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Molecular Cloning and Functional Analysis of Sialyltransferases[1]

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To elucidate the regulatory mechanism for carbohydrate expression and to understand the meaning of the carbohydrate-structural diversity, we started to clone sialyltransferase (ST) genes based on two different strategies, *i.e.* expression and homology cloning. So far, 13 STs have been cloned in our laboratory, 7 of which turned out to be new ones. The primary enzyme structures deduced from all the cloned ST genes suggest a putative domain structure with a type II transmembrane topology. There are no significant amino acid sequence similarities among these cloned STs, except for in two sialyl motifs, L and S, which are proposed to be the CMP-sialic acid recognition and/or catalytic sites. Northern blot analysis revealed the developmental stage-dependent and/or tissue-specific expression of most of the cloned STs. The cloned STs are classified into four families according to the carbohydrate linkages they synthesize, *i.e.* the ST3Gal-, ST6Gal-, ST6GalNAc-, and ST8Sia-families. Generally, enzymes in these families exhibit strong activity toward certain acceptor groups but show very weak activity toward other acceptor groups, and the substrate specificities of the enzymes overlap one another, as indicated by *in vitro* experiments. Enzymes in the ST3Gal-family are expressed mainly in a tissue-specific manner. However, those in the ST6GalNAc- and ST8Sia-families are expressed in a tissue- as well as developmental stage-specific manner. *In vivo* conditions are supposed to be more complex. Therefore, it is quite important to examine their substrate specificities *in vivo* and the mechanism of their expression to elucidate the physiological role of each enzyme and the meaning of the diversity in carbohydrate structure. Using cloned cDNAs and expressed enzymes, we have been studying how sialylcarbohydrate expression is regulated and what the functions of sialylcarbohydrate chains are. Recently, we found that transfection of the GD3 synthase, an α2,8-ST (ST8Sia I), gene triggers cholinergic neuritogenesis in Neuro2a cells through the *de novo* expression of GD3, suggesting that the GD3 synthase gene behaves as a neural differentiation inducer.

Key words: cloning, differentiation, glycosyltransferase, sialic acid, sialyltransferase.

Sialic acids and their derivatives are ubiquitous at the terminal positions of oligosaccharides of glycoproteins and glycolipids in tissues of various animal species. They play important roles in a large variety of biological processes, such as cell-cell communication, cell-matrix interactions, maintenance of serum glycoproteins in the circulation, and so on (*1*). The expression of sialyl-glycoconjugates changes during development, differentiation, and oncogenic transformation (*2*). Two basic questions need to be answered in order to understand the roles of sialyl-glycoconjugates, (i) how is their expression regulated and (ii) why do sialyl-glycoconjugates exhibit remarkably diverse structures. As shown in Table I, there are at least 12 sialyl-carbohydrate structures in glycoconjugates. Also, these questions need not only to be addressed for sialyl-carbohydrate structures, but also for other carbohydrate structures. Therefore, the strategy applied to sialyl-glycoconjugates should be applied to other glycoconjugates.

The transfer of a sialic acid from CMP-Sia to an acceptor carbohydrate is catalyzed by a family of glycosyltransferases called sialyltransferases (STs) (*3*). Studies involving biosynthetic analyses with membrane fractions suggested that each linkage is formed by an individual enzyme. Some enzymes are considered to discriminate aglycon-structures as well. Thus, more than 12 different STs are presumably required to synthesize all known sialyl-oligosaccharide structures. To answer the above questions, precise knowledge on the enzymes themselves and the gene structure of each ST is prerequisite. In this review, I briefly summarize the results regarding the structures and functions of STs.

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Abbreviations: The nomenclature for gangliosides follows the system of Svennerholm (*77*). In this review, I use abbreviations for the cloned sialyltransferases (see Table II). In each case, ST denotes sialyltransferase, the number shows the linkage, and the final part is the acceptor carbohydrate. Finally, the members of each group are distinguished using Roman numerals. S.G., submaxillary gland; PSA, polysialic acid; N-CAM, nuclear cell adhesion molecule.

TABLE I. **Commonly found sialyl-oligosaccharide structures.**

Linkage	Sialyl-oligosaccharide structure	Representative glycoconjugates
α2,3	NeuAcα2-3Galβ1-4/3GlcNAc	*N*-Glycan, polylactosamine, ganglioside
	NeuAcα2-3Gal(β1-4Glc)β1-1′-Cer	Ganglioside
	NeuAcα2-3Galβ1-3GalNAc	*O*-Glycan, ganglioside
α2,6	NeuAcα2-6Galβ1-4/3GlcNAc	*N*-Glycan, polylactosamine, ganglioside
	NeuAcα2-6GalNAc	*O*-Glycan
	Galβ1-3[NeuAcα2,6]GalNAc	*O*-Glycan, ganglioside
	Galβ1-4/3[NeuAcα2,6]GlcNAc	*N*-Glycan, ganglioside
α2,8	NeuAcα2-8NeuAcα2-3Gal	Ganglioside
	[NeuAcα2-8]$_n$NeuAcα2-3Gal	*N*-Glycan (N-CAM, α-subunit of N^+-channel), ganglioside

I. Molecular cloning and expression of STs

1. Strategy for new ST cloning

a) Sources: Since it is very difficult to interpret the different results of kinetic studies for one particular ST from different animal species or different tissues of the same species, it is important that the enzyme source is restricted to one particular animal or one particular tissue when we study the diversity of STs and their regulation. We initially planned to focus our studies on STs in mouse neural tissues. However, a large number of papers have reported the occurrence of different types of chick embryo STs and their embryonal stage-dependent regulation (*4*). Thus, we decided on the following strategy, to first clone some STs from chick embryos and then to find the mouse homologues using the chick clone information.

b) Cloning methods: To clone ST cDNAs using the sequence information on purified enzymes is a sound and basic, but difficult way, because of their low abundance and the instability of their activity. We used two different strategies, *i.e.* PCR and expression cloning. When we have all the materials, including antibodies, expression vectors, and recipient cells, expression cloning is indeed a powerful means of cloning a cDNA encoding a glycosyltransferase. However, with the lack of one of the materials it is extremely hard to use this method. On the other hand, PCR cloning is a powerful method when there is a highly conserved region in the gene that has been found in cloned members of the same gene family. The merits of the PCR cloning method (Fig. 1) can be summarized as follows: (i) the purification of STs and preparation of specific antibodies are not required, and (ii) it is possible to clone every member of an entire gene family once one member of the family has been cloned. On the other hand, the demerits are (i) cloning is not possible without highly conserved region(s), and (ii) it is absolutely necessary to express the enzymes and identify the enzymatic activities. The use of oligonucleotides corresponding to the consensus sequences encoding the DNA-binding domains of the steroid and thyroid hormone receptor superfamily as probes has led to the isolation of cDNAs of this superfamily (*5-7*). Similarly, homeobox-containing genes have been isolated using degenerate oligonucleotides corresponding to the most conserved amino acid sequences in the homeodomain (*8*). At the beginning of our project in 1991, the cDNA sequences of two STs (rat ST6Gal I and porcine ST3Gal I) were reported by Paulson and coworkers (*9, 10*). Comparison of these amino acid sequences revealed highly conserved regions (*11*), named sialyl motifs L and S (*12*), which are not found in other glycosyltransferases. The conservation of the sialyl motifs suggested that other members of the ST gene family might contain these motifs. Thus, we decided to use the PCR cloning strategy to hunt for cDNAs encoding new STs using the sialyl motif sequences.

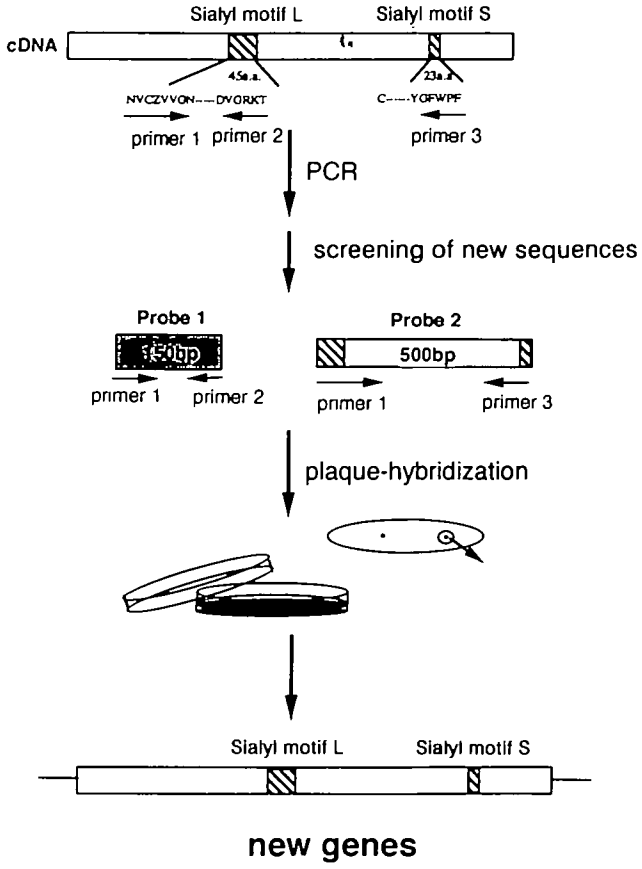

Fig. 1. **Brief illustration of how to perform PCR-cloning of the ST-family.** Using degenerate primers, PCR was performed using a mouse cDNA library as a template, followed by screening of new sequences. We have two criteria for the screening of PCR-fragments, as follows: First, the length of the fragments is 150 or 500 bp, depending on the primer combination. Second, do the new fragments contain other conserved amino acids or not? After screening of candidate fragments, the cDNA which has an open reading frame is obtained by plaque-hybridization.

c) Expression methods: When we have a cDNA with an open reading frame, expression of the enzymatic activity is necessary to determine the substrate specificity. The expression vector commonly used is pcD-SRα (*13*) or its modified form. When a vector containing the putative full

length coding region of the ST gene is expressed in COS-7 cells, the enzyme should be sorted into the Golgi apparatus, and thus its activity can be measured in a cell lysate. This method has the drawback that we need to prove whether the transferase activity is due to the transfected cDNA or COS-7 cells. Also, STs belong to the type II membrane protein family and the transmembrane region sometimes interferes with expression of the enzymes. To avoid these difficulties, we transfected an expression vector which includes only the stem and active domains fused with the IgM signal peptide sequence. With this method, the enzyme should be secreted into the culture medium, and thus its activity can be detected in the medium. When the enzyme activity is still low, we include the IgG-binding domain of protein A in the vector and concentrate the activity on an IgG-Sepharose column. These methods were applied to a transient expression system. In some cases, we also used a stable expression system, which includes a vector, pcD-SRα-Hyg or pSR-bsr, and hygromycin B or blasticidine selection.

The above expression strategy principally has the following possibility: the excluded regions, *i.e.* cytosolic-, transmembrane-, and occasionally stem-regions, might affect the substrate specificity. de Vries *et al.* reported that protein-A chimeric Fuc-T III and V showed increased activity toward glycoproteins, whereas they exhibited decreased activity toward glycosphingolipids, compared to the full-length enzyme (*14*). Whenever we try to determine the substrate specificity of an expressed enzyme, we always keep in mind the above possibility, and examine the effect of the fusions as far as we can.

2. General features of cloned STs

Using the above two cloning strategies, we have cloned more than 15 ST candidate species. After transfecting expression plasmids, we concentrated the enzyme activity and then carefully determined the substrate specificity. On the basis of the results, we were able to obtain 13 ST-cDNA clones, including 7 new ones, from mouse and four clones from chick. The STs so far cloned and reported are summarized in Table II. The underlines and asterisks indicate our works and new ones, respectively. Three of those listed, ST3Gal II, ST6GalNAc II, and ST8Sia III, were not previously known to exist and might only be obtained by the PCR cloning method, *i.e.* not by other methods. This indicates that the PCR cloning strategy is very powerful for cloning ST-cDNAs.

In the EMBL gene database, the sequence data for almost 40 clones of 13 species of STs from various animal species have been deposited. I present a dendrogram based on the available sequence data (Fig. 2). The dendrogram suggests an interesting notion. The primary criteria for classifying these clones are the linkage and acceptor specificities. The species is secondary. This ordered state suggests that a

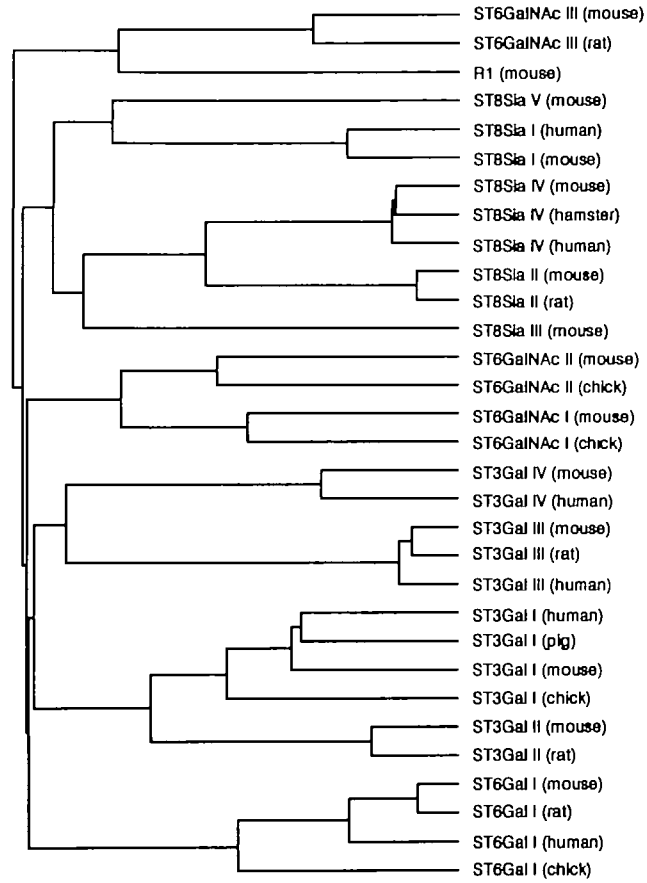

Fig. 2. **A dendrogram of cloned STs.** The dendrogram was constructed according to Higgins and Sharp (*78*) with sequence data in the EMBLE database and our unpublished data. R1 is a candidate ST, though we have not detected its activity yet. According to the sequence similarity, R1 is possibly a member of the ST6GalNAc-family.

TABLE II. **So far cloned and expressed sialyltransferases (STs).**

Enzyme	Abbreviation	So far used names	Sources
Galβ1-3GalNAc α2,3-ST	ST3Gal I	ST-3O, ST3GalA.1, ST-2	Pig (*10*), mouse (*15*), chick (*16*), human (*17*)
*Galβ1-3GalNAc α2,3-ST (2nd-type)	ST3Gal II	ST3GalA.2, SAT-IV	Mouse (*18*), rat (*18*)
Galβ1-3(4)GlcNAc α2,3-ST	ST3Gal III	ST-3N, ST-3	Rat (*19*), human (*20*), mouse[a]
Galβ1-4(3)GlcNAc α2,3-ST	ST3Gal IV	ST-Z, SAT-3, ST-4	Human (*21, 22*), mouse[a]
Galβ1-4GlcNAc α2,6-ST	ST6Gal I	ST-6N, SiaT-1, ST-1	Rat (*9*), human (*23*), mouse (*24*), chick (*25*)
*GalNAc α2,6-ST	ST6GalNAc I	ST6O-I	Chick (*26*), mouse[b]
*Galβ1-3GalNAc GalNAc α2,6-ST	ST6GalNAc II		Chick (*27*), human (*28*), mouse (*29*)
NeuAcα2-3Galβ1-3GalNAc GalNAc α2,6-ST	ST6GalNAc III	STY, ST6O-II	Rat (*30*), mouse[c]
*GD3 synthase	ST8Sia I	SAT-II (SAT-III (76))	Human (*31-33*), mouse (*34*)
Polysialic acid synthase	ST8Sia II	STX	Rat (*35*), mouse (*36, 37*)
*Siaα2-3Galβ1-4GlcNAc α2,8-ST	ST8Sia III		Mouse (*34*)
*Polysialic acid synthase	ST8Sia IV	PST-1	Hamster (*38*), mouse (*39*), human (*40*)
*α2,8-ST	ST8Sia V	SAT-V/SAT-III	Mouse[d]

Underlines and asterisks indicate our works and new ones, respectively. [a,d]Kono *et al.*, submitted for publication. [b,c]Kurosawa *et al.*, manuscript in preparation.

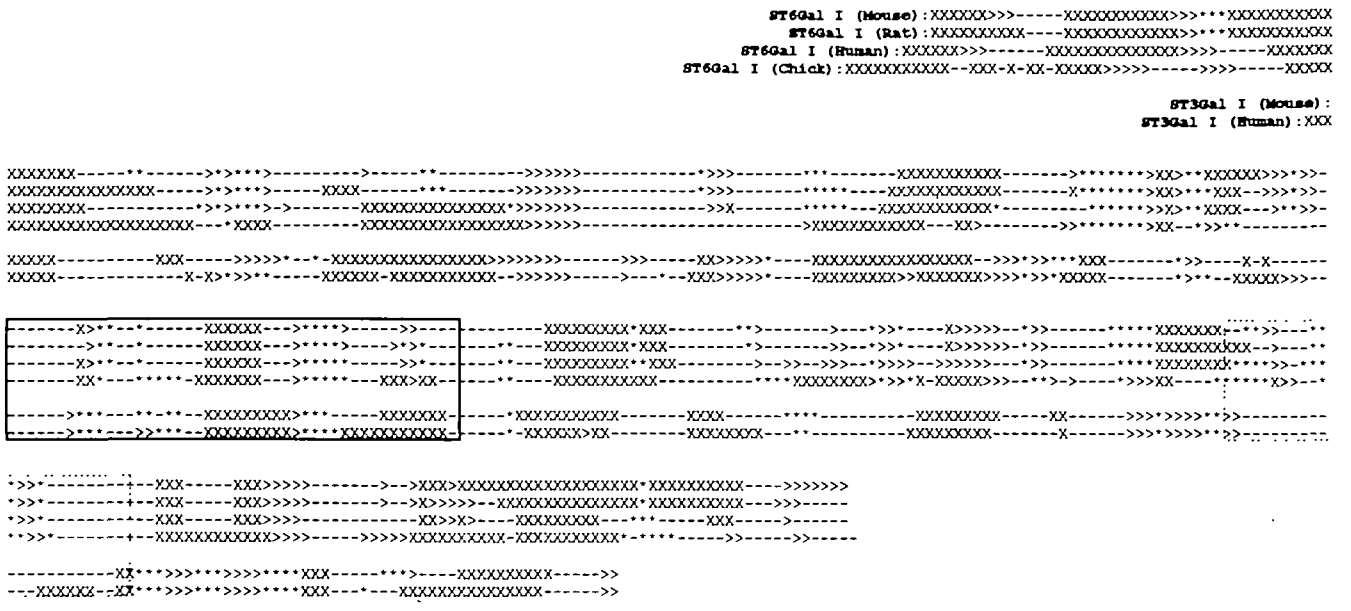

Fig. 3. **A semi-graphical representation of the predicted secondary structures of ST6Gal I of mouse, rat, man, and chick, and ST3Gal I of mouse and man.** The secondary structures were predicted according to Garnier *et al.* (*79*). The sequences are aligned so that the N-terminal positions of sialyl motif L are in line. The boxes with solid and dotted lines indicate the regions of sialyl motifs L and S, respectively. The symbols used are as follows: X, helical conformation; −, extended conformation; >, turn conformation; and *, coil conformation.

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Sialyl motif L
                .****...* *. *  **.** .. * * . ...      *** .* Residues
ST3Gal I        RCAVVGNSGNLKDSSYGPEIDSHDFVLRMNKAPTGG-FEADVGSRT (138-182)
ST6Gal I        RCAVVSSAGSLKNSQLGREIDNHDAVLRFNGAPTDN-FQQDVGTKT (180-224)
ST6GalNAc I     TCAVVGNGGILNDSRVGREIDSHDYVFRLSGAVIKG-YEQDVGTRT (392-336)
ST8Sia I        KCAVVGNGGILKmSGCGRQIDEANFVMRCNLPPLSSEYTRDVGSKT (136-181)
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Sialyl motif S
                .. * .          *. *  *.* Residues
ST3Gal I        PSTGILSIIFSIHICDEVDLYGF (264-286)
ST6Gal I        PSSGMLGIIIMMTLCDQVDIYEF (318-340)
ST6GalNAc I     PTTGALLLLTALHLCDKVSAYGF (447-469)
ST8Sia I        LSTGLFLVSAALGLCEEVSIYGF (272-294)
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Fig. 4. **The conserved region shared by the four mouse STs.** Alignment of the conserved region was conducted for the four sequences. Asterisks indicate perfectly conserved positions in the alignment, and dots indicate well conserved positions.

difference in animal source is not beyond a difference in the acceptor or linkage specificity. In other words, it may be possible that the major STs in each animal have the same substrate specificity. In fact, there are no differences in substrate specificity among ST6Gal I from chick, mouse, rat, and man, or among ST3Gal I from chick, pig, and mouse, though their amino acid sequences are widely different (80-64% identity) (*41*). The differences in amino acid sequence among ST6Gal I from various animals, however, comprise replacements with similar types of amino acids, though some exceptions were observed for the chick enzyme. Their predicted secondary structures, especially from the N-terminal of sialyl motif L to the C-terminal of an enzyme, are very similar (Fig. 3). Almost the same phenomenon was observed in the case of ST3Gal I. Thus, animals from chicken to man have almost the same enzymes. Evolutionally, the enzymatic diversity of STs was probably acquired before the divergence of fur and feather.

With the accumulation of more sequence information on cloned STs, it became clear that there is no significant similarity among them at the sequence level, except for that in the two sialyl motifs, L and S (Fig. 4). The sialyl motifs, L and S, shared by all these enzymes could principally contain either a CMP-Sia binding site or catalytic site, or both. The results of site-directed mutagenesis of two STs, ST3Gal I (unpublished data), and ST6Gal I (*42*), indicate that the sialyl motifs are involved in at least the binding of the donor substrate, CMP-Sia. The primary structures of all the cloned enzymes suggested a putative domain structure with a type II transmembrane topology, as in most other glycosyltransferases, consisting of a short NH_2-terminal cytoplasmic domain (3-11 a.a.), a transmembrane domain (13-18 a.a.), a proteolytically sensitive and proline-rich stem region (30-200 a.a.), and a large COOH-terminal active domain (about 300-350 a.a.) containing sialyl motifs L and S (Fig. 5).

NeuAc (*N*-acetylneuraminic acid) and NeuGc (*N*-glycolylneuraminic acid) are two major kinds of sialic acids in animals, their relative amounts varying among species and tissues. For example, NeuGc is not detected in normal man or chicken (*43*), while it is abundant in horse erythrocytes and mouse serum glycoproteins (*44*). NeuGc- and NeuAc-containing glycoconjugates are immunologically quite distinct, and it has been shown that significant amounts of NeuGc residues, the HD (Hanganutziu-Deicher)-antigen, are present in a variety of human and chicken cancer cells (*45*). The expression of NeuGc- and NeuAc-containing glycoconjugates is developmentally regulated (*46*), and under genetic control (*47*). Thus, it is very important to determine how the expression of NeuGc is regulated. It was reported that CMP-NeuAc is hydoxylated to CMP-NeuGc

in the cytosol (*48*), and subsequently transported into the Golgi apparatus and transferred to glycoconjugates without a significant difference compared to CMP-NeuAc (*49*). Higa and Paulson (*50*) noticed that CMP-NeuGc is also a good donor substrate for STs purified from various animal organs. However, it remains a matter of debate of whether a single enzyme or a mixture of closely resembling NeuGc-specific and NeuAc-specific isoforms catalyzes the two reactions. Using cloned ST3Gal I and ST6Gal I from both chicken and mouse, CMP-NeuAc and CMP-NeuGc were compared as donor substrates with pyridylamino-oligosaccharides as acceptors (*41*). ST6Gal I showed 4–7 times higher activity toward CMP-NeuGc than CMP-NeuAc, while for ST3Gal I there was no significant difference between them, irrespective of the origin of the enzyme. Also, the difference in donor substrate (*i.e.* NeuAc or NeuGc) had little effect on the preferences as to acceptor substrates of these enzymes. Thus, the cloned STs can utilize both CMP-NeuAc and CMP-NeuGc as donor substrates, and the preference difference between the STs, at least partly, explains the difference in the ratios of NeuAc and NeuGc in glycolipids and glycoproteins in individual tissues.

3. Comparison of the substrate specificities and expression of cloned STs, focusing on new members

3-1) β-Galactoside α2,3-STs (ST3Gal-family)

Four species of β-galactoside α2,3-STs have been cloned from various animal species by several groups (Table II). Among them, ST3Gal II was found by our group.

a) Substrate specificity: The reported substrate specificities of the four enzymes are somewhat contradictory, especially in the case of ST3Gal IV. ST3Gal IV, which was cloned from a human melanoma by means of lectin resistance selection, preferentially utilizes oligosaccharides containing a terminal Galβ1-4GlcNAc structure over ones containing the Galβ1-3GlcNAc structure. So this enzyme is presumably involved in the biosynthesis of the sialyl Lewis x [NeuAcα2-3Galβ1-4-(Fucα1-3)GlcNAc] determinant, which is a ligand for the E-, P-, and L-selectins, known as cell adhesion molecules (*21*). In contrast, ST3Gal IV cloned from human placenta exhibited a greater preference for the Galβ1-3GalNAc sequence than Galβ1-4GlcNAc (*22*). This difference may be due to the construct used for expression, or the assay conditions. We cloned cDNAs of four different STs from mouse brain cDNA libraries and compared their substrate specificities under the same conditions, *i.e.* source, construct, and assay conditions. The recombinant enzymes expressed in COS cells exhibited distinct but overlapping acceptor substrate specificities, and also showed a difference in acceptor substrate preference (Table III) (Kono, M. *et al.*, submitted for publication).

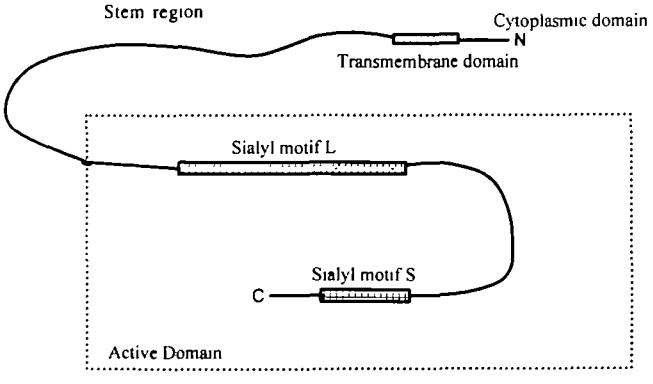


Fig. 5. **Illustration of the topology of so far cloned STs.**

Recombinant ST3Gal I and II produced in COS-cells exhibit the same acceptor substrate specificity, *i.e.* the highest activity toward Galβ1-3GalNAc- (type III), and very low activity toward Galβ1-3GlcNAc- (type I), but none toward the Galβ1-4GlcNAc- (type II) sequence in gangliosides and glycoproteins as well as in oligosaccharides (*51*). The existence of two different kinds of ST with the same acceptor substrate specificity in the same tissue (mouse brain) leads us to the assumption that these enzymes are responsible for the biosynthesis of the NeuAcα2-3Galβ1-3GalNAc sequence in gangliosides and *O*-glycosidically linked oligosaccharides of glycoproteins, respectively. A kinetic parameter and acceptor competition experiment on ST3Gal I and II clearly demonstrated their different substrate preferences, *i.e.* Galβ1-3GalNAc and asialofetuin serve as predominant acceptors for ST3Gal I, whereas gangliosides serve as predominant acceptors for ST3Gal II (Table III). Sandhoff's group reported that in a competition experiment, GM1b, GD1a, and GT1b synthases were found to be a single enzyme in Golgi vesicles from rat liver (*52, 53*). ST3Gal II, like ST3Gal I, is capable of the production of GM1b, GD1a, and GT1b from asialo-GM1, GM1a, and GD1b, respectively. It would be interesting to determine which of the two enzymes is predominant and more closely related to ganglioside biosynthesis. Northern blot analysis revealed that ganglioside-rich tissues, such as brain, liver, kidney and so on, mainly expressed ST3Gal II. These results suggest that the ganglioside in the brain is mainly synthesized by ST3Gal II.

Mouse ST3Gal III also has a strong preference for Galβ1-3GlcNAc over Galβ1-4GlcNAc, as reported for rat and human ST3Gal III (*19, 20*). On the other hand, the activities of ST3Gal IV toward Galβ1-3GlcNAc and Galβ1-4GlcNAc are opposite compared to in the case of ST3Gal III (Table III). Although some groups reported that human

TABLE III. **Comparison of the *in vitro* substrate specificities of the mouse ST3Gal-family.** Relative V_{max}/K_m values are shown. The highest V_{max}/K_m value for an oligosaccharide of each enzyme is taken as 100. * indicates less than 0.1.

	Oligosaccharide			Glycolipid		
	Galβ1-3GlcNAc (Type I)	Galβ1-4GlcNAc (Type II)	Galβ1-3GalNAc (Type III)	Lac-Cer (GA3)	Gg⁴Cer (GA1)	nLc⁴Cer
ST3Gal I	0.4	ND	100	ND	4.3	ND
ST3Gal II	0.76	ND	100	ND	109	ND
ST3Gal III	100	5.6	3.3	ND	*	*
ST3Gal IV	10.5	100	1.5	ND	*	*

Gg⁴Cer: Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′-Cer; nLc⁴Cer: Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′-Cer; ND: could not be detected.

ST3Gal IV exhibits activity toward Galβ1-3GalNAc and glycolipids (*22, 54*), *e.g.*, nLc4Cer, Gg4Cer (GA1), and LacCer (GA3), mouse ST3Gal IV does not exhibit significant activity toward Galβ1-3GalNAc, and exhibits very low activity toward glycolipids *in vitro*. The very low activity is almost the same as the level reported in the case of human ST3Gal IV (*54*), and as the negligible activity of ST3Gal III toward gangliosides compared to the activity toward glycoprotein (Kono, M. *et al.*, submitted for publication). As found in an *in vitro* experiment, the activity of ST3Gal IV toward glycolipids is negligible relative to those of ST3Gal I and II. This strongly indicates the existence of other unknown ST3Gal species with activity toward glycolipids, especially nLc4Cer and LacCer.

b) Tissue specific expression: Northern blot analysis revealed that the expression of each mRNA of the ST3Gal-family shows a unique tissue specificity rather than developmental stage dependency except ST3Gal II (Table IV). ST3Gal I is highly expressed in submaxillary gland (S.G.), intermediate in kidney, spleen, and liver, and very low in brain (*15*). On the other hand, the level of mRNA expression in the case of ST3Gal II is highly in fetal and adult brain, fetal liver, new born heart and kidney, adult spleen, and low in adult liver, heart, kidney, colon, thymus, S.G., and testis (*18* and Kono, M. *et al.*, submitted for publication). A large amount of ST3Gal III mRNA is highly expressed in brain, kidney, heart, spleen, and colon, and low in thymus and S.G. ST3Gal IV is expressed in almost all tissues, but low in colon and adult testis (Kono, M. *et al.*, submitted for publication). These differences in tissue specific expression suggest the expression of each ST3Gal influences the distribution of sialyl-glycoconjugates *in vivo*.

3-2) GalNAc α2,6-STs (ST6GalNAc-family)

O-Linked oligosaccharide synthesis is initiated by the addition of GalNAc, which is proposed to be added as early as in the rough endoplasmic reticulum. Glycosylation then proceeds through the addition of Gal and Sia (Fig. 6), most probably in the *trans*-Golgi region, and may sometimes be completed by the addition of fucose, GalNAc, GlcNAc, Gal, NeuAc, or sulfate.

We have cloned three types of GalNAc α2,6-ST, *i.e.* ST6GalNAc I and II from chick and mouse, and ST6GalNAc III from mouse, which transfer a sialic acid from CMP-Sia through the α2,6-linkage onto a GalNAc residue *O*-linked to Thr/Ser of a glycoprotein. ST6GalNAc I exhibits broad specificity toward GalNAc, Galβ1-3GalNAc and NeuAcα2-3Galβ1-3GalNAc, although ST6GalNAc II requires the β-galactoside residue linked to the GalNAc residue, whereas the sialic acid residue linked to the galactose residue is not essential for its activity (Table V). The third type of ST6GalNAc, *i.e.* ST6GalNAc III, was first cloned by Paulson's group, and requires the NeuAcα2-3Galβ1-3GalNAc-moiety of glycoproteins and glycolipids as an acceptor (*30*). Two types of GalNAc α2,6-ST, the S.G. and liver types, in microsome fractions have been reported (*55, 56*). One [EC 2.4.99.3] of them exhibits broad specificity toward GalNAc-Galβ1-3GalNAc and Neuα2-3Galβ1-3GalNAc, and the other [EC 2.4.99.7] exhibits a much narrower specificity, *i.e.* only toward the NeuAcα2-3Galβ1,3GalNAc moiety of glycoproteins. Thus, ST6GalNAc I corresponds to the former [EC 2.4.99.3], and ST6GalNAc III to the latter [EC 2.4.99.7]. ST6GalNAc II is a so far unidentified enzyme.

Mammalian cells contain at least three members of the ST6GalNAc-family, which have different but overlapping substrate specificities. The meaning of the diversity of the ST6GalNAc-family is not clear yet. Knowledge about their *in vivo* substrate specificities and the regulation mechanisms for their expression will be necessary to clarify this.

Among the three enzymes, only ST6GalNAc I can synthesize Siaα2-6GalNAcα1-Ser/Thr, which is known to attenuate further elongation (*57*), and is also known as the sialyl Tn antigen associated with adenocarcinomas and certain tumors (*58*). Northern analysis revealed that ST6GalNAc I is mainly expressed in chick embryonal stages. Thus, attenuation of the elongation of *O*-linked saccharides may be necessary for some embryonal stages

TABLE IV. **Tissue specificity of gene expression of the ST3Gal-family.**

Tissue	ST3Gal I	ST3Gal II	ST3Gal III	ST3Gal IV
Brain (fetal)	±	++	+++	++
Brain (adult)	±	++	+++	++
Liver (fetal)	++	++	++	++
Liver (adult)	+	±	+++	++
Heart	+	+	+++	++
Kidney	±	+	+++	++
Spleen	+++	++	+++	++
Thymus	++	±	+	++
Colon	−	±	++	+
Testis	−	±	+	+
S.G.	++++	±	±	++

Refs. *15* and *18*, and Kono, M. *et al.*, submitted for publication.

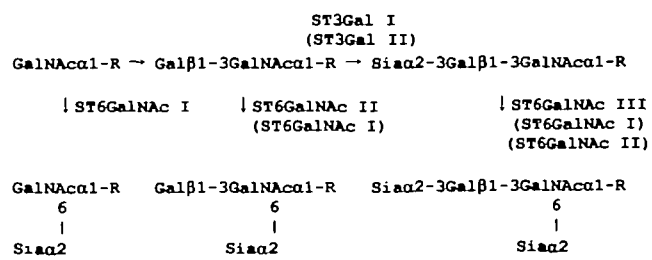


Fig. 6. **STs involved in the biosynthesis of *O*-linked carbohydrates.** R: Ser/Thr.

TABLE V. **Comparison of the ST6GalNAc-family.**

	Acceptor	Mainly expressed in	EC number
ST6GalNAc I	NeuAcα2-3Galβ1-3GalNAc-Ser/Thr	Embryo (chick)	EC 2.4.99.3
	Galβ1-3GalNAc-Ser/Thr		
	GalNAc-Ser/Thr		
ST6GalNAc II	NeuAcα2-3Galβ1-3GalNAc-Ser/Thr	Embryo, brain, kidney, *etc.*	
	Galβ1-3GalNAc-Ser/Thr		
ST6GalNAc III	NeuAcα2-3Galβ1-3GalNAc-Ser/Thr	Brain, liver	EC 2.4.99.7
	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′-Cer (GM1b)		

and some tumors.

Another point is that ST6GalNAc I has an extraordinarily long stem region (around 200 a.a.), which we do not have any clue about at present.

3-3) α2,8-STs (ST8Sia-family)

The Siaα2-8Sia sequence has been widely observed in various gangliosides, such as GT1a, GD3, and b- and c-series gangliosides, also being found, but more restrictedly in glycoproteins of mammals. It has been reported that Siaα2-8Sia-sequence is associated with only two proteins in mammals as sialic acid homopolymers (polysialic acids; PSA), the neural cell adhesion molecule (N-CAM) and the α subunit of the voltage-gated sodium channel in rat brain (*59, 60*). To clarify the mechanism underlying the expression of glycoconjugates containing Siaα2-8Sia-sequences, we have been making extensive attempts to clone cDNAs encoding various α2,8-STs. In 1994, three groups, including ours, independently cloned the human GD3 synthase, an α2,8-ST, gene (ST8Sia I) (*31-33*). After that, we cloned five species of α2,8-ST genes (ST8Sia I-V) from mouse by homology cloning using the sequence information on human GD3 synthase and rat STX (Table II). STX was reported by Livingston and Paulson (*35*) to be a developmentally regulated ST-like protein, and to exhibit 59% amino acid sequence identity to human GD3 synthase in sialyl motif L, suggesting the possibility that it belongs to the ST8Sia-family. ST8Sia I from mouse exhibits 90.4% identity to human GD3 synthase, and they also have the same substrate specificity. Presumably, they are homologous to each other.

ST8Sia III exhibits α2,8-ST activity toward the Siaα2-3Galβ1-4GlcNAc sequence in *N*-glycans and glycolipids (*34*). Under certain conditions, ST8Sia III could synthesize oligomeric α2,8-sialyl linkages bound to the Siaα2-3Galβ1-4GlcNAc-sequence, *i.e.* (Siaα2-8)$_n$Siaα2-3Galβ1-4GlcNAc (n=1-3), but not PSA (Kojima, N. *et al.*, unpublished result). The sequence of Siaα2-8Siaα2-3Galβ1-4GlcNAc on *N*-glycans is very rare, but was found in trout egg (*61*). ST8Sia III also exhibits activity toward GM3, GD3, and 2,3-SPG. The V_{max}/K_m values strongly indicate that 2,3-SPG is a much more suitable acceptor for ST8Sia III than GM3 or GD3. In addition, the V_{max}/K_m values for fetuin indicate that ST8Sia III prefers *N*-linked oligosaccharide, which contain the Siaα2-3Galβ1-4GlcNAc sequence. The mRNA expression of ST8Sia III in brain and testis is specific and developmentally regulated (Tables VI and VII). We are searching for the *in vivo* substrate.

ST8Sia V is expressed in brain at the late embryonal stage (E-20), and mainly in adult brain. ST8Sia V exhibits the activities of SAT-V (*62*) and SAT-III (*63*). With glycolipids as substrates, the substrate specificities of ST8Sia V and ST8Sia I are exactly opposite. ST8Sia I exhibits strong activity toward GM3, but almost negligible activity (if any, less than 10%) toward GD1a, GT1b, and GD3. However, ST8Sia V exhibits activity toward GD1a, GT1b, and GD3, but negligible activity toward GM3. At any rate, how the sialylation of glycolipids is performed by these three enzymes (ST8Sia I, III, and V) *in vivo* is a matter of interest.

TABLE VI. **Differential gene expression of the ST8Sia-family in mouse brain.**

	Fetal (days)			Newborn (days)			Adult
	7	14	20	3	7	10	
ST8Sia I	+	+	++	+	+	+	+
ST8Sia II	−	++	++++	++	+	−	−
ST8Sia III	−	−	+++	++	++	+	±
ST8Sia IV	−	±	+	±	±	±	±
ST8Sia V	−	±	+	+	++	+++	++++

3-4) Two types of polysialic acid synthase

PSA is found on the number 5 Ig-like domain of N-CAM and is one of the most characteristic features of N-CAM. The homophilic interaction between N-CAMs is modulated by the amount of PSA on N-CAM. The content of PSA is developmentally regulated and varies among tissue type. A remarkable decrease in the PSA content accompanies the conversion of the embryonic form of N-CAM to the adult form (*64*), suggesting that PSA on N-CAM influences the developmental process by regulating the adhesiveness between cells. The less adhesive nature of the embryonic form of N-CAM would play an important role in the early embryonic stages, when considerable cell migration occurs. In the later developmental stages, the less PSA form, *i.e.* higher affinity adult form, would help stabilize cell-cell contact. The degree of polysialylation on N-CAM is considered to be important for axon fasciculation and pathway finding (*65*), and for long term potentiation and memory formation (*66*). N-CAM mediated neuroplasticity continues to be present in defined brain regions of the adult, *i.e.* the hypothalmic-neurohypophysial axis, the piri-form region of the neocortex, and areas associated with ongoing neuritogenesis such as the olfactory system and hippocampal formation (*67*). Thus, it is quite important to determine how PSA is synthesized and how its expression is regulated for understanding of the role of PSA in neural development.

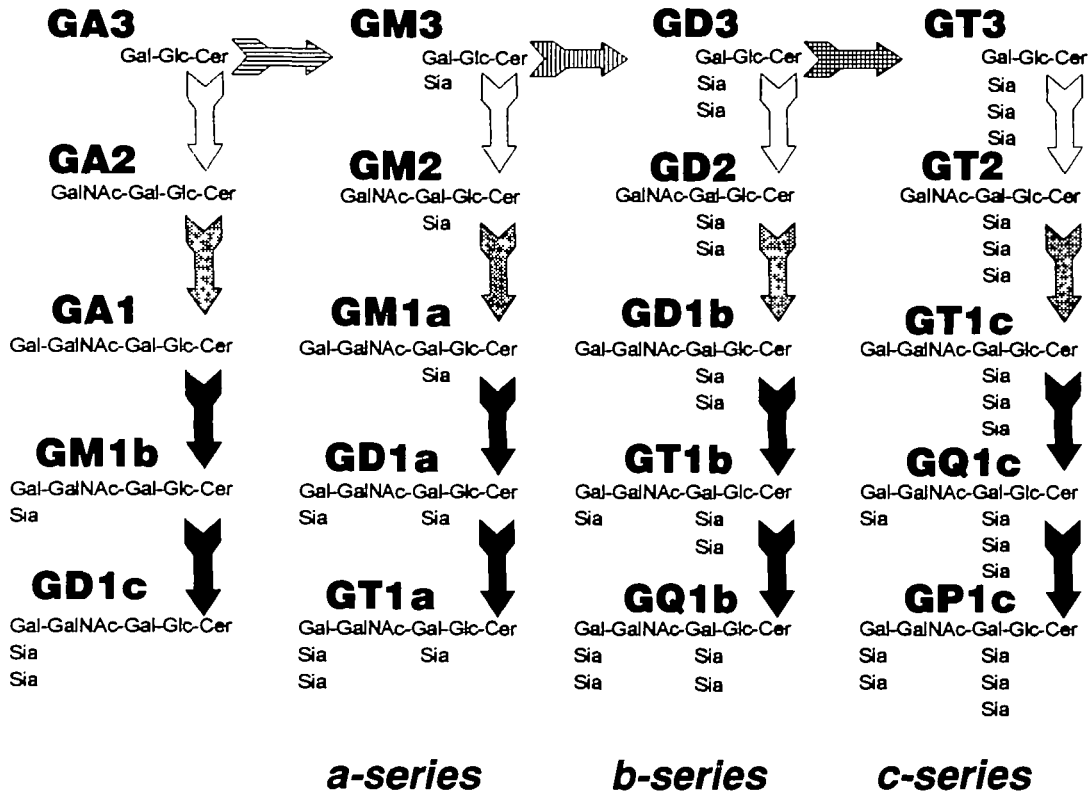
As shown in Table II, we have cloned two species of PSA synthase genes from mouse. ST8Sia II exhibits 99.2% identity to rat STX at the a.a. level, and its polysialylation activity is highly specific to N-CAM. Though it exhibits activity toward *N*-glycans of fetuin and α1-acid glycoprotein, the reaction velocity is far less than that toward N-CAM (*37*). Further characterization revealed that ST8Sia II requires core α1,6-linked fucose and a polypeptide chain for polysialylation, as seen for N-CAM, and a lack of this requirement leads to abortion of polysialylation, though ST8Sia II transfers some sialic acids to substrates (*68*). ST8Sia IV has almost the same substrate specificity as ST8Sia II. Incidentally, mouse ST8Sia IV exhibits high

TABLE VII. **Tissue specificity of gene expression of the ST8Sia-family.**

Tissue	ST8Sia I	ST8Sia II	ST8Sia III	ST8Sia IV	ST8Sia V
Brain (fetal)	+++	+++++	+++	±	+
Brain (adult)	+++	−	±	±	+++
Heart	−	−	±	+++	−
Liver	−	−	−	−	−
Lung	±	+	±	+++	−
Kidney	+++	−	−	−	−
Spleen	±	−	−	++	−
Thymus	++	−	−	−	−
Testis	+	++	++	±	−
Placenta	−	−	−	−	−
S.G.	−	−	−	−	−

amino acid sequence identity (99.2%) with hamster polysialyltransferase-1, which is necessary for polysialic acid expression (*38*). So far, polysialic acid synthesis is considered to involve a few enzymes, *i.e.* the initiator, which forms the first α2,8-sialyl linkage, elongator, and terminator enzymes. However, both ST8Sia II and IV can synthesize polysialic acid chains *in vitro* without an initiator ST. As mentioned above, the fact that ST8Sia III can form oligoα2,8-sialic acids attached to the Siaα2-3Galβ1-4GlcNAc sequence indicates the possibility that ST8Sia III is the initiator enzyme for PSA synthesis. But this is not the case, because the *N*-glycan acceptor of ST8Sia III is different from those of ST8Sia II and IV, as suggested by the results of Sephadex G-50 column chromatography of *N*-linked oligosaccharides of fetuin (Kojima *et al.*, unpublished observation).

The gene for ST8Sia IV is strongly expressed in lung, heart, and spleen, but only weakly in brain, and its expression does not change during embryonal development (*39*). On the other hand, the ST8Sia II gene is highly regulated



Enzyme	Corresponding cloned ST suggested on *in vitro* assaying
α2,3-ST (GM3 synthase, SAT-I)	(not cloned)
α2,8-ST (GD3 synthase, SAT-II)	ST8Sia I [0.03] > ST8Sia III [0.59]
α2,8-ST (GT3 synthase, SAT-III)	ST8Sia V [0.28] > ST8Sia III [3.3] > ST8Sia I [5.0]
β1,4-GalNAc transferase	-
β1,3-Gal transferase	-
α2,3-ST (SAT-IV)	ST3Gal II [0.67-0.83] > ST3Gal I [0.91-2.0]
α2,8-ST (SAT-V)	ST8Sia V [0.07-1.1] > ST8Sia I [2.0-5.0]

Fig. 7. **Hypothetical biosynthetic pathways for gangliosides.** Ganglioside synthesis in mammals is regulated through three main metabolic pathways, the a-series (comprising GM3, GM2, GM1, GD1a, and GT1a), the b-series (comprising GD3, GD2, GD1b, GT1b, and GQ1b), and the c-series (comprising GT3, GT2, GT1c, GQ1c, and GP1c). [ ] indicates K_m value(s) (mM) for corresponding substrate(s) (*31, 34*, and Kono, M. *et al.*, submitted for publication).

during the course of neural development, as reported by Livingston and Paulson (*35*). These differences suggest that ST8Sia II may be mainly involved in the biosynthesis of PSA on N-CAM. This hypothesis was supported by the results of Scheidegger *et al.* (*69*). They found that the ST8Sia II gene was expressed in a variant cell line of a human small cell carcinoma, NCI-H69/F3, with the PSA and N-CAM-positive phenotypes. But this was not found in a PSA-negative variant, the ST8Sia IV gene not being expressed in either of these two types of cells. In addition, transfection of human ST8Sia II cDNA into either COS cells or a PSA-negative variant led to surface expression of PSA. Another possibility is that both ST8Sia II and IV synthesize PSA on N-CAM, but their activities are differently regulated, *e.g.* it is possible that ST8Sia II acts mainly in the early developmental stages, and ST8Sia IV in later stages to maintain the homeostatic state on cell to cell interaction. Recently, we found that PSA on N-CAM is synthesized mainly by ST8Sia II during the neuronal differentiation of P-19 cells of a mouse embryonal carcinoma and MNS-8 cells, both of which were established by Nakafuku and Nakamura (*70*) from embryonic rat neuroepithelium by introducing the *mycer* fusion gene (*80*). A more critical experiment will be necessary to solve this problem.

II. Structural and functional analysis of STs

1. Establishment of technology for the large scale production of STs

Cloned STs have been expressed in cultured eukaryotic cells. However, there are several advantages in producing the enzymes in bacteria, including low cost, easy handling and mass-production. Mouse ST6Gal I was produced in an insoluble form in *E. coli* cells harboring expression plasmids (*71*). The insoluble protein was solubilized with 8M urea and then the solution was diluted for renaturation of the enzyme. The substrate specificity and kinetic parameters, except for the specific activity, of the renatured enzyme were similar to those of the enzyme obtained from rat liver. These results suggest that a bacterial expression system is a powerful tool for large scale production of STs and the elucidation of the molecular mechanisms of STs. However, the problem of the low enzyme specific activity with this method remains to be solved.

2. New evidence for the occurrence of a glycolipid-mediated signal transduction system: *de novo* GD3-expression triggers cholinergic neuritogenesis by Neuro2a cells (*72*)

GD3 synthase, an α2,8-ST, plays a key role in the biosynthesis of b- and c-series gangliosides (Fig. 7). Since the biosynthesis of b- and c-series gangliosides, and their expression have been shown to be correlated with the differentiation and synaptogenesis of neuronal cells, the expression of GD3 synthase might be a key event in neuronal cell differentiation. A murine neuroblastoma cell line, Neuro2a, expresses negligible amounts of GM3 and b-series gangliosides, but significant amounts of a-series gangliosides, GM1 and GD1a. If the GD3 synthase gene is transfected into and expressed in Neuro2a cells, the ganglioside biosynthesis pathway should be modified. An N2a-GD3 cell line was established by the transfection of a plasmid, pSRb-GD3, which contains a cDNA encoding GD3 synthase and the blasticidine deaminase gene. N2a-GD3 synthesized and expressed GD3 and/or b-series gangliosides including GQ1b but not c-series gangliosides. On the other hand, mock-transfected cells, N2a-bsr, did not express GD3 or other b- or c-series gangliosides.

Besides ganglioside synthesis and expression, N2a-GD3 exhibited the following characteristics: (i) it spontaneously grew axon-like neurites, which were stained by an antibody against phosphorylated neurofilaments (Fig. 8), (ii) cell growth was suppressed (Fig. 9), and (iii) acetylcholine esterase was induced (Fig. 10). The gene which induces these three phenomena is classified as a "cholinergic differentiation inducer" gene. So, the GD3 synthase gene qualifies as one such gene, and some of the induced gangliosides may be cholinergic differentiation inducer(s). Exogenous GM1 stimulated the neuritogenesis of N2a-bsr but not differentiated N2a-GD3, indicating that the mechanism of neurite sprouting in this system may overlap *en route* with that of exogenous GM1.

On the other hand, we found recently that when most gangliosides were converted to Fuc-GM1 by transfection of H-type α1,2-fucosyltransferase, the cells did not show ganglioside-stimulated axon-like neuritogenesis. However, these cells exhibited the potential to undergo differentiation with dendrite-like neurite sprouting (*73*). Ariga *et al.* reported that when mouse neuroblastoma F-11 cells were transfected with the *O*-acetylesterase gene of the influenza C virus, GD3 increased 150%, compared to the control cell

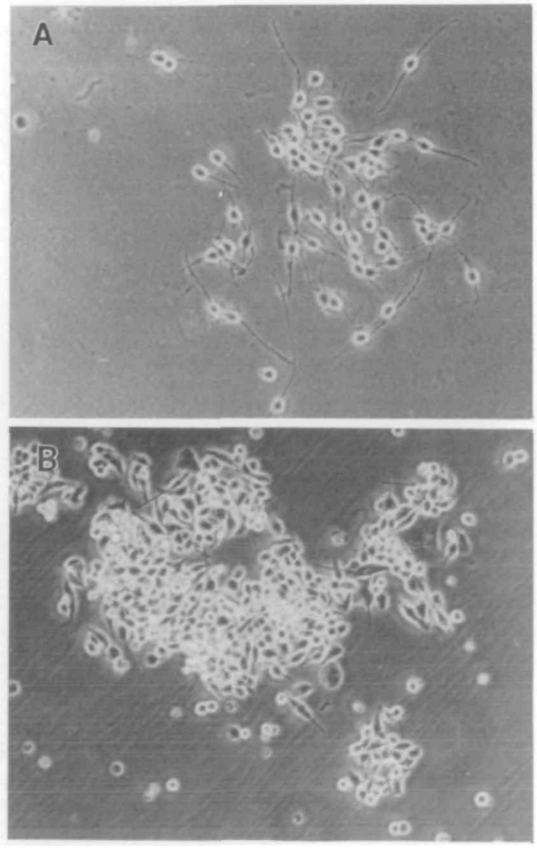


Fig. 8. **Morphology of GD3 synthase- (A) or mock- (B) transfected cells (*72*).**

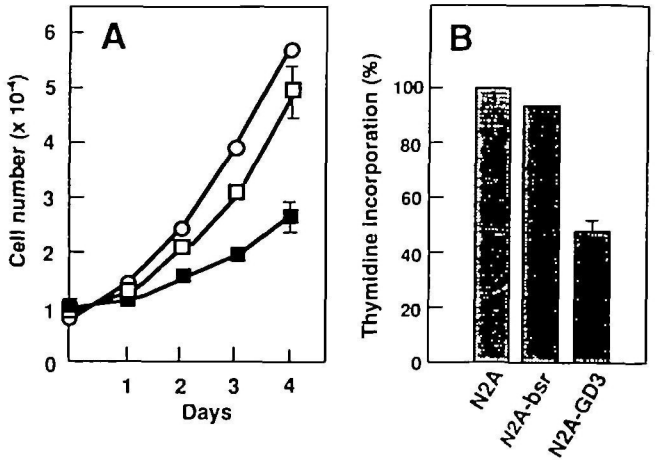


Fig. 9. **Cell growth (A) and thymidine incorporation (B) of N2a-GD3 (*72*).** A, cell growth of N2a-GD3 (■), N2a-brs (□), and Neuro2a (●).

level, and *O*-acetylated GD3 decreased to 27% of the control level (*74*). The neurites of the transfected F-11 cells were elongated.

These observations directly demonstrate the primary importance of the expression of some gangliosides on the cell surface for the development and differentiation of some neural cells. The next questions are what kinds of neural development and differentiation are contributed to by ganglioside, and whether the ganglioside contribution is necessary or sufficient for neural development and differentiation. Obviously, lots of work need to be done on this.

These observations led to the following two considerations regarding the role of gangliosides in cellular differentiation. The first explanation is a simple one. There is a specific ganglioside molecule which controls the differentiation of neural cells. Furthermore, the specific ganglioside may be a cofactor for some signal transduction system involving the *trk*-families, IP3, PKA, PKC, Cam-kinases, ceramide and so on, as second messengers, or a cofactor for some transcription factors coupled with differentiation. The problems with this hypothesis are what specific ganglioside at each cell stage is responsible, how does the differentiation proceed, and whether or not there is coupling to a known signal transduction system.

The second explanation is based on the set-theory of mathematics. The idea is based on the hypothesis that the ganglioside function is not due to a specific molecule but to a set of gangliosides, including their concentrations, and each cell has a monitoring system for this set of cell surface gangliosides as information transducers. A change in the ganglioside set may be a trigger for switching on or off of the next signal transduction system, or a transcription factor system for differentiation. The most important points about this hypothesis are what is the ganglioside monitoring system and how do the signals proceed.

To clarify the mechanisms underlying the above phenomena, we are now examining what happens during and after the expression of gangliosides using inducible promoter systems. Very recently, we established an inducible system involving tetracycline, and further investigation is now in progress. At any rate, we found that a cell surface glycoconjugate is coupled to the cell differentiation process,

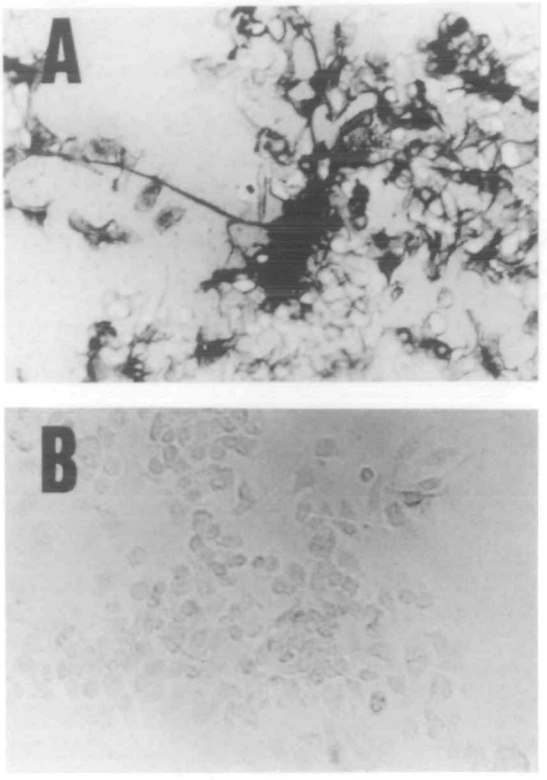


Fig. 10. **Acetylcholine esterase staining of N2a-GD3 after cessation of cell growth (A), and N2a-brs (B) (*72*).**

and that the transfection of the glycogene into cells is a useful experimental tool for investigation of the functions of glycoconjugates.

III. The controversial points

1. How to elucidate the *in vivo* substrate specificity

As for *in vitro* experiments, the substrate specificities of STs in the same family overlap each other, the differences being only a matter of degree. Generally, an enzyme exhibits strong activity toward certain acceptor groups but very weak activity toward other acceptor groups. For example, each enzyme of the ST3Gal-family qualitatively exhibits activity toward three types of oligosaccharides, *i.e.* Galβ1-3GlcNAc (Type I), Galβ1-4GlcNAc (Type II), and Galβ1-3GalNAc (Type III). The asterisk in Table III means negligible activity not none. Similar phenomena were observed for the ST6GalNAc- and ST8Sia-families. Much attention is now being focused on whether or not these *in vitro* "negligible" activities have any physiological meaning. Everyone agrees that the *in vivo* substrate specificity is more important for the determination of ST functions than the *in vitro* one. To elucidate the *in vivo* substrate specificity, a useful strategy is to determine what kinds of carbohydrate are expressed when a vector containing the cDNA of a ST is transfected into cells. However, the usual methods used for this strategy have the following problems: (a) the transfection of cDNA itself will not provide "physiological conditions," (b) a lack of a proper substrate or the production of a non-physiological product may lead to a non-physiological state, and (c) we tend to use vectors

which have a strong promoter system to significantly express exogenous cDNA. In fact, when we use a strong promoter, such as CAG, SRα, and so on, a large amount of enzyme is occasionally expressed in the cells. We do not know the precise mechanism by which the synthesis of the carbohydrate chain proceeds in the Golgi apparatus. The circumstances in the Golgi apparatus must be completely different from those in an *in vitro* assay system. In the Golgi apparatus, K_m, V_{max}, and so on will have no significance. If large amounts of an enzyme are expressed in the Golgi apparatus, the negligible *in vitro* activity may have some meaning. With only these methods, a wrong conclusion may be drawn, for example, enzyme A participates in the synthesis of carbohydrate B *in vivo*, even if this activity is very minor *in vitro*. Of course in the cases of some specific stages during the course of development and unusual physiological stages, such as cancer and so on, the negligible activity *in vitro* may also have some meaning. Recent reports from the groups of Scheidegger (*69*), Kannagi (*75*), and Nakayama (*76*) may be such cases. As stated above, Scheidegger *et al.* reported that the ST8Sia II gene was expressed in human small cell carcinoma line NCI-H69/F3 (*69*). Kannagi *et al.* reported that colon cancer tissues expressed significant amounts of ST3Gal I, which showed ST activity toward the Type I (Galβ1-3GlcNAc) sequence to yield sLe^a-precursors (*75*) (see Table III). Nakayama *et al.* reported that over-expression of GD3 synthase cDNA leads to the expression of GT3 (*76*). To avoid drawing the wrong conclusion, it is urgently necessary to establish more significant methods for elucidating the physiological substrate specificity *in vivo*.

2. Other points

Since the beginning of our project, we have cloned the cDNAs of many STs, though the cDNA cloning of some enzymes, for example, GM3 synthase and GlcNAc α2,6-STs, remains to be performed.

As stated above, most ST genes are expressed in development- and cell type-specific manners, and seem to play an important role in sialyl-glycoconjugate expression. Using these clones, we are now trying to clarify the regulatory mechanisms for carbohydrate expression in various ways, for example, genomic analysis, transcriptional analysis, determination of the molecular anatomy of an enzyme itself, and so on. The availability of ST cDNAs provides us with a useful and powerful means of elucidating the meaning of the diversity and the biological functions of sialyl-glycoconjugates.

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