish. I found through the genome database search that zebrafish contains orthologue genes of all mammalian isozymes except GalNAc-T15. Among these genes, I isolated one ubiquitous (GalNAc-T1) and three brain-specific orthologue genes (GalNAc-T9, -T13, and pt-GalNAc-T) from zebrafish. The zebrafish orthologue genes were highly homologous to the corresponding human isozyme with amino acid similarity of 96, 94, 94 and 80%, respectively. I investigated the expression of zebrafish pt-GalNAc-T by whole mount *in situ* hybridization (WISH). It was expressed throughout the central nervous system including telenthephalon, midbrain, and hindbrain in the embryos of 36 to 48 hpf. In these embryos, its strong expression in eyes was also observed. When the expression of pt-GalNAc-T in the embryos was suppressed with morpholino antisense oligonucleotides, malformation of the eyes and brain was observed during the development. In these

embryos, the pax2.1 gene expression was lost at the hindbrain, indicating that zebrafish pt-GalNAc-T may participate in the normal development of hindbrain. This indicates that pt-GalNAc-T, one of possible causative genes of Williams-Beuren Syndrome, is involved in the normal development of zebrafish hindbrain.

Keywords: mucin-type O-glycosylation, GalNAc-transferase, brain, zebrafish

Contents

1. Introduction

Glycosylation of proteins is an indispensable process for proteins to acquire correct three-

dimensional conformation and functions, and a number of studies report specific roles of carbohydrate moieties of glycoproteins. Mucin-type O-glycosylation, which is characterized by the sugar-peptide linkage GalNAcα1 → Ser (or Thr), is widely distributed in many secretory and membrane glycoproteins, and is also involved in important biological functions such as cell-cell recognition, host-pathogen interaction, and protection of proteins from proteolytic degradation [1,2]. The initial step of mucin-type O-glycosylation is catalyzed by the UDP-GalNAc : polypeptide N-acetylgalactosaminyltransferase (GalNAc-transferase) (EC. 2.4.1.41), which transfer GalNAc from UDP-GalNAc to Ser and Thr residues of proteins [3]. To date, 15 GalNAc-transferase isozymes have been reported in vertebrate [4-20]. The large number of isozymes in the family suggests that O-glycosylation in the cell is regulated through distinctive sets of the isozymes expressed in each tissue. In fact, it is reported that the cloned GalNAc-transferases are characteristically expressed in each tissue, and can be classified into two groups based on their tissue expression patterns : i) house-keeping enzymes, and ii) tissue-specific enzymes. Apparently, GalNAc-T1, -T2, -T8, and -T15 belong to the former group [6,12,20,21]. Their expression is ubiquitous and they are probably involved in the general synthesis of mucin-type carbohydrate chains in many cell types. On the other hand, the other isozymes are regarded as the latter-type isozymes. Their expression is restricted to several tissues. Of these isozymes, GalNAc-T9, which was cloned by our group, is particularly interesting in that it is specifically expressed in the brain [13]. Although several proteins in the brain are reported to carry mucin-type carbohydrate chains, the involvement of the brain-specific isozymes in the O-glycosylation has not been reported. Toba *et al.* previously cloned another novel brain-specific putative GalNAc-T from rat, which is highly homologous to GalNAc-T9, and its detailed expression in the brain. First, the author describes cloning and expression pattern of its human orthologue.

Recent studies demonstrated that the O-glycosylation of a cell is a coordinated work by the GalNAc-transferases expressed in it. It is, therefore, important to investigate expression and catalytic properties of each isozyme for elucidating the roles of O-glycans in the tissue. For this purpose, knockout mice were generated in which either of *GalNAc-T1*, *-T4*, *-T5*, or *-T13* was ablated, but these mice are normally developed and fertile with apparently normal phenotypes [18,22,23]. It seems that the other isozymes in the knockout mice complemented the functions of the ablated genes.

Zebrafish is one of the most excellent model organisms to study a role of genes in development for several reasons. First of all, its development is very rapid. All common vertebrate body specific features can be seen during 2 days after the fertilization. The embryos are relatively large and their development can be observed easily through the chorion. In addition, the method to knock-down

the function of specific genes is available, in which antisense morpholino-modified oligonucleotides are used to inhibit translation of a target mRNA with higher efficiency and specificity than antisense RNA or DNA. In fact, there are several studies, in which the mutants such as no tail, chordin, one-eyed-pinhead, nacre, and sparese are generated by the injection of morpholino oligonucleotides for a targeted gene [24]. Especially, it should be noted that this method has the advantage of knocking-down two or more genes simultaneously with the specific morpholino antisense oligo mixture [25,26]. As described above, the author cloned mammalian brain-specific GalNAc-transferase. However, their activity is not still detected in my laboratory, in spite of the assay using various kinds of synthetic peptides and recombinant isozymes expressed in both insect and mammalian cells. Thus, their function in the brain still remains to be elucidated. In this report, the author also describes the study on the roles of the brain-specific isozymes in zebrafish development. For this purpose, the author first cloned zebrafish orthologues for the mammalian brain-specific GalNAc-transferase. The author then knocked-down the expression of these isozymes in the zebrafish embryos using the morpholino-based knock-down approach and investigated the phenotypic changes of the injected embryos during the development. This study indicated that *zebrafish putative GalNAc-T gene* (*z-pt-GalNAc-T*) was likely to participate in normal brain morphogenesis. A possible involvement of *pt-GalNAc-T* in WBS was also described.

2. Materials and Methods

2.1 Cloning of human *pt-GalNAc-T*

We have previously reported the cloning of a novel brain specific clone highly similar to human GalNAc-T9 from rat, designated *rat putative GalNAc-T (r-pt-GalNAc-T)* [27]. To isolate the human orthologue of *r-pt-GalNAc-T*, the NCBI database was searched by the Blastn algorithm using the full-length *r-pt-GalNAc-T* sequence as a probe. A human brain double-stranded cDNA was synthesized from 1 μg of human brain total RNA (Origene) using a Marathon cDNA amplification kit (Clontech). For the amplification of *human putative GalNAc-T (h-pt-GalNAc-T)*, PCR was carried out with human brain template cDNA and the primers, hPTT-S (5'-AGCCCGAGGGGGCGCAG GTC-3') and hPTT-A (5'-GGGGAGGAGCCATGTCCTG-3'). AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems) was used for PCR. The conditions for PCR were as follows: 95°C, 12 min for the activation of AmpliTaq Gold^{TM} DNA polymerase; 94°C, 20 sec; 72°C, 3 min; 5 cycles; 94°C, 20 sec; 70°C, 3 min; 5 cycles; 94°C, 20 sec; 68°C, 3 min; 45 cycles. The amplified cDNA fragment was ligated into *pGEM-T easy* vector and sequenced.

2.2 Northern blot analysis of *h-pt-GalNAc-T*

Northern blot analysis for *h-pt-GalNAc-T* was performed with Multiple Tissue Northern blot II (Clontech) as described previously [13]. For this analysis, an RNA probe is used, which is a 500 base fragment including cytosolic domain, transmembrane domain, stem region, and a part of putative catalytic domain sequences.

2.3 Construction of an expression vector, *pFastHisGP67*

The following synthetic oligonucleotides were purchased from Invitrogen. FLAG-F; 5'-GAT CCCATGGATTATAAAGATGATGATGATAAA-3', FLAG-R; 5'-GCCGCCTTTATCATCAT CATCTTTATAATCCATGG-3', His-F; 5'-GGCGGCCATCATCATCATCATCACGGCGGCGG GAGCT-3', and His-R; 5'-CCCGCCGCCGTGATGATGATGATGATG-3'. A *PolH/GP67* DNA fragment, containing a baculovirus polyhedrin promoter and a gene coding for the signal sequence for the insect secretory protein, glycoprotein67, was isolated from *pAcGP67* plasmid vector (Phermingen) by digesting the vector with *Eco*RV and *Bam*HI and purifying the corresponding DNA fragments. *pFastBac*^{TM}*1*, a transfer vector for the Bac-to-Bac system (Invitrogen), was digested with *Acc*I and blunt-ended with Blunting High (TOYOBO), and the product was digested with *Sac*I, obtaining the product, *pFastBac*^{TM}*1/SacI/AccI-blunt* fragment. 5'-Ends of FLAG-F, FLAF-R, His-F, and His-R were phosphorylated by T4 polynucleotide kinase. Phosphorylated FLAG-F and FLAG-R were, then, incubated together at 60°C for 2 min to obtain the duplex coding for the FLAG epitope-tag, MDYKDDDDK. Similarly, the cDNA for the His epitope-tag consisting of successive 6 histidine residues was obtaind by incubating His-F and His-R under the same conditions. Then both cDNA were mixed with the *pFastBac*^{TM}*1/SacI/AccI-blunt* fragment and the *PolH/GP67* fragment and ligated by the T4 ligase (Promega). DH5α was transformed with the ligated product and screened by PCR. The isolated clone, designated *pFastHisGP67*, was sequenced to confirm that cDNA for the GP67 secretion signal and the FLAG/His tags was correctly inserted.

2.4 Constructoin of *pFastHisGP67/h-pt-T*

h-pt-GalNAc-T cDNA deleted with a sequence for the cytosolic domain and the transmembrane region was obtained by PCR using a primer pair, hPTT-Not (5'-ATAAGAATGCGGCCGCCA TCGCGGTGCGCAGCGGAGACGCCTTCC-3') and hPTT-Xba (5'-GCTCTAGAGACTACTTGATGGA GTTCTT-3'). The nucleotides underlined in the primers are *Not*I and *Xba*I restriction sites introduced into the amplified cDNA, respectively. The amplified fragment was digested with *Not*I and *Xba*I, inserted into *pFastHisGP67* digested with *Not*I and *Xba*I, and then ligated. DH5α was transformed with the ligated product and screened by PCR. The isolated clone, designated

pFastHisGP67/h-pt-T, was sequenced and used for transformation of the DH10Bac™ E. coli host strain, which harbors a baculovirus shuttle vector and a helper plasmid. The recombination between *pFastHisGP67h-pt-T* and the baculovirus shuttle vector in DH10Bac™ produced a bacmid DNA that contains cDNA for h-pt-GalNAc-T. The bacmid DNA was obtained from DH10Bac host cells, and introduced into Sf9 insect cells with Cellfectin™ and incubated for 3 days. Recombinant baculoviruses secreted into the medium were recovered and was amplified for the expression of the recombinant h-pt-GalNAc-T isozyme. Expression of the recombinant proteins was carried out according to the instructions described in the Bac-to-Bac® Baculovirus expression system manual (Invitrogen).

2.5 Enzyme purification and assays

Three days after the transfection of High Five cells with the recombinant vaculoviruses, conditioned medium was recovered and dialyzed against 25 mM Tris-HCl buffer (pH 7.2), containing 100 mM NaCl, and 5 mM imidazole. The dialyzed medium was centrifuged at 10,000 rpm at 4°C for 1 min. The supernatant thus obtained was mixed with Ni-NTA agarose (QIAGEN) and incubated at 4°C overnight under constant rocking to make a uniform suspension. The Ni-NTA agarose adsorbed with recombinant h-pt-GalNAc-T was washed three times with 25 mM Tris-HCl buffer (pH 7.2), containing 100 mM NaCl, and 10 mM imidazole and then with 25 mM Tris-HCl buffer (pH 7.2), containing 100 mM NaCl, and 25 mM imidazole, three times each. Finally, recombinant h-pt-GalNAc-T was eluted with 25 mM Tris-HCl buffer (pH 7.2), containing 100 mM NaCl, and 500 mM imidazole. The enzyme activity was determined as described previously [27] with the exception that the reaction was carried out at 37°C.

2.6 Identification of zebrafish orthologue genes in the database and their cDNA cloning

The amino acid sequences of all the GalNAc-transferases identified to date were used to perform a tBLASTn search against the *Danio rerio* whole genome shotgun sequence database, the *Danio rerio* EST database in NCBI, and the finished/unfinished genome sequence database in Sanger Institute. A number of sequences homologous to each isozyme were obtained, and the zebrafish contig sequences for each mammalian isozyme were generated by comparing the sequences found in the database. Then, primer pairs, zGT1-f (5'-GGTTTCTGTTCTCGTCTCGTGC-3'), zGT1-r (5'-CAT TCGTTCGTTGTTGG-3'), zGT9-f (5'-GCTGAAAGAACAGCTCCCAC-3'), zGT9-r (5'-GCTCTCCAGCT CAGGTTATTTAG-3'), zGT13-f (5'-GGTCCTCCAGTGGGATGACG-3'), zGT13-r (5'-GGAAAGCCAAA AACCTG ACTCC-3'), zPTT-f (5'-TTACCA TCAC ATCC CTG CTTGTC-3'), and zPTT-r (5'-CCC CTT CGGC TTGA GTTCTTC-3'), were designed from the contig and used for amplification of zebrafish

full-length *GalNAc-T1*, *GalNAc-T9*, *GalNAc-T13*, and *pt-GalNAc-T*, respectively. AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems) and cDNA, which was isolated from 48 hours post fertilization zebrafish embryos, were used for PCR. The conditions for PCR were as follows: 95°C, 12 min for the activation of AmpliTaq Gold^{TM} DNA polymerase; 94°C, 20 sec; 72°C, 3 min; 5 cycles; 94°C, 20 sec; 70°C, 3 min; 5 cycles; 94°C, 20 sec; 68°C, 3 min; 45 cycles. For the amplification of *zebrafish GalNAc-T13*, nested PCR was carried out on the PCR products using primer pair, zGT13-f2 (5'-TGTCCAGTCACACTGCCCCC-3'), zGT13-r2 (5'-CCAAAAACCTGACTCCAAGTCTTG-3'). The amplified fragments were subcloned into the *pGEM-T easy* vector and sequenced.

2.7 Maintenance of fish

Fish was maintained at 28.5°C on a 12 hour light/dark cycle. To prevent the pigment formation, the embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma).

2.8 Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out as previously described [28] with some modifications. Briefly, embryos fixed with phosphate-bufferd saline (PBS) containing 4% paraformaldehyde were dehydrated with methanol and rehydrated with PBS containing 0.1% Tween 20. They were then transferred into a hybridization mixture (5x SSC, containing 50 mg/ml of heparin, 100 mg/ml of calf thymus DNA, 10 mg/ml of tRNA, 50% formamide, and 0.1% Tween 20) and incubated for at least 1 hour at 55°C. The hybridization mixture was then replaced with the hybridization mixture containing 100 ng/ml of DIG-labelled RNA probe (Fig. 5) and the embryos were incubated at 57°C overnight. After a rinse with 5x SSC, they were washed in 2x SSC containing 50% formamide at 65°C for 30 minutes, 2x SSC for 10 minutes, and soaked in RNase buffer (10 mM Tris-HCl (pH 8.0), containing 0.5 M NaCl) for 10 minutes. Following the treatment with 20 mg/ml of RNase A at 37°C for 1 hour, a series of washings (2x SSC for 10 minutes; 2x SSC containing 50% formamide at 65°C for 30 minutes; 0.2x SSC at 55°C for 15 minutes) was performed. The embryos were rinsed with MAB (100 mM maleic acid (pH 7.5), containing 150 mM NaCl, and 0.1% Tween 20) and soaked in blocking solution (MAB, containing 1% blocking reagent (Roche), 2% fetal calf serum, 0.2% Tween 20, and 0.2% TritonX-100) for 2 hours at room temperature. The embryos were then incubated with AP-conjugated anti-DIG Fab fragments, which were diluted 1: 6000 in the blocking solution, at 4°C overnight. After washing three times with PBS containing 0.1% Tween 20 for 30 minutes, the embryos were rinsed three times with AP reaction buffer (100 mM Tris-HCl (pH 9.0), containing 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, and 1 mM levamisole) for 10 minutes. Detection was performed with BM-purple (Boehringer Mannheim). After stopping the coloring reaction, I washed the specimens three times

with the AP reaction buffer without levamisole and stored them in PBS containing 4% paraformaldehyde.

2.9 Morpholinos

Morpholino oligos were purchased from Gene Tools, LLC; MO-zT9, CTTGCGTGCCACAGC CATTAAAGCC; MO-zT13, CTTACAGTACACGAAGCGTCTCATG; MO-zPTT, CCATCTT CTCATCACAAAGGCCATG. A morpholino oligo stock solution was prepared by dissolving morpholino oligos in 1 x Danieau buffer (5.0 mM HEPES (pH 7.6), containing 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, and 0.6 mM Ca $(NO_3)_2$) at the concentration of 67.2 mg/ml. The working solution prepared by diluting the stock solution was injected into one to two-cell stage zebrafish embryos.

3. Results and Discussion

3.1 Cloning of human pt-GalNAc-T

To investigate the unique nature of brain-specific GalNAc-T9, we previously attempted to clone its rat orthologue gene. However, the obtained rat clone was not an orthologue, but a highly homologous clone of human GalNAc-T9. Since this clone had structural features conserved in the GalNAc-transferase family, it was designated *rat putative GalNAc-T (r-pt-GalNAc-T)* [27]. Interestingly, *r-pt-GalNAc-T* was also brain-specifically expressed. Northern blot analysis revealed a brain-specific expression of 2.0 kb mRNA. *In situ* hybridization also showed its expression in the restricted regions in the rat brain, such as cerebellum, hippocampus, hypothalamus, and the internal layers of cerebral cortex [27]. In order to study its expression in human, I cloned the human orthologue of *r-pt-GalNAc-T*. Blast search analysis of the database using the nucleic acid sequence of *r-pt-GalNAc-T* identified several human genomic and EST clones that contain sequences highly homologous to the rat clone. I generated the contig from these genome clones, and obtained the human clone by PCR using the nucleotide sequence of the contig (Fig. 1). The predicted amino acid sequence of the resultant clone was compared with human GalNAc-T9 and r-pt-GalNAc-T (Fig. 2). The human full-length cDNA clone contained an open reading frame encoding a type II membrane protein consisting of 598 amino acid residues with a 7 amino acid N-terminal cytoplasmic domain, a 20 amino acid transmembrane domain, a 92 amino acid stem region, and a 479 amino acid putative catalytic region. This clone had several characteristics commonly found in the GalNAc-transferase families: 1) a glycosyltransferase 1 (GT1) motif, a conserved sequence commonly found in glycosyltransferases [29], 2) a DXH sequence in the GT1 motif, a putative binding site for a sugar donor and/or a metal ion [30], 3) a Gal/GalNAc transferase motif consisting of about 40 amino acid re-

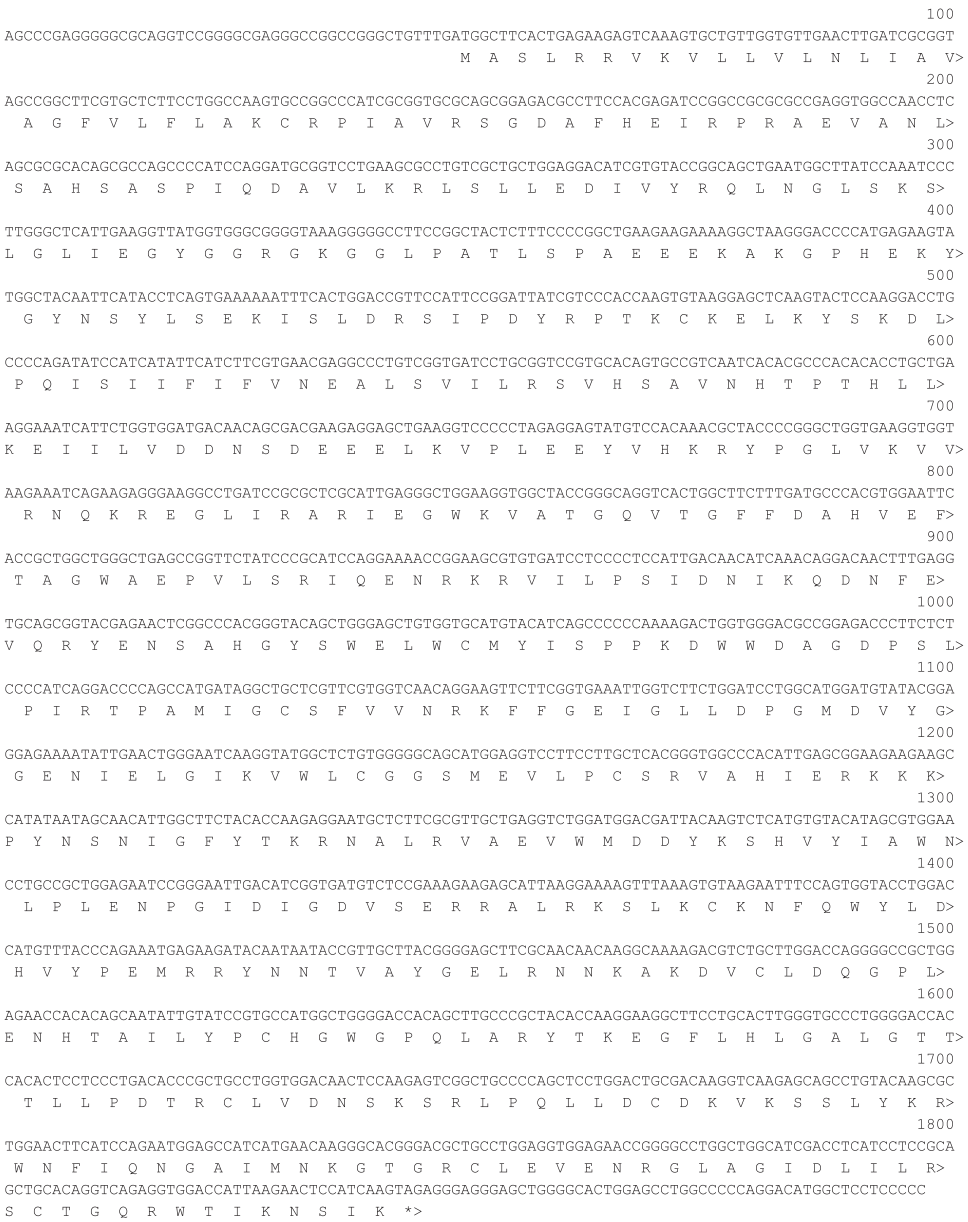


Fig. 1 Nucleic and amino acid sequences of human pt-GalNAc-T

The upper and lower lines indicate nucleic and putative amino acid sequences, respectively.

sidues [29], 4) conserved cysteine residues [29,31,32], and 5) a C-terminal lectin-like domain [33]. Among the known GalNAc-transferases, the cloned gene was most homologous to r-pt-GalNAc-T with 98% amino acid similarity. It also had 77% similarity to human GalNAc-T9. Judging from the sequence similarity to the other clones, the human clone obtained is regarded as an orthologue of

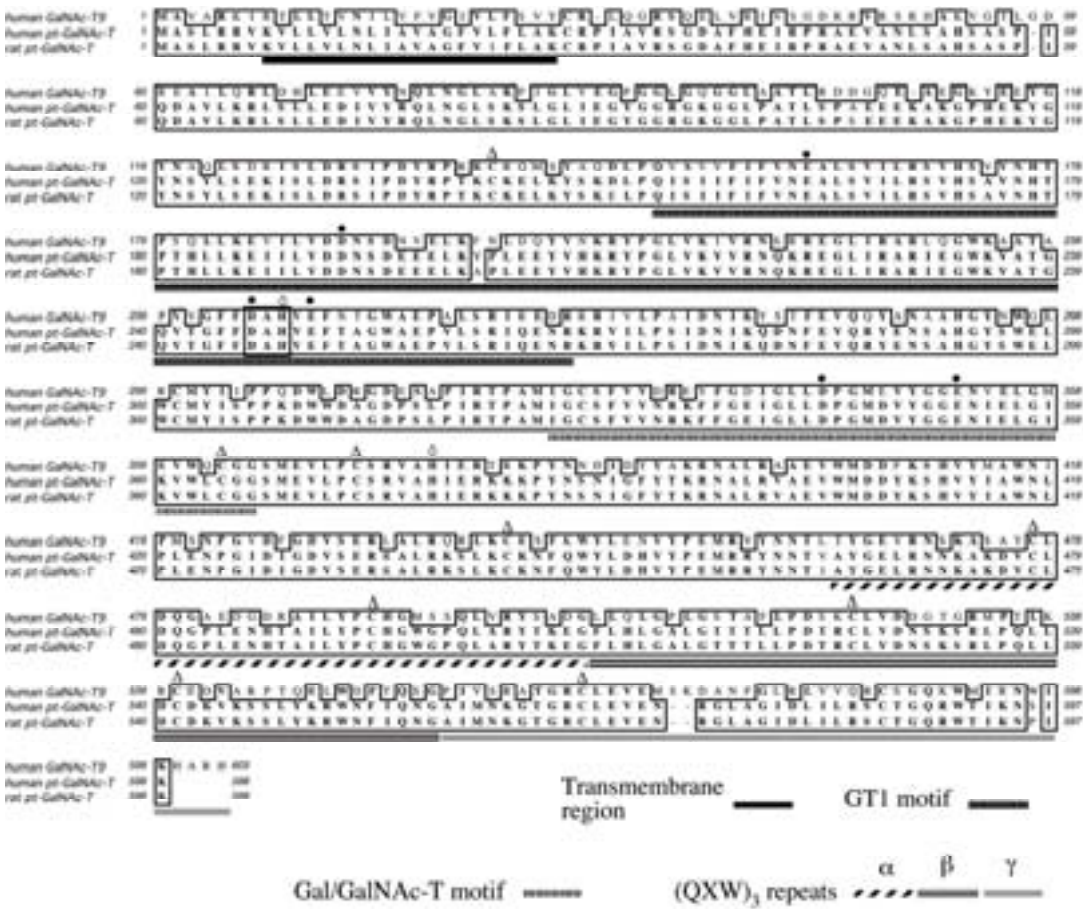


Fig. 2 Amino acid alignment of rat and human pt-GalNAc-T, and human GalNAc-T9. Amino acid alignment was performed using the pairwise and multiple Clustal W (1.4) method in MacVector. The parameters for alignment were: slow alignment, open gap penalty=10, extend gap penalty=0.05, matrix=blosum 30, delay divergence=10%, and no hydrophilic gap penalty. The DXH sequence in the glycosyltransferase 1 motif is outlined. Conserved acidic, histidine, and cysteine residues are indicated by*, ◇, and Δ, respectively.

rat pt-GalNAc-T. Hence, this clone was designated *human putative GalNAc-T (h-pt-GalNAc-T).*

3.2 Northern blot analysis of human pt-GalNAc-T

Williams-Beuren syndrome (WBS) is caused by the hemizygous deletion of a region of approximately 1.6 Mb of chromosome band 7q11.23 [34] and is a rare neurodevelopmental disorder characterized by cardiovascular abnormalities (particularly supravalvular aortic stenosis), transient juvenile hypercalcemia, abnormal weight gain and growth, and unusual facial features [35]. Recently, *h-pt-GalNAc-T* was found to be identical to one of the genes identified in the Williams-Beuren syndrome critical region (*WBSCR*). This gene, *WBSCR17*, was also most abundant in brain, with sig-

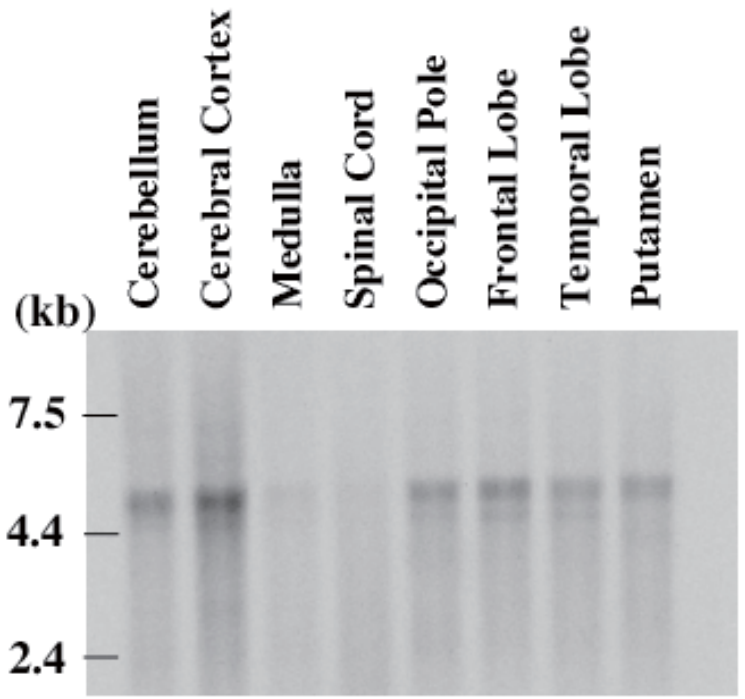


Fig. 3 Northern blot analysis of h-pt-GalNAc-T

Adult human brain multiple tissue Northern blot II (Clontech) was hybridized with a digoxigenin-labeled probe of h-pt-GalNAc-T, and detected as previously described [19].

Table 1 Synthetic undecpeptides used for assay

Origin	Sequences
human MUC1a	AHGVTSAPDTR
human MUC5AC	GTTPDPVPTTG
human MUC7	TTAAPPTPSAG

nificant amount in heart [36]. *h-pt-GalNAc-T* is, therefore, a potential candidate for the genes causing WBS. I investigated the expression of h-pt-GalNAc-T in human brain in more detailed by Northern blot analysis. Fig. 3 shows that h-pt-GalNAc-T was most strongly expressed in cerebral cortex. Its expression in cerebellum, occipital pole, frontal lobe, temporal lobe, and putamen was moderate. This is in contrast with human GalNAc-T9, which is most abundantly expressed in cerebellum, with the lesser amount in cerebral cortex [13]. In human, a single 5 kb mRNA of *pt-GalNAc-T* was observed, and this transcript being larger than rat one (2 kb) appears to result from difference of untranslated sequences.

3.3 Expression of human pt-GalNAc-T in insect cells

I constructed an expression vector for a soluble recombinant protein coded by *h-pt-GalNAc-T* and expressed a recombinant isozyme in High Five insect cells in order to examine the acceptor specificities of pt-GalNAc-T. After purification of recombinant h-pt-GalNAc-T with Ni-NTA agarose, an enzymatic activity of the recombinant molecule was examined using synthetic peptides derived from the potential O-glycosylation sites of mammalian mucins (Table 1). However, no transferase activity has been detected so far. This may result from several causes. First of all, pt-GalNAc-T may be a member of so-called follow-up type isozymes, which requires partial glycosylation of the acceptors in order to recognize them as substrates. In fact, prior glycosylation of the acceptor peptides was reported to be a prerequisite for glycosylation by GalNAc-T4, -T7, and -T10 [7,10,14]. Secondly, the transmembrane region of pt-GalNAc-T may be involved in the activity. Expression of the recombinant soluble molecules, therefore, may have generated inactive enzymes. Thirdly, pt-GalNAc-T may belong to other glycosyltransferase families involved in transferring different monosaccharide. It is, however, unlikely that pt-GalNAc-T catalyzes reactions other than the GalNAc transfer, since it has motif structures of GalNAc-transferase families, exhibiting very high homology (77%) to GalNAc-T9. Finally, judging from their restricted expression in the brain, pt-GalNAc-T may have a narrow substrate specificity for catalyzing brain-specific glycosylation, and may not glycosylate typical mucin-like sequences used in this study. Although I demonstrated presence of the brain-specific GalNAc-transferases such as GalNAc-T9 and pt-GalNAc-T, the catalytic activities have not been detected yet as described above. There are some examples of O-glycosylated glycoproteins in the brain. Tenascin-R is a brain glycoprotein with a disialylated core-1 structure, Siaα2 → 3Galβ1 → 3 (Siaα2 → 6) GalNAc, as a major carbohydrate chain, and is expressed in the spinal cord, cerebellum, hippocampus and olfactory bulb [37,38], colocalizing with r-pt-GalNAc-T except for in the olfactory bulb. Chromogranin A (CgA) is also found in the brain and spinal cord, as well as in the endocrine and immune systems, such as the pituitary gland, pancreas, spleen, and thymus [39]. *In situ* hybridization of CgA mRNA in rat brain sections revealed the strongest expression in the pyramidal cell layer of the hippocampus and the subiculum [39], where r-pt-GalNAc-T is abundantly expressed as well. β-Amyloid precursor protein (APP) is another example of O-glycosylation in the brain [40]. Both CgA and APP are known to accumulate in the extracellular β-amyloid plaque in Alzheimer's disease [39]. CgA is also reported to accumulate in Parkinson's disease and Pick's disease [41-43]. O-Glycosylation of α-synuclein is also reported [44]. α-Synuclein is a major component of intracellular fibrillary aggregates and regarded as implicated in the pathogenesis of Parkinson's disease [45]. Aberrant O-glycosylation may cause the conformational change in α-synuclein, resulting in deposition of abnormal filaments. Although the relation-

ship of h-pt-GalNAc-T with these pathological conditions is not clear, it is possible that the deposition of these proteins in the brain is related to altered O-glycosylation by pt-GalNAc-T. Some of these glycoproteins colocalize with pt-GalNAc-T in the brain, thus raising the possibility that they might be endogenous substrates of pt-GalNAc-T. Studies using synthetic peptides derived from the potential O-glycosylation sites of these glycoproteins are in progress.

3.4 Detabase analysis

To investigate biological function of a GalNAc-transferase, the author attempted to clone GalNAc-transferase orthologues from zebrafish and examine their expression and biochemical properties. First, the database of zebrafish genome and expressed sequence tags was examined to isolate zebrafish orthologue genes for the mammalian GalNAc-transferases. The search revealed many genome and EST sequences homologous to each human isozyme with the exception of GalNAc-T15. These sequences were compared to generate contigs and the obtained contig sequences were aligned with the known GalNAc-transferases using the ClustalW program. The alignment shows that all the GalNAc-transferase cloned so far except GalNAc-T15 were conserved between human and zebrafish (Fig. 4). It is often observed that zebrafish has two homologous sequences of the mammalian equivalent [46], probably due to the additional genome duplication in zebrafish. In zebrafish, the function of ancestral genes may be allotted to two genes and each gene sometimes has a more restricted function than the original gene. Thus, the function of a zebrafish gene is often easier to study than mammalian ones [46]. In fact, zebrafish *GalNAc-T4*, *-T8*, and *-T11* genes appear to be duplicated, since two homologous genes for each mammalian isozyme have been identified in the database. Fig. 4 shows the phylogenetic tree generated based on the homology of amino acid sequences of the putative catalytic domain of the isozymes. Presence of the GalNAc-transferase family has been reported in some model organisms, such as nematode [47], and *Drosophila* [16,48,49], but it is often difficult to relate an isozyme in those organisms to those in mammals due to the low homology. By contrast, the zebrafish isozymes are highly homologous to mammalian ones. This indicates that zebrafish is a useful model organism to study biological roles of the mammalian GalNAc-transferases.

3.5 Isolation of zebrafish orthologues for the mammalian brain-specific GalNAc-transferases

Zebrafish is useful in studying a gene function as well, because morpholino-oligo based gene knock-down technique is available, in which two or more genes can be knocked-down simultaneously. To elucidate roles of O-glycans in brain, the author cloned orthologues of the mammalian brain-specific isozymes (*GalNAc-T9*, *-T13*, and *pt-GalNAc-T*) from zebrafish (Fig. 5). The ze-

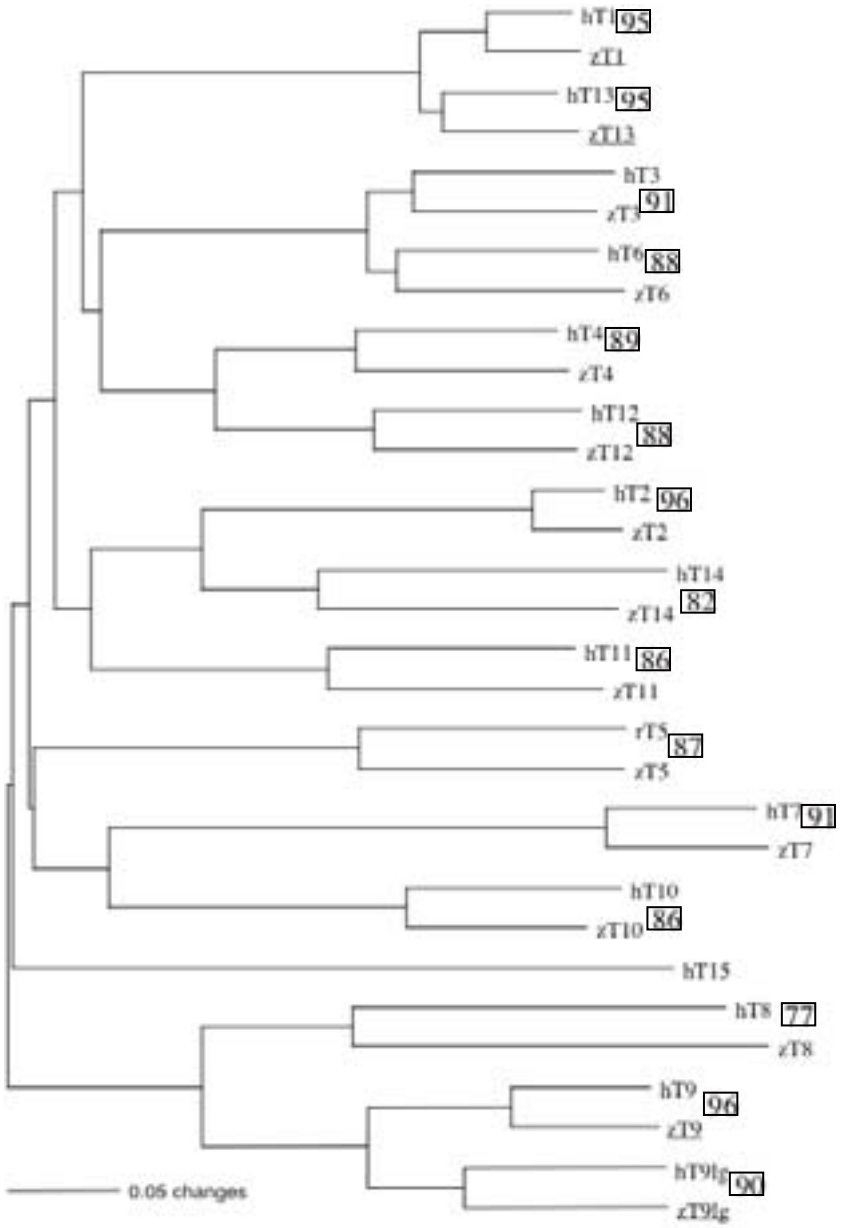


Fig. 4 Phylogenetic relationship between zebrafish and human GalNAc-transferases

A phylogenetic tree was constructed by comparing the putative catalytic sequences of the isozymes. An NJ image method of the PAUP* version 4.0 was used for analysis. The scores outlined show amino acid similarity of the conserved region between zebrafish and human orthologues. As described in the text, there were two zebrafish orthologue genes for GalNAc-T4, -T8, and -T11. For these isozymes, only the gene with the higher homology to the human equivalent was included in the tree.

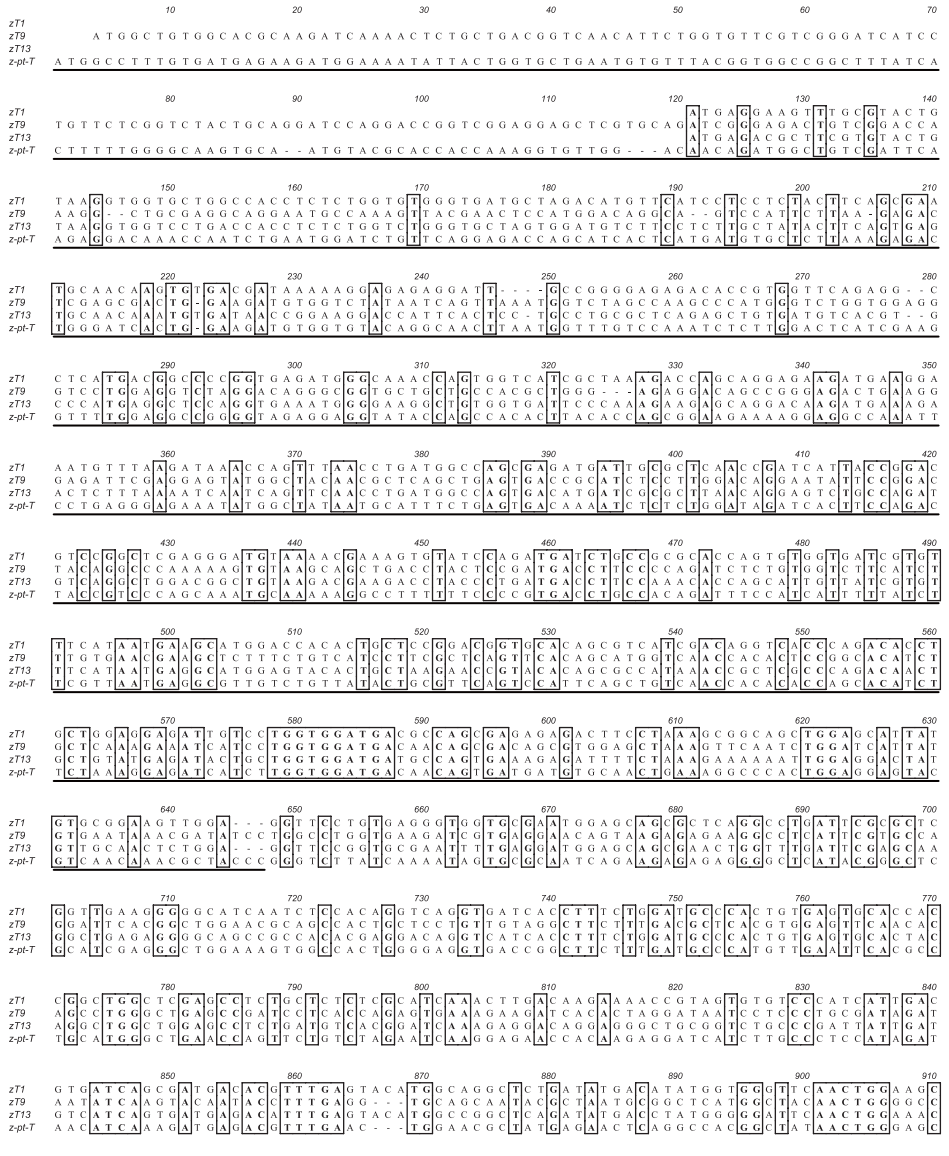
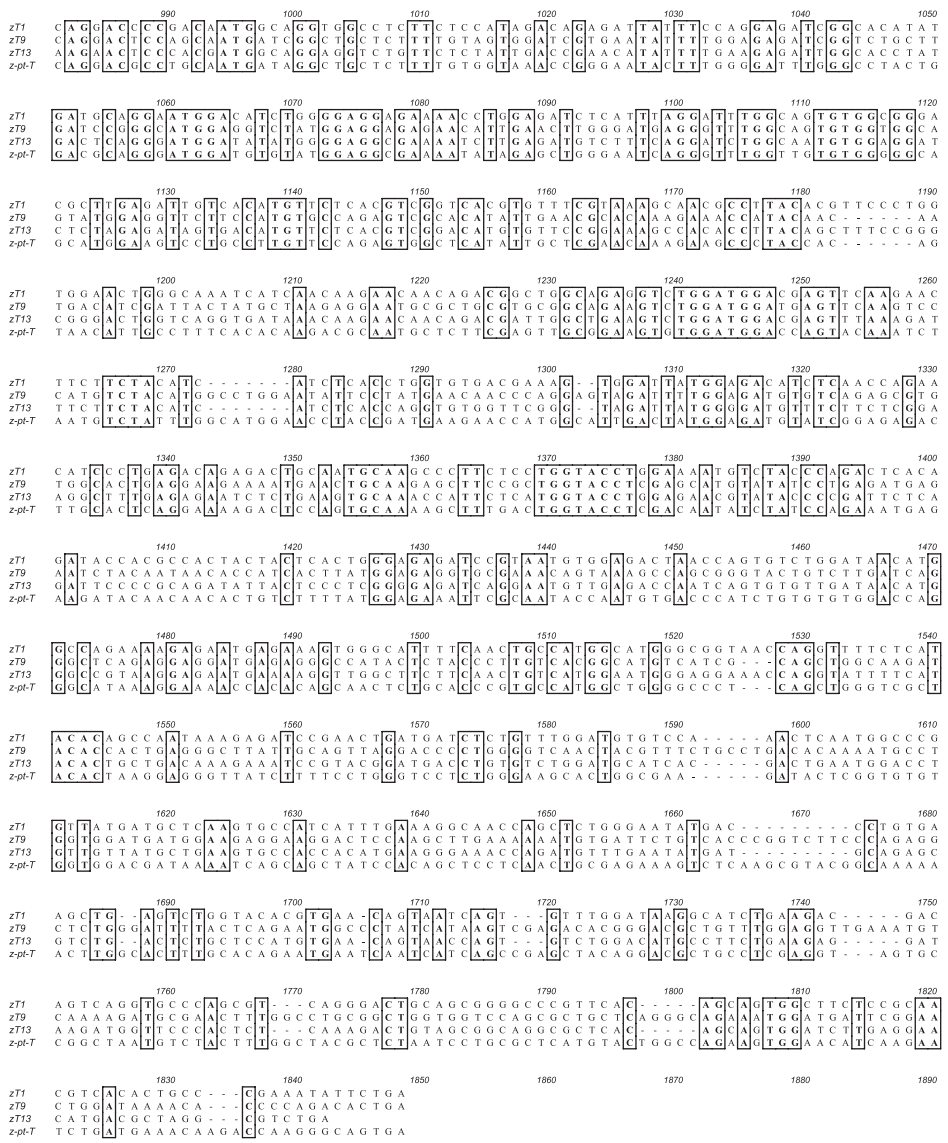


Fig. 5 Nucleic acid alignment of *zebrafish GalNAc-Ts*, Nucleic acid alignment was performed using the pairwise and multiple Clustal W (1.4) method in MacVector. The parameters for alignment were: slow alignment, open gap penalty=10, extend gap penalty=5.0, and delay divergence=40%. Underlined sequences correspond to *z-pt-GalNAc-T* antisense RNA probe used in Whole-mount *in situ* hybridization.

brafish orthologue for *GalNAc-T1* was also cloned (Fig. 5) which, among the isozymes, is most ubiquitously and abundantly expressed in the tissue including the brain [6]. The zebrafish clones were isolated by PCR, based on the sequence information obtained from the database. Figs. 6A to -



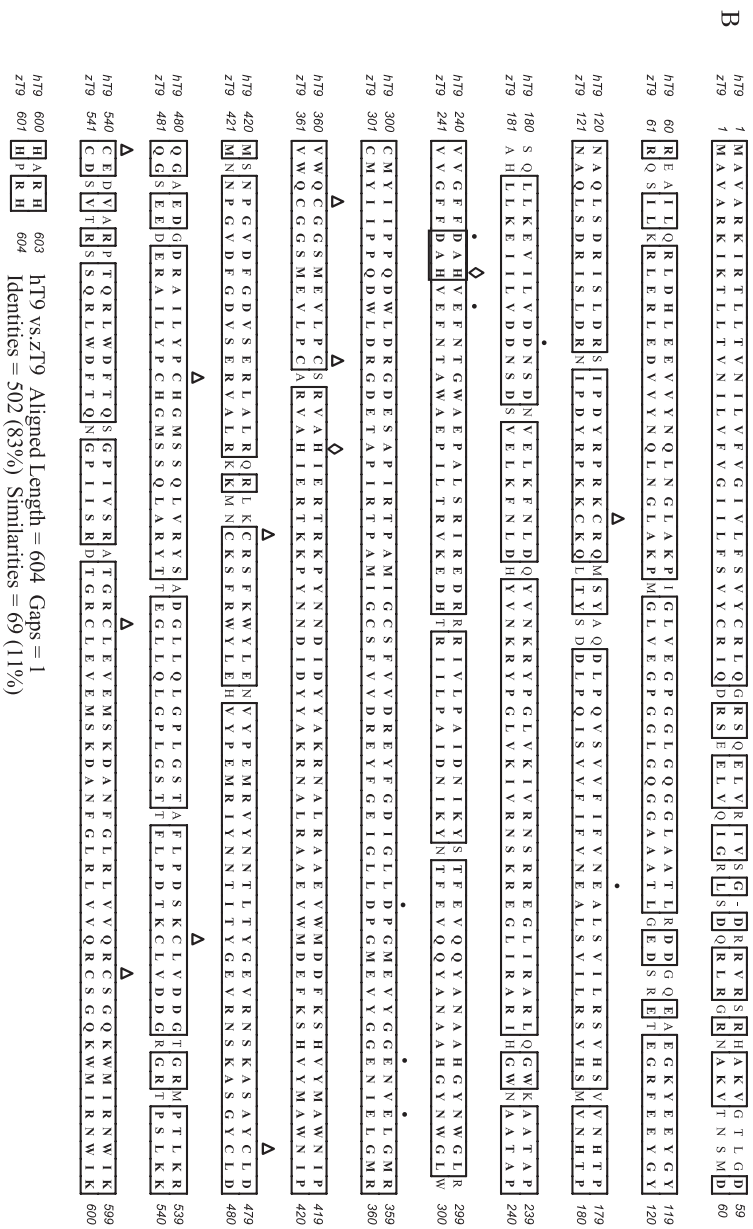
6D show the amino acid sequences of the obtained zebrafish clones, in comparison with their human orthologues. Amino acid sequence comparison between zebrafish and mammalian isozymes indicates high homology, with 96, 94, 94, and 80% homology to human GalNAc-T1, -T9, -T13, and pt-GalNAc-T, respectively. It is, therefore, expected that analysis of zebrafish orthologues helps uncover the biological roles of the mammalian GalNAc-transferases in the brain.

3.6 Whole-mount in situ hybridization of zebrafish pt-GalNAc-T

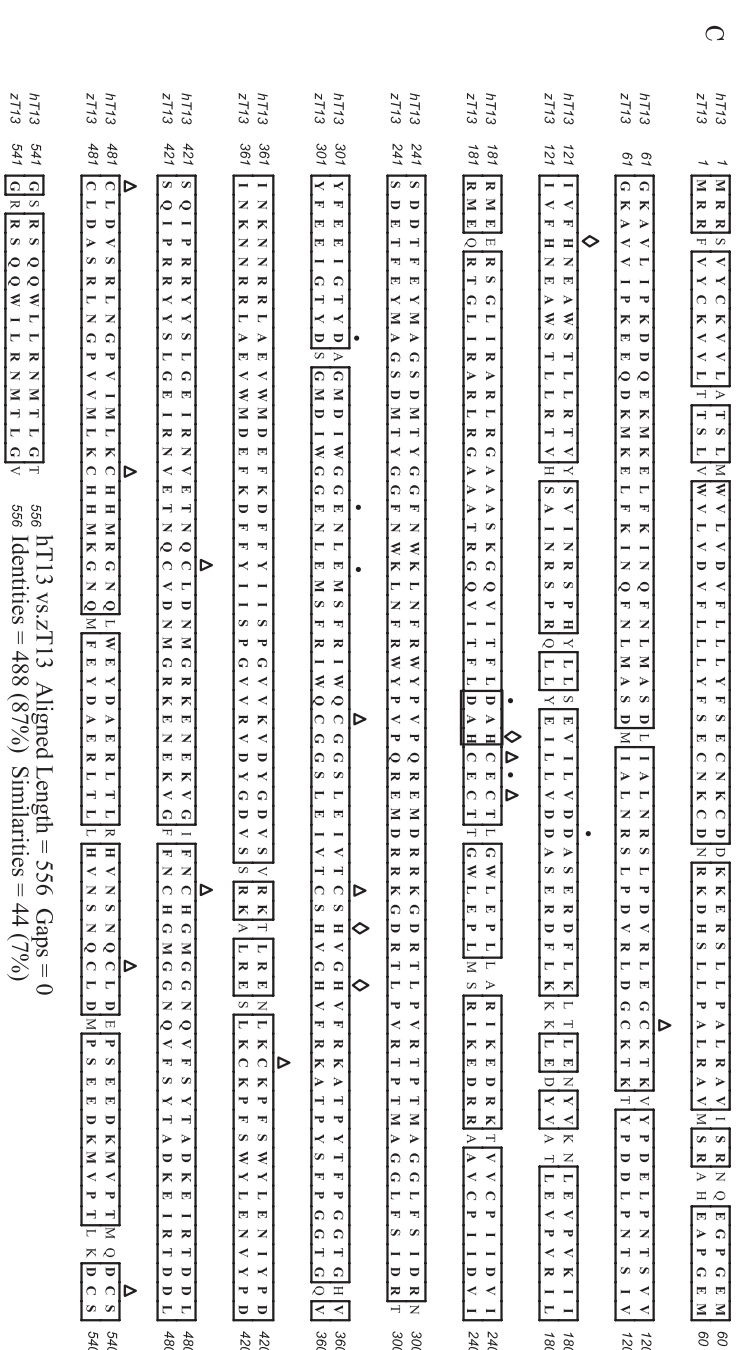
The author first investigated the expression of pt-GalNAc-T in the zebrafish embryos. Whole-

Fig. 6 Amino acid alignment of zebrafish and human orthologues

Amino acid alignment was performed as described in the legend to Fig. 2. The DXH sequence in the glycosyltransferase 1 motif is outlined. Conserved acidic, histidine, and cysteine residues are indicated by •, ◇, and Δ, respectively. The orthologues between human and zebrafish were compared. A, GalNAc-T1, B, GalNAc-T9, C, GalNAc-T13, and D, pt-GalNAc-T.



mount *in situ* hybridization of z-pt-GalNAc-T indicated that this gene is expressed throughout central nervous system including telenthephalon, midbrain, hindbrain, and eyes from 36 to 48 hours post fertilization (hpf) (Fig. 7). The author previously reported that rodent and human orthologues of this clone is specifically expressed in the brain as well [27]. The similar expression patterns of these isozymes in different organisms may suggest their functional similarity in each organism.



3.7 Knock-down analysis of zebrafish pt-GalNAc-T expression

The author, then, analyzed the function of z-pt-GalNAc-T in the development by the morpholino oligo-mediated knock-down of z-pt-GalNAc-T expression. Injection of 5 ng morpholino antisense

oligos into the two-cell stage embryos led to a drastic change in the phenotype in 7 out of 10 injected embryos, with characteristically smaller eyes and heads (Fig. 8A, B). Although preliminarily, the author knocked-down the expression of zebrafish GalNAc-T9 and -T13 in the embryos. Interestingly, the knock-down of GalNAc-T9, a highly homologous clone to pt-GalNAc-T, gave the similar phenotypic changes with the characteristically small eyes. Contrary to this, no phenotypic changes

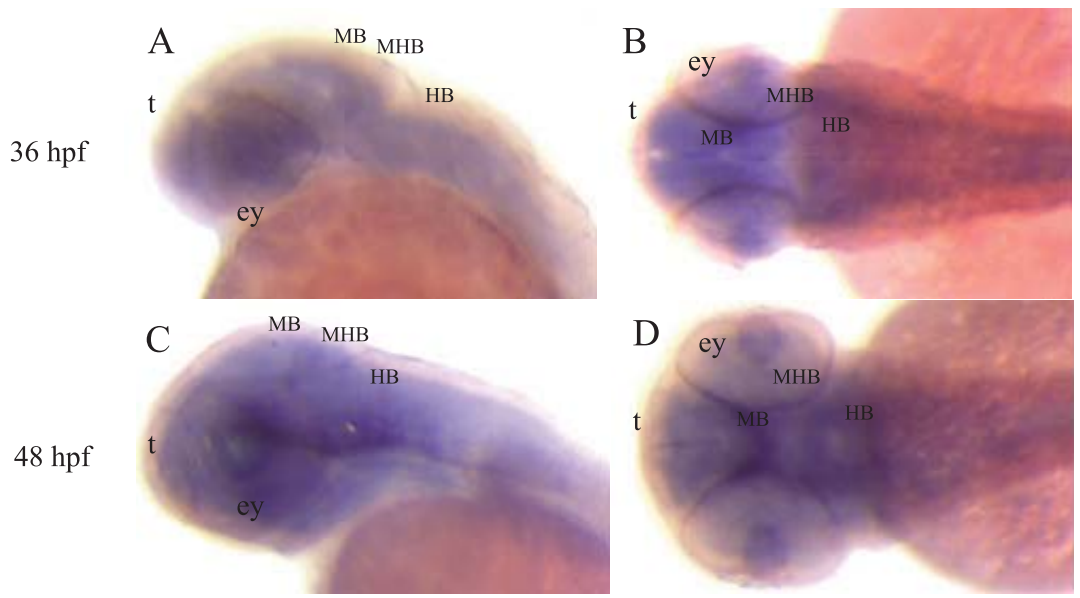


Fig. 7 Whole-mount *in situ* hybridization analysis of z-pt-GalNAc-T

Zebrafish embryos at the 36 hpf (A, B), 48 hpf (C, D) were hybridized with labeled RNA probes specific for z-pt-GalNAc-T. Orientation: (A, C) lateral views, dorsal at the top; (B, D) dorsal views, anterior at the top. Abbreviations : t, telencephalon; ey, eye; MD, midbrain; MHB, midbrain-hindbrain boundary; HB, hindbrain

were observed in the GalNAc-T13 knock-down embryos. It is reported that the knockout mice of GalNAc-T13 also had no remarkable phenotypic changes [18]. To observe the brain structure in detail, the embryos, injected with morpholino oligos, were depigmented by treating them with 0.2 mM PTU that inhibits melanization of the embryos (Figs. 8C-G). The transparent embryos thus obtained clearly demonstrated the brain malformation of the z-pt-GalNAc-T morphants, with the most manifest deformation in the hindbrain (Fig. 8D, F). Some of these morphant embryos also represented an enlarged ventricle in addition to the eye and brain disorders (Fig. 8G).

3.8 Marker analysis of zebrafish pt-GalNAc-T morphants

To examine whether z-pt-GalNAc-T is indeed implicated in the brain formation, the author investigated the expression of pax2.1 in the 36, 48 hpf embryos, which is a paired-box transcription factor and is one of the earliest genes to be specifically activated in the development of midbrain and midbrain-hindbrain boundary (MHB) [50,51]. After the 20 somite stage, pax2.1 is expressed in the posterior hindbrain [52]. The author found that the z-pt-GalNAc-T knock-down zebrafish lost the pax2.1 gene expression at the hindbrain, but with the expression in the choroid fissure unchanged (Fig. 9). These data indicate that z-pt-GalNAc-T may be related to normal development of hindbrain.

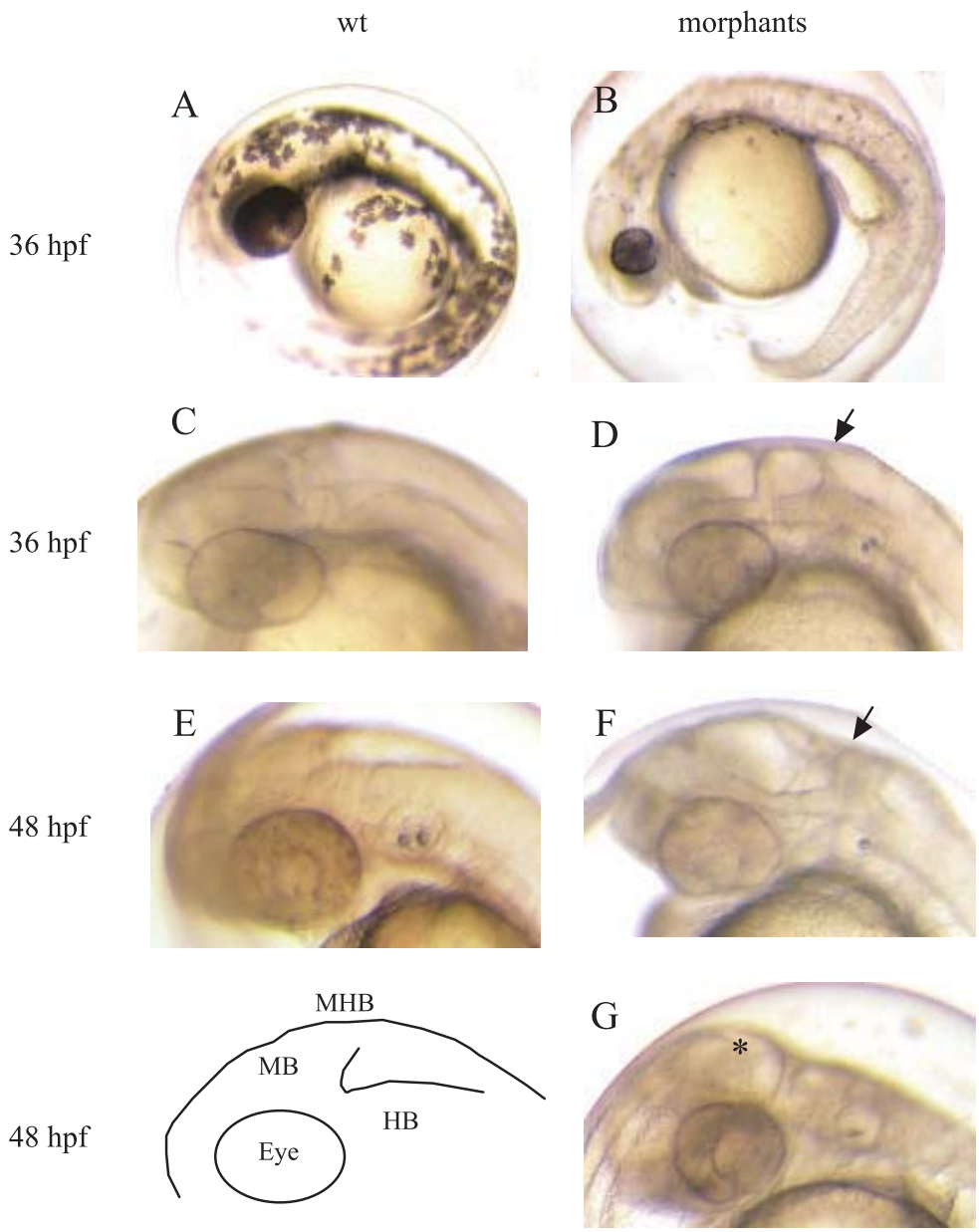


Fig. 8 Morpholino-induced knock-down of z-pt-GalNAc-T

Morphological analysis of embryos injected with 5 ng of z-pt-GalNAc-T morpholino oligos was carried out. In all embryos, anterior is towards the left and dorsal is upwards. (A, C, and E) Wild-type, (B, D, F, and G) z-pt-GalNAc-T morpholino-injected embryos. The melanization of embryos (C to G) was inhibited by treatment of PTU. Arrow and asterisk indicate hindbrain malformation (D, F) and the enlarged ventricle (G), respectively. Abbreviations; MB, midbrain; MHB, hindbrain; HB, hindbrain

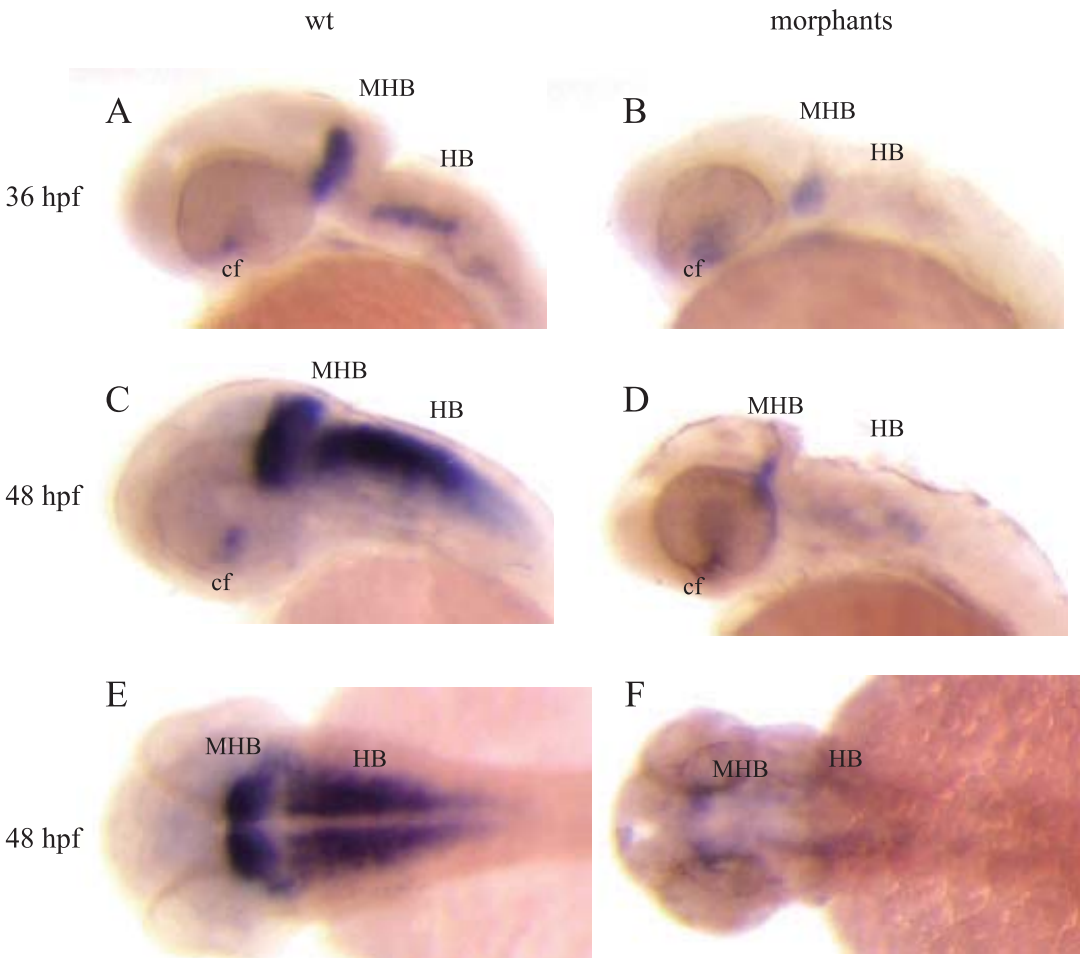


Fig. 9 Expression of pax2.1 in the zebrafish embryos injected with the z-pt-GalNAc-T morpholino oligos

Expression of pax2.1 at 36 hpf and 48 hpf is shown in Wild-type (A, C, and E) and the embryos injected with 5 ng of z-pt-GalNAc-T morpholino oligos injected embryos (B, D, and F). Expression of pax2.1 is absent in the hindbrain of the morphant embryos. Orientation: (A-D) lateral views, dorsal at the top; (E, F) dorsal views, anterior at the top. In all embryos, anterior is towards the left and dorsal is upwards. Abbreviations: cf, choroid fissure; MHB, midbrain-hindbrain boundary; HB, hindbrain.

For normal development of vertebrate brain, partitioning of neural epithelium into domains is essential, which involves constriction formation of the neural epithelium in the posterior forebrain, at the midbrain-hindbrain junction, and in the hindbrain [53]. In anterior-posterior patterning, spatially restricted expression of transcription factors that specify regional identity plays an important role. For example, transcription factors such as val/Kr, Krox20, Hox, and Notch, are required for a hierarchial segmentation of the zebrafish hindbrain [54-60]. The author will investigate whether the knock-down of z-pt-GalNAc-T affects the expression of these molecules in the developing brain

of the morphants.

3.9 Relationship of pt-GalNAc-T with Williams-Beuren Syndrome

Merla et al. reported that human pt-GalNAc-T is expressed in heart and brain in adults [36]. In this report (Fig. 3), the author demonstrated its expression pattern in human brain in more detail by Northern hybridization. It was most strongly expressed in cerebral cortex, with the lesser amount in cerebellum, occipital pole, frontal lobe, and temporal lobe. As for the rat pt-GalNAc-T, *in situ* hybridization of coronal sections of the adult rat brain showed the mRNA expression in hippocampus, cerebral cortex, and cerebellum [27]. In embryonic day 19 mice, this gene was strongly expressed in dienthephalon, cerebellar primordium, and dorsal root ganglion [27]. As described above, it was recently reported that human pt-GalNAc-T is located at the WBSCR, chromosome 7q11.23 [36]. WBS is an autosomal dominant disorder characterized by heart and vascular disease, mental retardation, and dysmorphic facial features, called elfin face, etc [61]. The brain-specific expression of pt-GalNAc-T in mammals and zebrafish, together with the fact that the gene for h-pt-GalNAc-T was located at the critical region of WBS and that patients with WBS have severe neurodevelopmental disorder, raises the possibility that h-pt-GalNAc-T may be involved in the pathogenesis of WBS. The increased volume of cerebrospinal fluid in individuals with this syndrome is reported [62]. It is noticeable that the enlarged ventricle size was also observed in the z-pt-GalNAc-T morphants (Fig. 8G). Patients with WBS have dysmorphic facial disorder called elfin face including characteristic eye features, with a stellate pattern of iris and strabismus, which are one of the useful diagnostic clues [63-66]. The abnormal eye formation of the z-pt-GalNAc-T morphants also supports the relationship of this isozyme with WBS. Taken together, it is concluded that z-pt-GalNAc-T is involved in the normal development of zebrafish brain and eyes. The possible involvement of human pt-GalNAc-T in WBS was also discussed.

Acknowledgments

I am extremely grateful to Dr. Akira Kurosaka, Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, for great help on the entire study. I am very grateful to Dr. Nobuyuki Itoh, Graduate School of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyoto University for helpful comments on the manuscript. I am thankful to Dr. Shinya Toba, Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, for advice and help on this study. I am grateful to Dr. Ayumi Miyake and Dr. Morichika Konishi, Graduate School of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyoto University for kindly advice on the zebrafish experiments and molecular biology. This work was in part supported by Grants-in-

Aid for Scientific Research from Japan Society for the Promotion of Science, and the Foundation for Bioventure Research Center from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Footnotes

Abbreviation: AAP, s-amyloid precursor protein; CgA, Chromogranin A; GalNAc, N-acetylgalactosamine; hpf, hours post fertilization; MHB, midbrain-hindbrain boundary; NJ, neighbor-joining; PBS, phosphate-bufferd saline; PCR, polymerase chain reaction; PTU, 1-phenyl-2-thiourea; SSC, standard saline citrate; WBS, Williams-Beuren syndrome; WBSCR, Williams-Beuren syndrome critical region;

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Zebrafish を用いた脳特異的 UDP-GalNAc : polypeptide N-acetylgalactosaminyltransferase の解析

中　村　直　介

要　旨

ムチン型糖鎖の生合成の開始反応を触媒する UDP-GalNAc : polypeptide N-acetylgalactosaminyltransferase（以降 GalNAc 転移酵素）はペプチド鎖上のムチン型糖鎖の位置と数を決定する重要な酵素である．GalNAc 転移酵素は大きな遺伝子ファミリーを形成しており，現在までにほ乳類で15種類のアイソザイム遺伝子がクローニングされている．これらのアイソザイムは互いに重複しながらも特有の基質特異性を示し，さらに時・空間的にも異なった発現様式を示す．このうち，筆者は，脳特異的に発現するアイソザイムの生物学的役割の解明を目的として，モデル生物を用いてこれらのアイソザイムに関する研究を行った．

筆者はまず，遺伝子の相同性に基づいて PCR を行い，脳特異的なアイソザイムである GalNAc-T9 に対して高い相同性を持つ，新規ヒト cDNA クローンを脳より単離した．この cDNA のヌクレオチド配列を解析したところ，この分子は GalNAc 転移酵素ファミリーに特徴的な構造を持つ事が分かった．従って，このクローンを pt-GalNAc-T と呼ぶ事にする．また，ヒト pt-GalNAc-T は，心臓と脳にて強く発現しており，神経発生疾患である Williams-Beuren 症候群患者の染色体欠失領域に存在する WBSCR17 遺伝子と同一のものである事が分かった．脳での pt-GalNAc-T の発現をさらに詳細に調べるために，ノーザンブロット法にて pt-GalNAc-T mRNA の発現を調べたところ，大脳皮質で最も多く，さらに小脳にて5.0 kb の mRNA を検出した．この分子の生化学的特徴を解析するために，可溶性の組換え分子を昆虫細胞にて発現させた．しかしながら，組換え h-pt-GalNAc-T は，ほ乳類のムチン由来の数種類のペプチドに対しては糖転移活性を示さなかった．pt-GalNAc-T は脳特異的な発現様式を示す事から，この分子が限定された基質特異性を持ち，脳内のタンパク質への糖鎖付加反応を触媒している可能性が考えられた．

次に，筆者は脳特異的アイソザイムの生物学的役割を調べるために，モデル生物のゼブラフィッシュにおいて脳特異的 GalNAc 転移酵素発現のノックダウン実験を行った．著者はまずゼブラフィッシュの脳特異的 GalNAc 転移酵素のオーソログ遺伝子のクローニングを試みた．ほ乳類アイソザイムに対応するゼブラフィッシュ遺伝子をデータベースから検索したところ，GalNAc-T15 を除く，14種類のオーソログ遺伝子を見いだした．そのうち，多くの組織で発現する GalNAc-T1 cDNA と脳特異的な GalNAc-T9，-T13，pt-GalNAc-T cDNA をゼブラフィッシュより単離した．これらの遺伝子は，哺乳類の相同遺伝子に対してアミノ酸配列で，それぞれ96, 94, 94, 80％の相同性を示した．筆者は，これらのアイソザイムの中で，pt-GalNAc-T のゼブラフィッシュにおける発現を whole mount *in situ* hybridization（WISH）により調べた．zebrafish pt-GalNAc-T の mRNA は受精後36.48時間胚にて前脳，中脳，後脳を含む中枢神経系に広く発現していた．また，眼における強い発現も観察された．さらに，モルホリノアンチセンスオリゴを用いて，pt-GalNAc-T の発現を阻害した時，眼や後脳で発生異常が見られた．さらに，これらの胚の後脳では，

pax2.1の発現が消失しており，本酵素がゼブラフィッシュの後脳の発生に関与している可能性が示唆された．以上より，Williams 症候群の原因遺伝子の 1 つと考えられる pt-GalNAc-T が後脳発生に関わっている事が分かった．

キーワード：ムチン型 O-グライコシレーション，GalNAc 転移酵素，脳，ゼブラフィッシュ