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# β1-4Galactosyltransferase Activity of Mouse Brain as Revealed by Analysis of Brain-Specific Complex-Type *N*-Linked Sugar Chains

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**We previously reported two brain-specific agalactobiantennary *N*-linked sugar chains with bisecting GlcNAc and α1-6Fuc residues, (GlcNAcβ1-2)$_{0 or 1}$Manα1-3(GlcNAcβ1-2Manα1-6)(GlcNAcβ1-4)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc [Shimizu, H., Ochiai, K., Ikenaka, K., Mikoshiba, K., and Hase, S. (1993) *J. Biochem.* 114, 334-338]. Here, the reason for the absence of Gal on the sugar chains was analyzed through the detection of other complex type sugar chains. Analysis of *N*-linked sugar chains revealed the absence of Sia-Gal and Gal on the GlcNAc residues of brain-specific agalactobiantennary *N*-linked sugar chains. We therefore investigated the substrate specificity of galactosyltransferase activities in brain using pyridylamino derivatives of agalactobiantennary sugar chains with structural variations in the bisecting GlcNAc and α1-6Fuc residues as acceptor substrates. While the β1-4galactosyltransferases in liver and kidney could utilize all four oligosaccharides as substrates, the β1-4galactosyltransferase(s) in brain could not utilize the agalactobiantennary sugar chain with both bisecting GlcNAc and Fuc residues, but could utilize the other three acceptors. Similar results were obtained using glycopeptides with agalactobiantennary sugar chains and bisecting GlcNAc and α1-6Fuc residues as substrates. The β1-4galactosyltransferase activity of adult mouse brain thus appears to be responsible for producing the brain-specific sugar chains and to be different from β1-4galactosyltransferase-I. The agalactobiantennary sugar chain with bisecting GlcNAc and α1-6Fuc residues acts as an inhibitor against "brain type" β1-4galactosyltransferase with a $K_i$ value of 0.29 mM.**

**Key words: brain, β1-4galactosyltransferase, sugar chain, pyridylamination, substrate specificity.**

The appearance of glycans on neural cell surfaces changes dramatically during brain developmental stages (*1*). The occurrence of such temporally precise transitions suggests specific roles for glycans in brain tissue formation. We previously reported two *N*-linked sugar chains, GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)(GlcNAcβ1-4)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (BA-2) and Manα1-3-(GlcNAcβ1-2Manα1-6)(GlcNAcβ1-4)Manβ1-4GlcNAc-β1-4(Fucα1-6)GlcNAc (BA-1), that are specifically and strongly expressed in mouse brain (*2*). BA-1 and BA-2 were found to be mainly bound to membrane glycoproteins in brain tissue. The amounts of these sugar chains changes in a brain development-dependent manner, reaching a maximum on postnatal day 7 in cerebrum and on postnatal day 21 in cerebellum (*3*). BA-1 lacks one GlcNAc residue linked to C-2 of the Manα1-3 residue of BA-2. Results obtained using *Hexa* and *Hexb* gene-disrupted mice (*4*) indicate that BA-2 is hydrolyzed to BA-1 by the action of β-*N*-acethylhexosaminidase B present in cerebrum. As well as the BA-2 noted above, the structure of BA-2 has been reported to be a major *N*-linked sugar chain in rat brain (*5*), hamster scrapie prion protein (*6*), β-trace glycoprotein (*7*), and asialo-transferrin obtained from brain tissue (*8*). However, the mechanism by which these sugar chains are expressed specifically in brain tissue is unknown. One approach to elucidating the mechanism of their formation lies in analyzing the substrate specificities of β1-4galactosyltransferase (β1-4Gal-T) activities. Six β1-4Gal-T genes of bovine, chicken, human, and rat origin have so far been cloned and characterized (*9-17*). Among these mRNAs, those of β1- 4Gal-T-III, -V, and -VI are expressed in brain (*14, 15*), while β1-4Gal-T-VI is not an enzyme that synthesizes Galβ1-4GlcNAc linkages (*16*). However, little is known about their acceptor specificities.

To elucidate the reason why BA-2 is specifically expressed in brain, we analyzed the amounts and types of galactosylated *N*-linked sugar chains in mouse brain, paying special attention to the structure of BA-2, as well as to the

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Abbreviations: Fuc, L-fucose; Gal, D-galactose; β1-4Gal-T, β1-4galactosyltransferase; GlcNAc, *N*-acetyl-D-glucosamine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Man, D-mannose; M3-M9, Man$_3$GlcNAc$_2$-PA-Man$_9$GlcNAc$_2$-PA, respectively; PA-, pyridylamino; Sia, sialic acid. The abbreviations of the oligosaccharides used in this study are listed in Table I.

substrate specificities of $\beta$1-4Gal-Ts using agalactobiantennary sugar chains as acceptors.

## MATERIALS AND METHODS

*Materials*—$\beta$-Galactosidase (*Aspergillus* sp.) was purchased from Toyobo (Osaka), $\beta$-galactosidase (*Streptococcus* 6646k) from Seikagaku Corp (Tokyo), pronase from Calbiochem (La Jolla, CA), a BCA reagent kit from Pierce (Rockford, IL), bovine serum albumin, $\alpha_1$-acid glycoprotein, $\alpha$-lactalbumin, and UDP-Gal from Sigma (St. Louis, MO), and sialidase (*Arthrobacter ureafaciens*) and human transferrin from Nacalai Tesque (Kyoto). Hen egg yolk IgY was prepared as described previously (*18*). The following Pyridylamino (PA-) sugar chains, prepared as reported (*19*), were used: G2Bi, G2BiF, G2BiB, G2BA-2, BA-2, BA-1, BA-1′, Bi, BiB, BiF, GaBi, GbBi, GaBiF, GbBiF, GaBiB, GbBiB, GaBA-2, GbBA-2, 226-T, 224-T, Tetra, TetraF, and $Man_3GlcNAc_2$-PA-$Man_9GlcNAc_2$-PA. A mixture of sialo-PA-sugar chains was prepared from $\alpha_1$-acid glycoprotein by hydrazinolysis-*N*-acetylation followed by pyridylamination as described previously (*2*). A Mono Q 5/5 column (0.5×5 cm) was purchased from Pharmacia (Uppsala), Bio-gel P-2 from Bio-Rad (Richmond, CA), Toyopearl HW-40F and a TSK-gel Sugar AX-I column (0.46×15 cm) from Tosoh (Tokyo), Cosmosil 5C18-P from Nacalai Tesque, Shodex NH2P-50 from Showa Denko (Tokyo), and a Shim-Pack CLC-ODS column (0.6×15 cm) from Shimadzu (Kyoto).

*Preparation of PA-Sugar Chains from Mouse Tissues*—PA-sugar chains were prepared from tissues of three 8-week-old ICR mice as described previously (*2*). Briefly, a lyophilized tissue sample (2 mg) was heated with 0.2 ml anhydrous hydrazine at 100°C for 10 h, and the sugar chains released were *N*-acetylated with 400 $\mu$l saturated sodium bicarbonate solution and 16 $\mu$l acetic anhydride by incuba-

TABLE I. **Structures and abbreviations of PA-sugar chains.**

| Abbreviation | Structure |
|---|---|
| BA-2 | GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 ; Fucα1-6 |
| GaBA-2 | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 ; Fucα1-6 |
| GbBA-2 | GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 ; Fucα1-6 |
| G2BA-2 | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 ; Fucα1-6 |
| BA-1 | GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Manα1-3 ; Fucα1-6 |
| BA-1′ | Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 ; Fucα1-6 |
| Bi | GlcNAcβ1-2 Manα1-6<br>Manβ1-4 GlcNAcβ1-4 GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GaBi | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>Manβ1-4 GlcNAcβ1-4 GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GbBi | GlcNAcβ1-2 Manα1-6<br>Manβ1-4 GlcNAcβ1-4 GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| G2Bi | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>Manβ1-4 GlcNAcβ1-4 GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| BiF | GlcNAcβ1-2 Manα1-6 ; Fucα1-6<br>Manβ1-4 GlcNAcβ1-4 GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GaBiF | Galβ1-4GlcNAcβ1-2 Manα1-6 ; Fucα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GbBiF | GlcNAcβ1-2 Manα1-6 ; Fucα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| G2BiF | Galβ1-4GlcNAcβ1-2 Manα1-6 ; Fucα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| BiB | GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GaBiB | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2 Manα1-3 |
| GbBiB | GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| G2BiB | Galβ1-4 GlcNAcβ1-2 Manα1-6<br>GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4 GlcNAcβ1-2 Manα1-3 |
| 226-T | Galβ1-4GlcNAcβ1-6 Manα1-6<br>Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4GlcNAcβ1-2Manα1-3 |
| 224-T | Galβ1-4GlcNAcβ1-2Manα1-6<br>Galβ1-4GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4GlcNAcβ1-2 Manα1-3 |
| Tetra | Galβ1-4GlcNAcβ1-6 Manα1-6<br>Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4GlcNAcβ1-4 Manα1-3<br>Galβ1-4GlcNAcβ1-2 |
| TetraF | Galβ1-4GlcNAcβ1-6 Manα1-6 ; Fucα1-6<br>Galβ1-4GlcNAcβ1-2 Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Galβ1-4GlcNAcβ1-4 Manα1-3<br>Galβ1-4GlcNAcβ1-2 |

tion at room temperature for 30 min. The reducing ends of the sugar chains were then pyridylaminated using 20 μl of a pyridylamination reagent and 70 μl of a reducing reagent prepared as described previously (*20*). Excess reagents were removed by gel filtration on a Toyopearl HW-40F column (1×25 cm) using 0.01 M ammonium acetate buffer, pH 6.0, as an eluent. The PA-sugar chain fraction that eluted between 4 and 20 ml was collected and freeze-dried.

*Preparation of Glycopeptide-Bi and -BA-2*—Glycopeptide-Bi was prepared from human transferrin (*21, 22*). Human transferrin (200 mg) was digested with 10 mg pronase at 37°C for 90 h in 20 mM Tris-HCl buffer, pH 8.5. The digestion was terminated by heating at 100°C for 3 min, and the reaction mixture was centrifuged. The supernatant was heated with 0.1 M hydrochloric acid at 80°C for 45 min. The desialylated glycopeptides thus obtained were digested with β-galactosidase (*Aspergillus* sp.) at 37°C for 48 h in 50 mM ammonium-acetate buffer, pH 5.5, and the reaction was terminated by heating at 100°C for 3 min. The reaction mixture was centrifuged, and the supernatant was separated by gel filtration on a Bio-Gel P-2 column (1.5×95 cm) using water as the eluent. Glycopeptides were detected by the anthron-$H_2SO_4$ method (*23*). The anthron-positive fraction was collected and used as glycopeptide-Bi. Glycopeptide-BA-2 was prepared in the same way from hen egg yolk IgY (300 mg) (*24, 25*). The structures of the glycopeptides were analyzed as follows. PA-sugar chains were prepared from the glycopeptides as described above, and the amounts of sugar chains (Bi and BA-2) in the preparations were quantitated using the correction factor described below. Glycopeptide-Bi contained mostly Bi, and glycopeptide-BA-2 contained BA-2 and high mannose-type sugar chains.

*Quantification of PA-Sugar Chains*—PA-sugar chains (100-200 pmol) were hydrolyzed with 4 M hydrochloric acid at 100°C for 8 h and then *N*-acetylated with 100 μl saturated sodium bicarbonate solution and 4 μl acetic anhydride. The solution was placed on a Dowex 50 column ($H^+$, 5×30 mm), the column was washed with 5 ml water, and the PA-monosaccharides were eluted with 5 ml of 1.5 M aqueous ammonia (*26*). The eluate was freeze-dried. PA-monosaccharides in the residue were applied to a TSK-gel Sugar AX-I column (0.46×15 cm) and eluted with 0.8 M potassium borate buffer, pH 9.0, containing 10% (v/v) acetonitrile at 73°C at a flow rate of 0.3 ml/min (*26*). The amount of PA-GlcNAc obtained was measured by comparing the peak area with that of a known amount of standard PA-GlcNAc. The amounts of PA-sugar chains were calculated from the amount of PA-GlcNAc found applying a correction factor obtained using a known amount of $Man_5GlcNAc_2$-PA.

*HPLC of PA-Sugar Chains*—PA-sugar chains were detected by fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm). All HPLC procedures were carried out at 25°C.

Reversed-phase HPLC on a Cosmosil 5C18-P column (0.15×25 cm) was performed with two eluents, 20 mM ammonium acetate buffer, pH 4.0 (Eluent A) and the same buffer supplemented with 0.5% 1-butanol (Eluent B). The column was equilibrated with 15% Eluent B. After injecting a sample, the proportion of Eluent B was increased linearly from 15 to 85% in 90 min at a flow rate of 0.15 ml/min.

Reversed-phase HPLC was also performed on a Cosmosil 5C18-P column (1.0×25 cm) using 20 mM ammonium acetate buffer, pH 4.0, containing 0.15% 1-butanol as an eluent at a flow rate of 3 ml/min.

Reversed-phase HPLC using a ShimPak CLC-ODS column was performed with 0.1 M phosphate buffer, pH 3.8, containing 0.1% 1-butanol at a flow rate of 2 ml/min as reported (*27*).

Size-fractionation HPLC was performed on a Shodex NH2P-50 column (1.0×10 cm) at a flow rate of 1.0 ml/min using eluents, acetonitrile:water:acetic acid (95:5:3, v/v) titrated to pH 7.0 with triethylamine (Eluent C) and water:acetic acid (100:3, v/v) titrated to pH 7.0 with triethylamine (Eluent D). The column was equilibrated with 3% Eluent D. After injecting a sample, the proportion of Eluent D was increased linearly to 33% in 3 min, and then to 70% in 32 min.

Size-fractionation HPLC was also performed on a Shodex NH2P-50 column (0.46×5 cm) at a flow rate of 0.8 ml/min using acetonitrile:water:acetic acid (800:200:3, v/v) titrated to pH 7.0 with 7 M aqueous ammonia as an eluent.

Size-fractionation HPLC was then repeated on the Shodex NH2P-50 column (0.46×25 cm) using eluents, acetonitrile:water:acetic acid (900:100:3, v/v) titrated to pH 7.0 with 7 M aqueous ammonia (Eluent E) and acetonitrile:water:acetic acid (200:800:3, v/v) titrated to pH 7.0 with 7 M aqueous ammonia (Eluent F), at a flow rate of 0.8 ml/min. The column was equilibrated with 5% Eluent F. After injecting a sample, the proportion of Eluent F was increased linearly to 14% in 3 min, to 25% in 17 min, to 50% in 60 min, and then to 75% in 5 min.

Mono Q HPLC was performed on a Mono Q HR 5/5 column at a flow rate of 1.0 ml/min using two eluents, water titrated to pH 9.0 with 1 M aqueous ammonia (Eluent G) and 0.5 M ammonium acetate, pH 9.0 (Eluent H). The column was equilibrated with Eluent G. After injecting a sample, the proportion of Eluent H was increased linearly to 10% in 3 min, to 40% in 14 min, and then to 100% in 5 min.

*Preparation of Enzyme Solution and Procedures for the Assay of β1-4Gal-T Activity*—Mouse tissues obtained on embryonic day 13 or postnatal day 10 were homogenized in a 9-fold volume of 10 mM HEPES-NaOH buffer, pH 7.2, containing 65 mM NaCl, 2.7 mM KCl, 0.5 mM phenylmethanesulfonyl fluoride, and 0.1% Triton X-100. The homogenate was centrifuged at 600×*g* for 10 min to remove cell debris and the nuclear fraction, and the supernatant was used as the enzyme solution. Part of the supernatant was further centrifuged at 21,600×*g* for 40 min, and the precipitate, which contained the Golgi membrane fraction, was suspended in homogenizing buffer and used as a membrane enzyme solution.

Enzyme assay using 20 μM of PA-sugar chain as an acceptor was performed at 37°C for 2-20 h in a total volume of 25 μl of 10 mM HEPES-NaOH buffer (pH 7.2), 65 mM NaCl, 2.7 mM KCl, 10 mM $MnCl_2$, 1 mM UDP-Gal, and enzyme solution (100 μg protein). The enzymatic reaction was terminated by heating at 100°C for 3 min, and the solution was centrifuged. Part of the supernatant was analyzed by reversed-phase HPLC. The amounts and structures of the products were assessed based on the elution positions of standard PA-sugar chains. The enzymatic reaction was also performed using 20 μM (based on

the Bi or BA-2 content) of a glycopeptide fraction as an acceptor in the same manner as described for PA-sugar chains. The enzymic reaction was terminated by heating at 100°C for 3 min, and the solution was freeze-dried. Sugar chains were released by hydrazine and pyridylaminated as described above. Excess reagents were removed by phenol-chloroform extraction (*28*). The PA-sugar chains obtained were separated on a Cosmosil 5C18-P column (1.0×25 cm), and fractions at the elution positions of the transfer products were collected. The amounts and structures of the transfer products were further confirmed by size-fractionation HPLC.

The amount of protein in an enzyme solution was measured using a BCA protein assay kit with bovine serum albumin as a standard.

*Enzymatic Hydrolysis of PA-Sugar Chains*—PA-sugar chains (100-200 pmol) were digested with 5 munits of β-galactosidase (*Streptococcus* 6646k) according to the manufacturer's protocol in 50 μl of 50 mM ammonium acetate buffer, pH 5.5, at 37°C for 17 h; or with 5 munits of sialidase in 50 μl of 50 mM ammonium acetate buffer, pH 5.0, at 37°C for 17 h. The enzymatic reactions were terminated by heating at 100°C for 3 min.

## RESULTS AND DISCUSSION

*Analysis of Galactose Residues on N-Linked Sugar Chains in Mouse Tissues*—In our previous papers, we reported the detection of BA-2 as a brain-specific sugar chain (*2, 3*). To analyze the mechanism of this specific expression, it is necessary to know whether Gal and/or Sia-Gal are bound to BA-2. *N*-linked sugar chains were released from 2 mg of lyophilized mouse cerebrum, liver, or kidney tissue by hydrazinolysis followed by *N*-acetylation, and the *N*-linked sugar chains thus obtained were pyridylaminated. The PA-sugar chains were then separated by Mono Q chromatography into a neutral fraction (N) and five acidic fractions (A1-A5) according to the elution positions of standard sialylated PA-sugar chains. Fraction N was analyzed by two-dimensional sugar mapping based on size-fractionation and reversed-phase HPLC. Fractions N3-N9 obtained by size-fractionation HPLC were collected according to the elution positions of $Man_3GlcNAc_2$-PA-$Man_9GlcNAc_2$-PA, respectively, and each fraction was further separated by reversed-phase HPLC (Fig. 1). The structure of each peak was assessed by comparing its elution position with those of standard PA-sugar chains. Peaks appearing at around 20 min in Fractions N5-N9 were of the high mannose-type (Fig. 1). The peak at around 40 min in Fraction N9 was Tetra. No BA-2 containing one or two Gal residues (Arrowheads F and G, respectively, in Fig. 1) was detected; it may be that such structures are present in very small amounts, as their presence has been reported in rat brain (*5*). Similar results were obtained for cerebellum (data not shown).

Galactosylated *N*-linked sugar chains in Fraction N were detected by comparing the elution profiles of reversed-phase HPLC before and after the digestion of Fraction N with β-galactosidase (Fig. 2). BiB increased after digesting Fraction Ns obtained from brain, liver, and kidney with β-galactosidase, signifying the existence of galactose bound to the BiB structure, whereas BA-2 increased only in Fraction N from kidney, and then only slightly. These findings indicate that BA-2, which accounts for about 7% of the total *N*-linked sugar chains (the sum of Fractions N, A1-A5), contained no (or possibly very little) Gal substitutions.

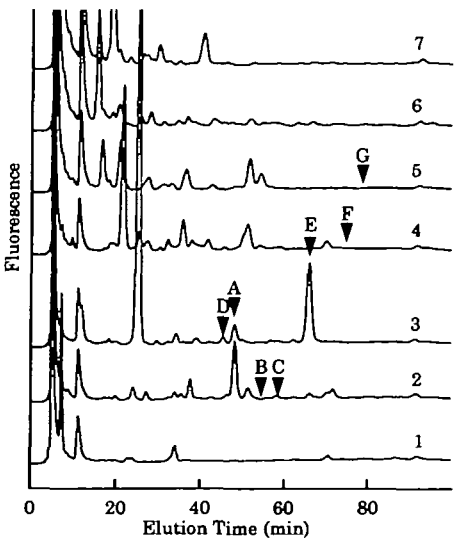



Fig. 1. **Reversed-phase HPLC of fractions obtained by size-fractionation HPLC of PA-sugar chains from cerebrum.** 1-7, elution profiles of fractions N3-N9 collected by size-fractionation HPLC according to the elution positions of $Man_3GlcNAc_2$-PA-$Man_9GlcNAc_2$-PA, respectively. HPLC was carried out on a Cosmosil 5C18-P column (0.15×25 cm). Arrowheads indicate the elution positions of standard PA-sugar chains: A, BA-1; B, BA-1′; C, BiB; D, BiF; E, BA-2; F, GaBA-2 and GbBA-2; G, G2BA-2.

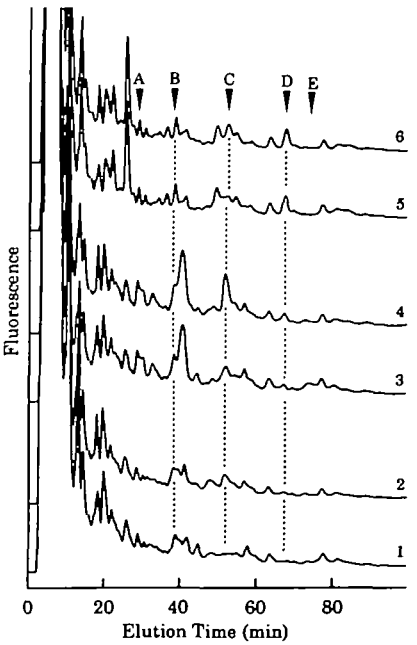



Fig. 2. **Reversed-phase HPLC of fraction Ns obtained from mouse tissues before and after digestion with β-galactosidase.** HPLC was carried out on a Cosmosil 5C18-P column (0.15×25 cm). 1, 3, and 5, HPLC of fraction Ns from liver, kidney, and brain, respectively. 2, 4, and 6, β-galactosidase (*Streptococcus* 6646k) digests of 1, 3, and 5, respectively. Arrowheads indicate the elution positions of standard PA-sugar chains: A, Bi; B, BiF; C, BiB; D, BA-2; E, G2BA-2.

The presence of BA-2 structures containing Sia-Gal was next analyzed in the acidic fractions. As the amount of the acidic fraction (the sum of Fractions A1-A5) in brain per milligram of protein was almost the same as the amounts in liver and kidney (Table II), it is considered that galactosylated *N*-linked sugar chains are expressed in brain at the same level as in liver and kidney. Fractions A1N-A5N obtained by digesting Fractions A1-A5, respectively, from cerebrum with sialidase were analyzed by reversed-phase HPLC (Fig. 3). G2Bi and G2BiF were detected in Fraction A2N, and 226-T, 224-T, and TetraF in Fractions A4N and A5N. Galactosylated BA-2 (Arrowheads G and H in Fig. 3, 1 and 2), however, occurred in only a very small amount, if at all (*29*). Similar results were obtained for cerebellum (data not shown). These findings again indicate that in mouse brain sialylated and galactosylated BA-2s are hardly present, if at all, while sialylated and galactosylated *N*-linked sugar chains not based on the BA-2 structure are expressed.

*β1-4Gal-T Activity in Mouse Tissues*—The fact that most *N*-linked sugar chains are galactosylated in mouse brain but galactosylated BA-2 was not detected suggests that the β1-4Gal-T activity in brain is specific. The substrate specificity of β1-4Gal-T in brain tissue was thus analyzed using an enzyme solution prepared from 8-week-old ICR mice and four biantennary PA-sugar chains in which the bisecting GlcNAc and α1-6Fuc residues were varied (BA-2, BiB, BiF, and Bi). Each acceptor has two Gal binding sites, the GlcNAc residue on the Manα1-6 branch and that on the Manα1-3 branch. The ratios of the initial transfer rates to the α1-3 branch to those to the α1-6 branch (branch specificity) were assayed (Table III). Enzyme solutions prepared from kidney and liver were also used as enzyme sources in control experiments. The bisecting GlcNAc residue (BiB) influenced the branch specificity of the β1-4Gal-Ts when the liver or kidney enzymes were used, but not with the brain enzyme. These results indicate that β1-4Gal-T activity in brain differs from those in liver and kidney. The branch specificities of the mouse liver and kidney β1-4Gal-Ts were similar to that of bovine milk β1-4Gal-T-I when the enzyme activity was analyzed using Bi as an acceptor (*30*).

The initial rates of Gal transfer were next compared. The initial transfer rate of brain β1-4Gal-T per milligram of protein was 1/10 the rates of the liver and kidney transferases when Bi was used as a substrate. The relative β1-4Gal-T activities of the three enzyme preparations using BiF as an acceptor were almost the same as those obtained using Bi; however, the bisecting GlcNAc residue reduced the initial rate of transfer (Fig. 4, column 3). When the β1-4Gal-T activity was analyzed using BA-2 as an acceptor, no activity was detected for the brain enzyme while the activities of the liver and kidney enzymes were similar to those using BiB as an acceptor (Fig. 4, column 4). These results and the detection of galactosylated tri- and tetra-antennary sugar chains (Fig. 3) indicate that "brain-type" β1-4Gal-T transfers galactose to tri- or tetra-antennary sugar chains, but does not transfer galactose to BA-2.

The preceding results were obtained using PA-sugar chains as acceptor substrates. Since the reducing ends of the PA-sugar chains were modified chemically and the structures of the reducing end residues differed from those of glycoproteins, it was thought that the substrate specificity of β1-4Gal-T might be affected by the structural changes at the reducing ends of the acceptors. To exclude this possibility, we next used glycopeptide-Bi and -BA-2 prepared in the manner described in "MATERIALS AND METHODS" as acceptors. After the enzymatic reactions, the

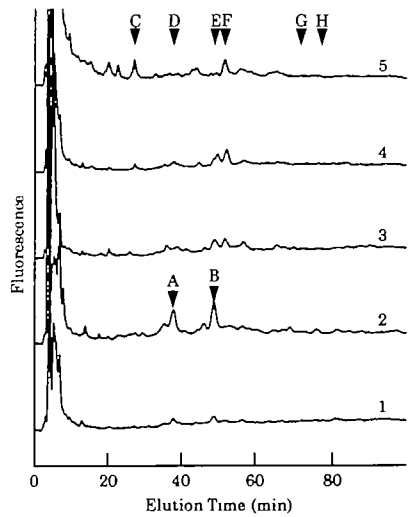



Fig. 3. **Reversed-phase HPLC of neutral fractions obtained by Mono Q HPLC of desialylated fractions A1-A5 from cerebrum.** HPLC was carried out on a Cosmosil 5C18-P column (0.15×25 cm). 1, elution profile of the neutral fraction obtained by Mono Q chromatography of the sialidase digests of fraction A1; 2, fraction A2; 3, fraction A3; 4, fraction A4; 5, Fraction A5. Arrowheads indicate the elution positions of standard PA-sugar chains: A, G2Bi; B, G2BiF; C, 226-T; D, Tetra; E, TetraF; F, 224-T; G, GaBA-2 and GbBA-2; H, G2BA-2.

TABLE II. **Amounts of *N*-linked sugar chains found in tissues.** The amounts of *N*-linked sugar chains were measured by quantitating PA-GlcNAc released by acid hydrolysis followed by *N*-acetylation as described in "MATERIALS AND METHODS." Values are expressed as pmol/mg of lyophilized tissue.

| | Fraction N | Sum of fraction A1-A5 |
|---|---|---|
| Liver | 1,090 | 300 |
| Kidney | 620 | 380 |
| Brain | 470 | 350 |

TABLE III. **Branch specificity of β1-4Gal-Ts.** The reaction mixtures were separated by a Shim-Pak CLC-ODS column under conditions in which GaBA-2, GbBA-2, GaBi, GbBi, GaBiF, GbBiF, GaBiB, GbBiB could be separated. The ratios of galactosylation on the Manα1-3 branch to that on the Manα1-6 branch are shown.

| Tissue | PA-acceptor | Manα1-3/Manα1-6 |
|---|---|---|
| Liver | Bi | 1.6 |
| | BiF | 1.6 |
| | BiB | 1.0 |
| | BA-2 | 1.0 |
| Kidney | Bi | 1.6 |
| | BiF | 1.6 |
| | BiB | 1.0 |
| | BA-2 | 1.0 |
| Brain | Bi | 1.0 |
| | BiF | 1.0 |
| | BiB | 1.0 |
| | BA-2 | ND[a] |

[a]ND, not detected.

$N$-linked sugar chains were released by hydrazinolysis-$N$-acetylation, and the reducing ends of the $N$-linked sugar chains were pyridylaminated. The PA-sugar chains thus obtained were separated by reversed-phase HPLC using a Cosmosil 5C18-P column (1.0×25.0 cm) (data not shown). With glycopeptide-Bi as the acceptor substrate, the fraction at the elution position of galactosylated Bi (GaBi and GbBi) was collected and further purified by size-fractionation HPLC using a Shodex NH2P-50 column (0.46×5 cm) (Fig. 5a, 1 and 3). A peak was detected at the elution position of GaBi and GbBi. The identity of the product was further confirmed by the detection of a peak at the elution position of Bi after β-galactosidase digestion (Fig. 5a, 2 and 4). Similar HPLC procedures were carried out using glycopeptide-BA-2 as the acceptor substrate. A peak appeared at the elution position of GaBA-2 and GbBA-2 when an enzyme solution prepared from liver was used (Fig. 5, b-1). This peak disappeared and a new peak appeared at the elution position of BA-2 as a result of β-galactosidase digestion (Fig. 5, b-2). When an enzyme solution prepared from brain tissue was used, however, no peak appeared at the elution position of GaBA-2 and GbBA-2 on either reversed-phase (data not shown) or size-fractionation HPLC (Fig. 5, b-3). Using the enzyme preparation from brain, Gal was not transferred from UDP-Gal to the acceptor with both bisecting GlcNAc and α1-6Fuc residues, and, as a consequence, a relatively large amount of the BA-2 structure was detected.

It is known that α-lactalbumin forms a lactose synthase complex with β1-4Gal-T-I. This complex transfers galactose to glucose (*31-33*), thus stimulating lactose synthesis, while galactosylation on the GlcNAc residues of Bi is suppressed (*34, 35*). We examined α-lactalbumin modulation of the transferase activity toward Bi using liver and brain enzyme solutions. The "brain-type" β1-4Gal-T was not suppressed by the addition of α-lactalbumin (data not shown). These results also indicate that the β1-4Gal-T(s) expressed in mouse brain differs from β1-4Gal-T-I.

β1-4Gal-T-I–VI have been cloned from bovine (*9, 10*),

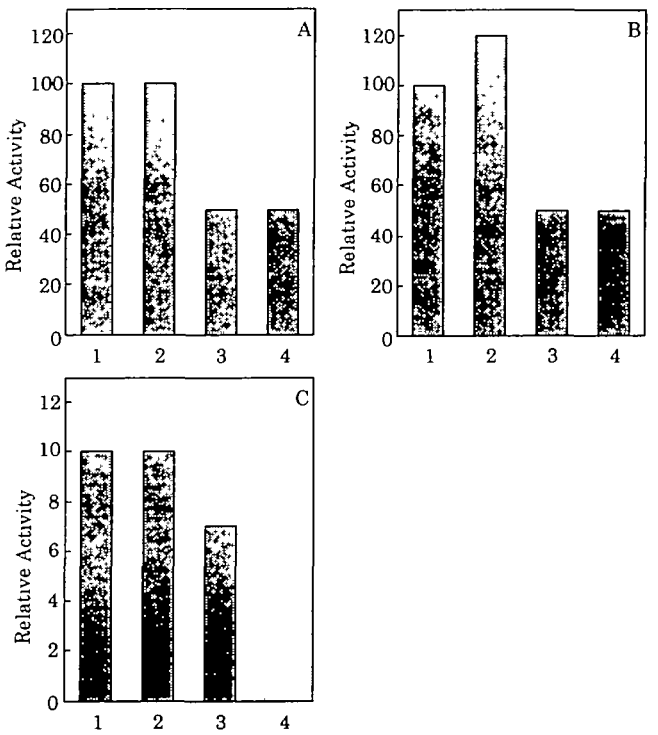



**Fig. 4. Acceptor specificities of β1-4Gal-Ts from mouse liver (A), kidney (B), and brain (C).** Transferase activities using (1) Bi, (2) BiF, (3) BiB, and (4) BA-2 as acceptor substrates. The initial rates of transfer were compared, and the value per mg protein obtained using Bi and liver enzyme was taken as 100.

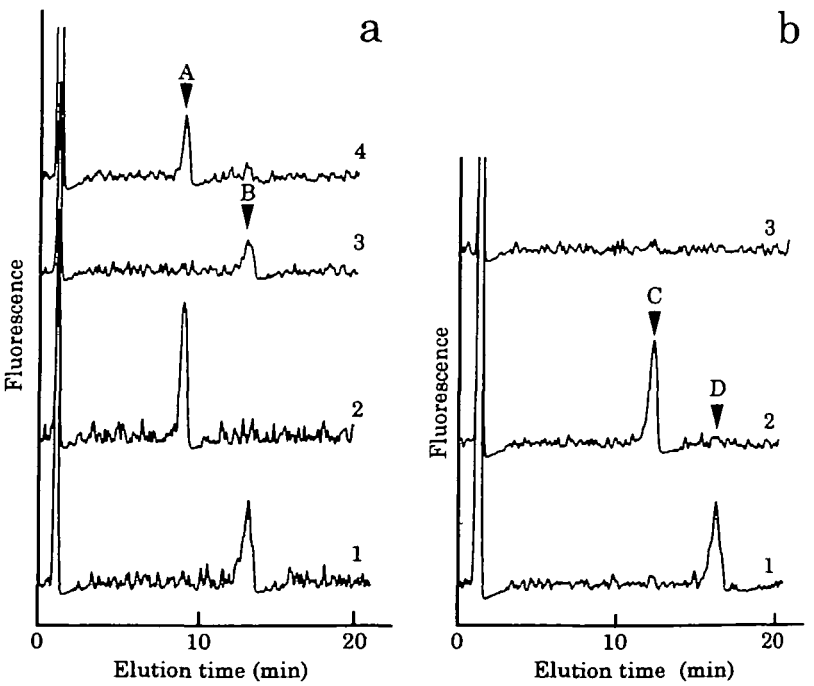



**Fig. 5. Size-fractionation HPLC of PA-sugar chains obtained from transfer products with β1-4Gal-T from mouse liver and brain using glycopeptide-Bi and glycopeptide-BA-2 as acceptor substrates.** PA-sugar chains prepared from the reaction mixture as described in "MATERIALS AND METHODS" were first separated by reversed-phase HPLC using a Cosmosil 5C18-P column (1.0×25 cm) (data not shown), and the fraction appearing at the elution position of GaBi, GbBi or GaBA-2, GbBA-2 was collected. Part of this fraction was further purified by size-fractionation HPLC using a Shodex NH2P-50 column (0.46×5 cm). a-1 and -3, HPLC of PA-sugar chains obtained with liver and brain β1-4Gal-T, respectively, using glycopeptide-Bi as an acceptor substrate. One-twentieth of the reaction mixture was injected. a-2 and -4, β-galactosidase (*Streptococcus* 6646k) digests of a-1 and -3, respectively. One-tenth of the reaction mixture was injected. b-1 and -3, HPLC of PA-sugar chains obtained with β1-4Gal-T from liver and brain, respectively, using glycopeptide-BA-2 as an acceptor substrate. One-twentieth of the reaction mixture was injected. b-2, β-galactosidase (*Streptococcus* 6646k) digest of b-1. One-tenth of the reaction mixture was injected. Arrowheads indicate the elution positions of standard PA-sugar chains: A, Bi; B, GaBi and GbBi; C, BA-2; D, GaBA-2 and GbBA-2.

human (*11, 12, 15, 17*), and a human breast tumor cell line (*14, 36*). Among these β1-4Gal-Ts, the mRNAs of β1-4Gal-T-III and -V are strongly expressed in human brain (*14, 15*), while β1-4Gal-T-VI is strongly and specifically expressed in human and rat brain (*15, 16*) and is reported to be responsible for the galactosylation of glycosphingolipids (*16*). To disclose whether the "brain-type" β1-4Gal-T is β1-4Gal-T-III, β1-4Gal-T-V, or an unknown β1-4Gal-T, further analysis is needed as the above reports did not describe the substrate specificities of the transferases using BA-2 as an acceptor (*12, 14, 15, 36*).

*Comparison of β1-4Gal-T Activities in Embryonic and Postnatal Mouse Brain*—Northern blot analysis recently revealed the β1-4Gal-T-I mRNA to be expressed in the brain tissues of embryonic mice, but not in those of adult mice, and the corresponding transferase activity using Bi as an acceptor substrate was found in embryonic brain (*37*). We, therefore, compared the β1-4Gal-T activities in mouse brain on embryonic day 13 and postnatal day 10 using BA-2 as an acceptor substrate. Gal was not transferred to BA-2 in the case of the membrane enzyme solution prepared from postnatal day 10 brain, but it was transferred when a membrane enzyme solution prepared from embryonic day 13 brain was used (Table IV). $(Gal)_{1-2}$-BA-2 were expressed in postnatal day 7 cerebrum, and their amounts were 1/5 that of BA-2 (unpublished data). These findings suggest that BA-2 was galactosylated by β1-4Gal-T-I in mouse embryonic brain, as the β1-4Gal-T-I mRNA still remains in postnatal day 7 cerebrum (*37*).

TABLE IV. **Transferase activities of the membrane enzyme solutions from the cerebral hemispheres using BA-2 and Bi as acceptor substrates.** E13, the membrane enzyme solution from embryonic day 13 brain, and P10, that of postnatal day 10 brain, were used as the enzyme sources. Values are expressed as enzyme activity per mg protein, and relative to that of galactosylation on Bi using a liver enzyme solution obtained from an 8-week-old mouse, which was taken as 100.

| enzyme solution (100 μg protein) | Galactosylation on BA-2 | Galactosylation on Bi |
|---|---|---|
| E13 | 30 | 150 |
| P10 | ND[a] | 10 |

[a]ND, not detected.

*Effect of BA-2 on β1-4Gal-T Activity in Mouse Brain*—We have shown evidence indicating the existence of a "brain-type" β1-4Gal-T(s) that can transfer Gal to Bi, BiF, and BiB but not to BA-2. The facts that the transferase recognizes both the α1-6Fuc and bisecting GlcNAc residues, and that the structure of BA-2 is quite similar to the structures of substrates such as Bi, BiF, and BiB suggest that BA-2 binds to the "brain-type" β1-4Gal-T. To confirm this, the effect of BA-2 on brain and liver β1-4Gal-T activity in 8-week-old mice was analyzed using Bi as an acceptor substrate. Inhibition of the transferase activity of the "brain-type" β1-4Gal-T was observed (Fig. 6a). To quantitate the products precisely, the fraction at the elution position of Bi and galactosylated Bi (GaBi and GbBi) was collected, and Bi and galactosylated Bi were analyzed by size-fractionation HPLC on a Shodex NH2P-50 column

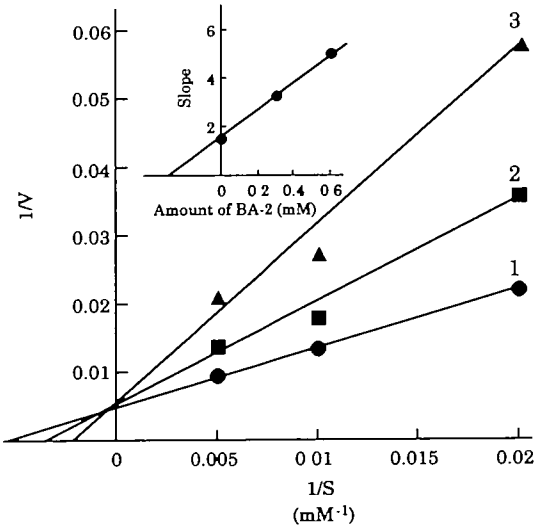
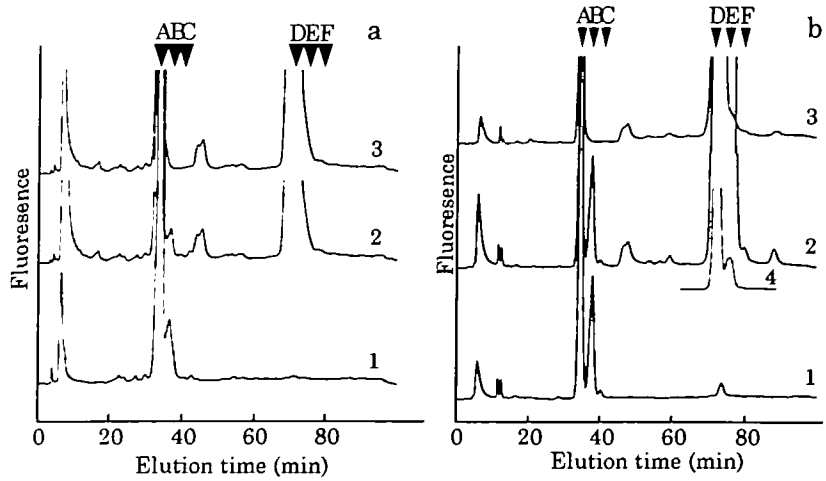



Fig. 7. **Lineweaver-Burk plots for the transferase activity of mouse brain β1-4Gal-T in the presence of BA-2.** Products were quantitated as described in the text. 1, β1-4Gal-T activities using Bi as an acceptor substrate; 2, the same as in 1 but in the presence of 0.3 mM BA-2; 3, the same as in 1 but in the presence of 0.6 mM BA-2. The transfer rates ($V$) are expressed in terms of the peak areas on the chromatograms. S, substrate concentration. Inset, slopes of lines 1-3 plotted *versus* the inhibitor concentration.

Fig. 6. **Effect of BA-2 on β1-4Gal-T activity in mouse brain and liver.** The enzymatic reactions were carried out as described in "MATERIALS AND METHODS" but in the presence of BA-2. HPLC was carried out on a Cosmosil 5C18-P column (0.15×25 cm). a-1, Reversed-phase HPLC of part of the reaction mixture obtained with mouse brain β1-4Gal-T using Bi as an acceptor substrate; a-2, the same as in a-1 but in the presence of 0.6 mM BA-2; a-3, the same as in a-2 but without UDP-Gal. b-1, Reversed-phase HPLC of part of the reaction mixture obtained with mouse liver β1-4Gal-T using Bi as an acceptor substrate; b-2, the same as in b-1 but in the presence of 0.6 mM BA-2; b-3, the same as in b-2 but without UDP-Gal; b-4, elution profile of 1/10 of b-2. Arrowheads indicate the elution positions of standard PA-sugar chains: A, Bi; B, GaBi and GbBi; C, G2Bi; D, BA-2; E, GaBA-2 and GbBA-2; F, G2BA-2.



(0.46×25 cm) under conditions whereby Bi and galactosylated Bi could be completely separated (data not shown). From the initial transfer rates observed, the $K_i$ value was obtained according to the reported method (*38, 39*) and found to be 0.29 mM (Fig. 7). The $K_m$ value of Bi was 0.15 mM. However, the liver β1-4Gal-T activity was not affected by the presence of BA-2 under the same reaction conditions (Fig. 6b). These results suggest that the affinity of liver β1-4Gal-T for BA-2 is much lower. Considering that glycoproteins with the BA-2 structure or their cluster are expressed mainly in membrane fractions (*3*), the BA-2 structure might be one of the ligands for "brain-type" β1-4Gal-T on the cell surface.

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