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The Molecular Cloning and Expression of α2,8-Sialyltransferase (GD3 Synthase) in a Rat Brain[1]

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We have cloned the cDNA for a GD3 synthase (α-2,8-sialyltransferase, [EC 2.4.99.8]) from a rat embryonic brain cDNA library. Mammalian cells transfected with the cloned cDNA expressed GD3 on the cell surface and showed GD3 synthase activity. The deduced protein (342 amino acid residues) was predicted to have a type II membrane topology containing the "sialyl motif" and was found to be 91% similar to its human homologue. Analysis of the acceptor specificity of GD3 synthase protein indicated that this enzyme catalyzed the biosynthesis of GT1a and GQ1b as well as GD3. Northern blot analyses showed that the GD3 synthase gene is preferentially transcribed in the brain and the spleen. The expression of GD3 synthase mRNA was developmentally regulated, with the highest level in the brain during embryonic days 15 to 18. *In situ* hybridization analyses demonstrated that the GD3 synthase is strongly expressed in the ventricular/subventricular zone of the embryonic rat brain and retina. In the adult rat, GD3 synthase mRNA was detected in the cerebral cortex, hippocampus, thalamus, and cerebellum. These studies show that the spatio- and stage-restricted expression of GD3 in the developing rat brain may be regulated in part by the level of GD3 synthase mRNA.

Key words: central nervous system, gangliosides, GD3 synthase, sialyltransferase.

Gangliosides are a family of sialic acid-containing glycosphingolipids which are amphiphilic components of the outer leaflet of plasma membranes (*1*). Much attention has been paid to the expression of gangliosides in the central nervous system (CNS) because they are highly enriched in the brain and their molecular species change during development of the bird and mammalian brain (*2*). Furthermore, their distributions in the CNS are spatio- and stage-specific (*3*), suggesting that they may play functional roles in the development and function of the CNS.

Numerous biochemical studies indicate that GD3 is the predominant ganglioside of the early, immature nervous systems of birds and mammals, but its amount decreases in contrast with the accumulation of higher sialylated gangliosides during maturation (*4*). The spatial and temporal expression of GD3 in the CNS has also been demonstrated by means of immunohistochemical staining using specific monoclonal antibodies (*5-10*). GD3 was intensely expressed in growing endothelia, in the immature neuro- and glioblasts of the ventricular and subventricular layers of the embryonic rat cerebellum, and in the dentate gyrus and hippocampus. In extraneural tissues, GD3 is implicated in cell attachment (*11*) and cell-to-cell interactions during embryogenesis (*12*). These observations suggest that GD3 may play an important role not only in brain development but in extraneural tissues.

The gangliosides are synthesized *via* four primary biosynthetic pathways by a family of glycosyltransferases (*13*). The diversity of ganglioside composition among vertebrates reflects the differential regulation of ganglioside biosynthesis through different pathways. GD3 is important as a precursor of the b and c series gangliosides and is synthesized by GD3 synthase (2,8ST), *i.e.*, CMP-*N*-acetylneuraminate:GM3 α2,8-sialyltransferase, which is responsible for the ganglioside pattern in tissues (*14*). To clarify the mechanism underlying the regulated expression of GD3, it is essential to elucidate the enzymatic basis of GD3 metabolism. The cDNA of human GD3 synthase has been cloned by the expression cloning strategy by us (*15*) and others (*16, 17*). The chromosomal localization of the 2,8ST gene was determined in human and mouse (*18*).

To address the questions of how the expression of GD3 is

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Abbreviations: 2,8ST, CMP-NeuAc:NeuAcα2→3Galβ1→4Glcβ1→1′ ceramide α2,8-sialyltransferase (GD3 synthase); HPTLC, high performance thin layer chromatography; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pairs.

regulated during brain development and what role GD3 plays in brain function, we isolated a rat 2,8ST cDNA by using PCR based on the deduced amino acid sequences of the human 2,8ST cDNA and demonstrated the distribution of mRNA in the embryo and adult brain. Rat 2,8ST mRNA is expressed by a limited number of tissues and its expression is regulated in a stage- and region-specific manner. These data suggest that the expression of the 2,8ST gene is involved in the developmentally specific and tissue-type specific expression of gangliosides in the nervous system.

MATERIALS AND METHODS

Cloning of the Rat GD3 Synthase cDNA—Based on the sequence information of the conserved sialyl motifs of human 2,8ST (*15, 19*), two degenerate oligonucleotides were synthesized. The sequence of the 5′ and 3′ primers were 5-CCNYTNAARAARTGYGCNGT (nucleotides correspond to 448-467) and 5′-SWRAANGGCCARAANCCRTA (nucleotides correspond to 925-944) (N=A+G+T+C, R=A+G, W=A+T, S=C+G, Y=C+T), respectively. Poly(A)$^+$ RNA was purified from rat brain at embryonic day (E) 15 by using a Fast Track mRNA isolation kit (Invitrogen). For PCR amplification, first strand cDNA synthesized from rat embryo E15 brain poly(A)$^+$ RNA was combined with each primer. Thirty cycles (95°C for 1 min, 50°C for 1 min, and 73°C for 2 min) were run using Taq polymerase (Stratagene), and the products were subcloned into pGEM-T vector (Promega). Several clones obtained from rat embryo E15 brain were sequenced by a dideoxynucleotide chain termination method with the Autocycle Sequencing kit (Pharmacia) using a Pharmacia A.L.F. DNA Sequencer. One clone, pGMR2, was judged to contain the sialyl motif, by homology with the human sequence (*15*). To clone the full-length rat 2,8ST cDNA, a 0.5-kbp *Apa*I-*Sac*I fragment of the clone pGMR2 was used as a probe for screening a rat E15 brain cDNA library. This cDNA library was constructed from poly(A)$^+$ RNA prepared from rat E15 brain as described above. Random-primed cDNA was inserted into the expression vector pCEV18 as described previously (*15*). Approximately 0.76 million colonies were screened with the cloned PCR fragment described above. One positive clone, pCEVr2,8ST, was isolated and sequenced as described above. Nucleotide sequences were analyzed by the Gene Works program (Intelli Genetics).

Expression of Rat 2,8ST in CHOP Cells—CHO cells that stably express the polyoma large T antigen (CHOP cells) were kindly provided by Dr. J. Dennis. CHOP cells (1×10^6 cells) were seeded on a petri dish (9 cm diameter) a day before transfection. The cells were transfected with one of the expression plasmids pCEVr2,8ST or pCEV18 (9 μg each) using LipofectAMINE™ (BRL) according to the manufacturer's instructions. After 72 h, cells were harvested and used for the sialyltransferase assay.

Construction of a Soluble Form of Rat 2,8ST—To obtain the *Staphylococcus aureus* protein A-rat 2,8ST fusion protein, a *Bsr*FI^{194}-*Pst*I^{1131} fragment of the rat 2,8ST encoding the C-terminal 287 amino acid residues of the predicted protein was blunt-ended and ligated with *Eco*RI linker. The resultant fragment was cloned in-frame into the unique *Eco*RI site of the pPROTA vector (*20*), kindly provided by Dr. M. Fukuda, just downstream of the C-terminal of the IgG-binding domain of protein A as described previously (*15*).

Expression of the Soluble Form of Rat 2,8ST—Ten micrograms of plasmid (pPROTAr2,8ST or pPROTA) was transfected into COS-7 cells (5×10^6 cells) by electroporation using a Bio-Rad Gene Pulser at 300 V, 960 μFD. After 72 h, the cell culture media were collected and used for the sialyltransferase assay.

Flow Cytometry Analysis—CHOP cells transfected with pCEVr2,8ST or with a control vector were harvested 72 h after transfection and stained with the anti-GD3 monoclonal antibody, R24. The cells were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG and analyzed on a FACScan (Becton-Dickinson). Approximately 60% of the population of CHOP cells transfected with pCEVr2,8ST expressed GD3 on the cell surface.

Northern Blot Analysis—Poly(A)$^+$ RNAs were isolated from rat embryo or adult brain by the method described above. Multiple Tissue Northern Blot of rat poly(A)$^+$ RNA was purchased from Clontech Laboratories. The blots were hybridized with a ^{32}P-labeled 0.5-kbp *Apa*I-*Sac*I fragment of the clone pGEMR2 described above or a 1.7-kbp rat EF1α cDNA (*21*) at 42°C in hybridization buffer (5×SSPE, 5×Denhardt's solution, 50% formamide, 0.2% SDS, 0.1 mg/ml salmon sperm DNA, 5% dextran sulfate). After hybridization, the filters were washed in 1×SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) plus 0.1% SDS twice for 1 h at 68°C.

Sialyltransferase Assays—Sialyltransferase activity was measured as described previously (*22*), with minor modifications. The reaction mixture (20 μl) contained 10 μl of cell homogenate, 1.5 mM CMP-[^{14}C]-NeuAc (5×10^4 cpm), 0.4% Triton CF54, 10 mM $MgCl_2$, 100 mM sodium cacodylate, pH 6.5, 1 mM 2,3-dehydro-2-deoxy-NeuAc, which inhibits endogenous sialidase activity, and 0.3 mM acceptor substrate. After incubation at 37°C for 2 h, the reaction was stopped with 10 μl of methanol. An aliquot (10 μl) of the reaction mixture was applied to a C18 reversed phase HPTLC plate (RP-18WE, E. Merck), which was developed with water for 10 min. The radio-labeled products were scraped off from the origin on the plate and extracted with 300 μl of chloroform-methanol (1 : 1, v/v) with sonication. The extract was dried and applied to a silica gel 60 HPTLC plate (E. Merck). After developing the plate in a solvent system of chloroform/methanol/0.5% $CaCl_2$ (55 : 45 : 10, v/v/v), the glycolipids were visualized with orcinol reagent and the mobility was compared to those of the standard gangliosides. The position and the radioactivity of each radio-labeled reaction product were estimated with a Fujix BAS 2000 Bio-imaging Analyzer (Fuji Photo Film). For the soluble form of rat 2,8ST, an aliquot (10 μl) of the 50% suspension of IgG-Sepharose (Pharmacia) was added to 1 ml of culture medium and rotated overnight at 4°C. After washing three times with PBS and two times with 100 mM cacodylate, pH 6.5, these beads were used as an enzyme source. The assay conditions were the same as described above except that 20 μM CMP-[^{14}C]-NeuAc (25×10^4 cpm) was used. Reactions were allowed to proceed at 37°C for periods of time adjusted to yield linear rates (<20% of CMP-NeuAc consumed during the course of the reaction; 2 h).

In Situ Hybridization—Embryos were obtained from female Wistar rat. Embryonic age was determined accord-

ing to the vaginal plug (day 0). Embryonic tissues were fixed with 4% paraformaldehyde in phosphate buffer at 4°C for 24 h, dehydrated and embedded in paraffin. Tissues from postnatal and adult rats were also fixed with 4% paraformaldehyde solution by cardiac perfusion and embedded in paraffin. Four-micrometer-thick sections were used for an *in situ* hybridization study. A 0.45-kb fragment (nucleotides 39 to 491) containing a 5′ untranslated region was amplified by PCR using a 5′ primer (5′-CACCGAGGCTGCGATGAGCC) and a 3′ primer (5′-TTCAGAATCCCACCGTTTCC). The PCR product was subcloned into pGEM-T vector (Promega). The resultant plasmid was used to generate [α-^{33}P]UTP-labeled antisense and sense-strand riboprobes using SP6 and T7 RNA polymerase (Promega), respectively. Treatment of the tissue slides and hybridization conditions were the same as described previously (*23*). Briefly, sections were pretreated with proteinase K (Boehringer-Mannheim) and acetylated with acetic anhydride/triethanolamine HCl. Tissue sections were hybridized with probes overnight in a moist chamber at 55°C. After hybridization, the sections were treated with 12.5 μg/μl of RNase A in 10 mM Tris HCl, pH 7.6, 1 mM EDTA, and 500 mM NaCl at 37°C for 30 min and then washed with 2×SSC and 0.2×SSC at 55°C. Dried slides were dipped in NTB-3 emulsion (Eastman Kodak) diluted (1 : 1) with 2% glycerol, dried and exposed for 2 weeks at 4°C in the desiccated slide boxes. After development and fixation, the sections were stained with hematoxylin and eosin.

RESULTS

Isolation and Analysis of Rat 2,8ST cDNA—To clone the rat 2,8ST cDNA, we designed two degenerate primers that corresponded to conserved sequences within the sialyl motif of human 2,8ST. The primers were used to generate PCR fragments using a first-strand cDNA template derived from the rat E15 embryo. We used the PCR fragments as a probe to screen a rat E15 brain cDNA library and obtained a full-length clone of rat 2,8ST cDNA (Fig. 1). The nucleotide sequence of the cloned cDNA has 87% identity with human 2,8ST cDNA. There are two in-frame ATGs at the 5′ end of the rat 2,8ST cDNA sequence, 42 nucleotides apart. However, by comparison with the human 2,8ST cDNA, the second ATG is more likely to be the initiation codon. Using the second in-frame ATG, the deduced open reading frame encodes a 342-amino acid polypeptide with a predicted molecular mass of 38,985 Da. The predicted polypeptide exhibited 91% identity with the human 2,8ST protein and had one amino acid insertion in the C-terminal putative catalytic domain. Analysis of the primary structure of the deduced rat 2,8ST protein suggests that the protein having a transmembrane domain at the N-terminal portion (residues 13-43) and sialyl motifs (sialyl motif 1: residues 120-168, sialyl motif 2: residues 258-280), is a member of the sialyltransferase family that has type II membrane topology.

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                                                CCGGCGTTGTGCTGAGGCTGCCACCACTGACCCTGGGACACCGAGGCTGCGATGAGCCCCTGCGGGCGGGCCCTACATACATCCAGAGGGGCC      93

    ATGGCCATGCTGGCGCGGAAATTCCCGCGTACCCGGCTACCCGTGGGAGCCAGTGCCCTCTGTGTGGTGGTCCTCTGTTGGCTCTATGTCTTCCCTGTCTACCGGCTGCCCAACGAGAAG     213
1   MetAlaMetLeuAlaArgLysPheProArgThrArgLeuProValGlyAlaSerAlaLeuCysValValValLeuCysTrpLeuTyrValPheProValTyrArgLeuProAsnGluLys

    GAGATCGTGCAAGGAGTGCTGGCGCAGCGCACAGCTTGGAGGAGGAACCAGACGTCAGCCAGGCTCTTCAGGAGACAAATGGAAGACTGCTGCAACCCCGCCCATCTCTTTGCAATGACG     333
41  GluIleValGlnGlyValLeuAlaGlnArgThrAlaTrpArgArgAsnGlnThrSerAlaArgLeuPheArgArgGlnMetGluAspCysCysAsnProAlaHisLeuPheAlaMetThr

    AAGGTGAACTCCCCCATGGGGAAGAGCCTGTGGTATGACGGGGAGTTTCTATACTCGCTCACCATCGACAACTCCACGTACTCCCTCTTCCCCCAGGCAACCCCCTTCCAGCTGCCATTG     453
81  LysValAsnSerProMetGlyLysSerLeuTrpTyrAspGlyGluPheLeuTyrSerLeuThrIleAspAsnSerThrTyrSerLeuPheProGlnAlaThrProPheGlnLeuProLeu

    AAGAAATGTGCGGTGGTGGGAAACGGTGGGATTCTGAAGATGAGTGGCTGTGGCCGTCAAATAGATGAAGCAAATTTTGTCATGCGGTGTAACCTTCCTCCCTTGTCAAGTGAATACACC     573
121 LysLysCysAlaValValGlyAsnGlyGlyIleLeuLysMetSerGlyCysGlyArgGlnIleAspGluAlaAsnPheValMetArgCysAsnLeuProProLeuSerSerGluTyrThr

    AGAGATGTGGGTTCCAAGACTCAGTTGGTGACAGCTAACCCCAGCATAATTCGCCAGAGATTTGAAAACCTGCTGTGGTCCAGAAAGAAGTTTGTGGACAACATGAAGATCTATAACCAC     693
161 ArgAspValGlySerLysThrGlnLeuValThrAlaAsnProSerIleIleArgGlnArgPheGluAsnLeuLeuTrpSerArgLysLysPheValAspAsnMetLysIleTyrAsnHis

    AGTTACATATACATGCCTGCCTTTTCTATGAAGACGGGAACAGAGCCGTCTCTCCGTGTGTACTACACCCTGAAAGACGCTGGAGCCAATCAAACAGTGCTTTTTGCTAACCCCAACTTT     813
201 SerTyrIleTyrMetProAlaPheSerMetLysThrGlyThrGluProSerLeuArgValTyrTyrThrLeuLysAspAlaGlyAlaAsnGlnThrValLeuPheAlaAsnProAsnPhe

    CTTCGAAACATTGGAAAGTTCTGGAAGGGTAGGGGGATCCATGCCAAGCGCCTGTCTACAGGACTCTTTTTGGTGAGTGCAGCCTTGGGCCTTTGTGAGGAGGTGTCCATCTACGGCTTC     933
241 LeuArgAsnIleGlyLysPheTrpLysGlyArgGlyIleHisAlaLysArgLeuSerThrGlyLeuPheLeuValSerAlaAlaLeuGlyLeuCysGluGluValSerIleTyrGlyPhe

    TGGCCCTTCTCCGTGAACATGCAAGGGGAACCTATCAGCCACCATTACTATGACAACGTCTTGCCCTTCTCAGGCTTCCATGCCATGCCTGAGGAGTTTCTTCAACTCTGGTATCTTCAT    1053
281 TrpProPheSerValAsnMetGlnGlyGluProIleSerHisHisTyrTyrAspAsnValLeuProPheSerGlyPheHisAlaMetProGluGluPheLeuGlnLeuTrpTyrLeuHis

    AAAATGGGTGCACTGAGGATGCAACTGGACCCATGTGAGGAGCCGTCGCCGCCACAGCCCACTTCCTAGGGACTGCAGAAGAGGAAAAGACTGGATGGGGGTATTTTTTTGCTAGATTTT    1173
321 LysMetGlyAlaLeuArgMetGlnLeuAspProCysGluGluProSerProProGlnProThrSer

    TTTTTATGTGACTTTCAAAAGGAGCTGCCAAAGTGTGTCATGGATTGGCG                                                                          1223
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Fig. 1. **Nucleotide sequence of the rat GD3 synthase cDNA and the deduced amino acid sequence.** The putative transmembrane domain is underlined. Four putative *N*-glycosylation sites are indicated with closed circles. The domains showing high similarity with the sialyltransferases cloned to date (sialyl motifs) are indicated by dashed lines.

Cloned 2,8ST cDNA Directs GD3 Expression and GD3 Synthase Activity in Transfected Cells—Flow cytometric analysis using anti-GD3 monoclonal antibody R24 showed that GD3 was expressed on the cell surface of the CHOP cells transfected with the expression plasmid, pCEVr2,8ST (Fig. 2A). To confirm that the transfected cells exhibited 2,8ST activity, an enzyme assay was performed using the cell homogenate of the transfectants and exogenous acceptor GM3, as described under "MATERIALS AND METHODS." The pCEVr2,8ST transfected CHOP cells contained a high level of 2,8ST activity (2,000 pmol/h/mg protein) (Fig. 2B). No GD3 was produced with the cell homogenate transfected with pCEV vector alone. Due to the endogenous GM3 synthase activity of CHOP cells, a small amount of radio-labeled GM3 was observed in both of the transfectants.

Further, to prove that the protein molecule encoded by the cloned 2,8ST cDNA is not a *trans*-acting factor that affected 2,8ST activity but 2,8ST itself, we analyzed the sialyltransferase activity of the soluble 2,8ST-protein A fusion protein. The recombinant plasmid was constructed by inserting the putative catalytic domain of rat 2,8ST cDNA (residues 35–342) to the pPROTA vector just downstream of the C-terminus of the IgG-binding domain of protein A. The expression plasmid pPROTAr2,8ST was transfected into COS-7 cells and sialyltransferase activity was examined as described under "MATERIALS AND METHODS." Cultured medium prepared from the cells transfected with pPROTAr2,8ST contained 2,8ST activity (0.1 pmol/ml/h), whereas the medium from the cells transfected with pPROTA vector alone had no significant 2,8ST activity (data not shown).

Substrate Specificity of Rat 2,8ST Protein—To examine the acceptor substrate specificity of the 2,8ST protein, various gangliosides were added exogenously as acceptor molecules for the enzyme assay. The expression plasmid pCEVr2,8ST, which contains full-length 2,8ST cDNA, was transfected to CHOP cells and cell homogenates were prepared as described by Nara *et al.* (*15*). Table I shows that the most favorable acceptor was GM3. GD1a and GT1b, which has a terminal NeuAcα2-3Gal, served as an acceptor to some extent. The reaction product when GD3 was used as an acceptor appeared to be a GT3 from comparison of its R_f value with those of standard gangliosides.

TABLE I. **Substrate specificity of rat GD3 synthase.** CHOP cells were transfected with pCEVr2,8ST and cultured for 3 days. The cell homogenate (100 μg protein) of transfected cells was mixed with reaction solution containing 0.3 mM acceptor and assayed as described in "MATERIALS AND METHODS." 100% of activity was equal to 2,000 pmol of NeuAc incorporation. The specific NeuAc incorporation was estimated by subtracting the background incorporation into endogenous substrate from the radioactivity in the presence of exogenous acceptor.

Acceptor (0.3 mM)	Relative activity (%)[a]
GM3	100
GM2	ND[b]
GM1a	ND
GD1a	13.2
GD3	3.1
GD2	ND
GD1b	ND
GT1b	13.8

[a]Activity is indicated as % incorporation of NeuAc into GM3. [b]ND, not detected.

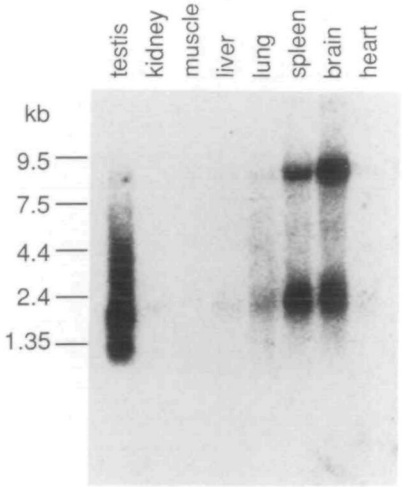

Fig. 3. **Northern blot analysis of GD3 synthase mRNA expression in adult rat tissues.** Each lane contains 2 μg of poly(A)$^+$ RNA from the indicated tissues. The blots were hybridized with a cDNA probe for rat GD3 synthase as described under "MATERIALS AND METHODS."

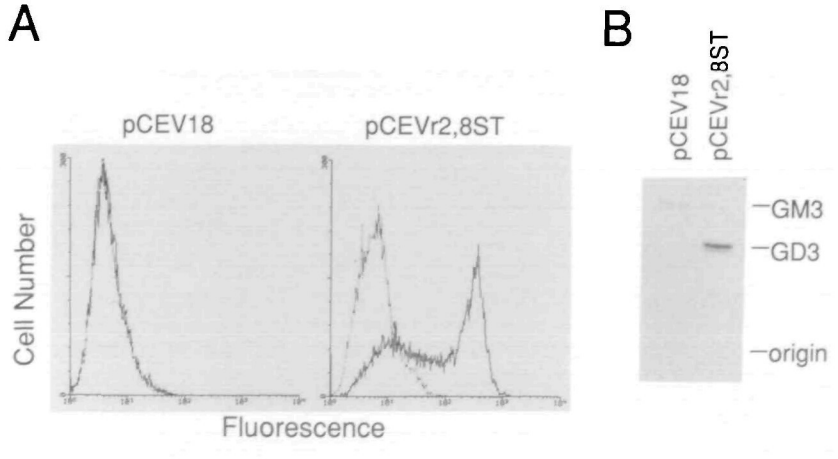

Fig. 2. **(A) Flow cytometry of CHOP cells transfected with pCEVr2,8ST or pCEV18 vector.** Cells were stained with anti-GD3 monoclonal antibody R24 followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (thick lines). Cells stained with FITC-conjugated anti-mouse IgG antibody alone (thin lines) are indicated as a control. **(B) GD3 synthase activity in CHOP cells transfected with pCEVr2,8ST.** GD3 synthase activity transiently expressed in CHOP cells was measured by using CMP-[^{14}C]NeuAc and GM3 as donor and acceptor substrates as described under "MATERIALS AND METHODS."

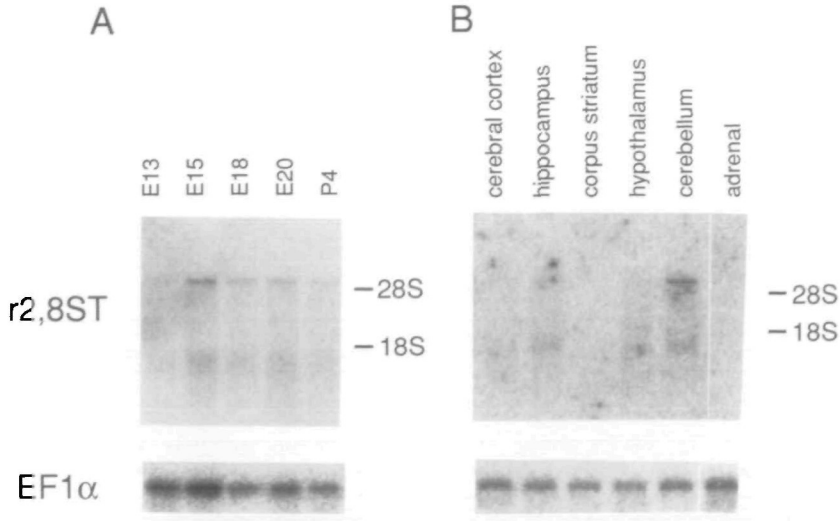

Fig. 4. **Northern blot analysis of GD3 synthase mRNA in developing and adult brain.** Each lane contains 1 μg of poly(A)$^+$ RNA from (A) developing rat brain and (B) adult rat brain tissues. The blots were hybridized with a cDNA probe for rat GD3 synthase and rat EF1α, respectively, as described under "MATERIALS AND METHODS."

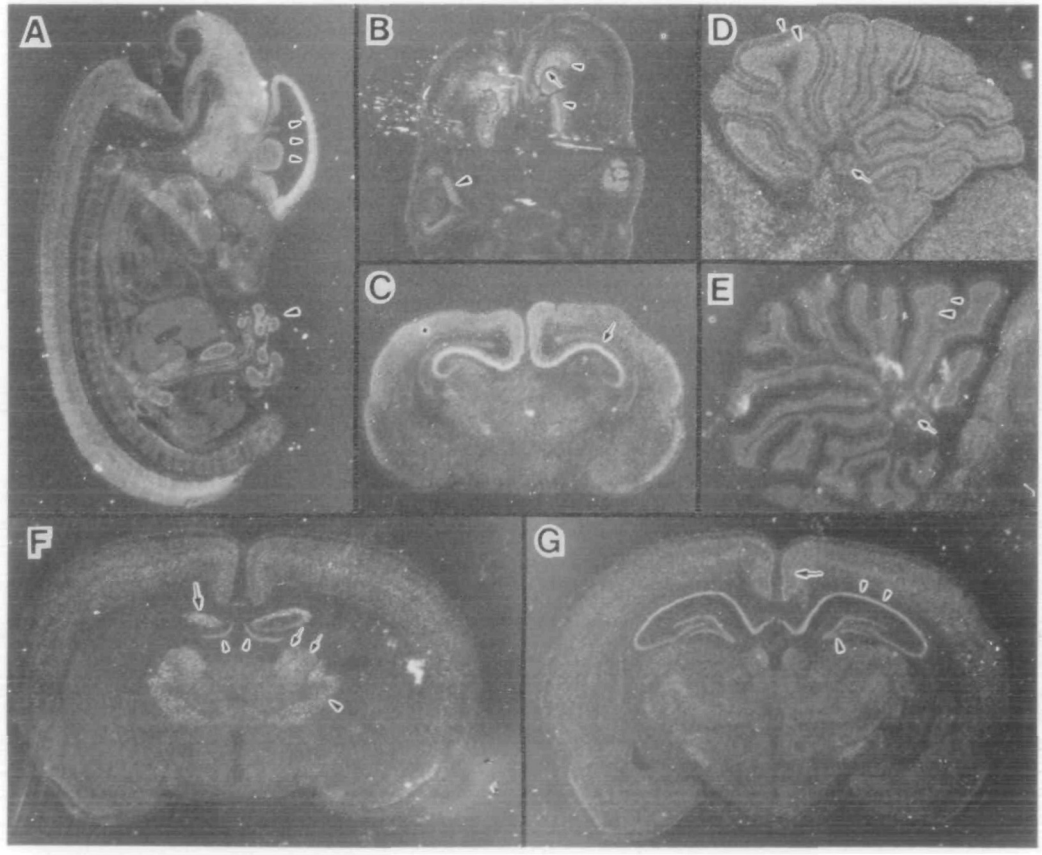

Fig. 5. **Expression of GD3 synthase mRNAs in the developing rat embryo and brain.** Dark field images of rat tissue sections hybridized with GD3 synthase antisense cRNA probe as described under "MATERIALS AND METHODS." (A) A sagittal section of an E15 rat embryo. The 2,8ST hybridization signal was localized in the central nervous system, and was strongest in the ventricular and subventricular zones (small arrowheads) and intestine (large arrowhead). (B) A coronal section of an E18 rat embryo brain. The 2,8ST expression was localized in the ventricular zone and subventricular zone (small arrowheads) and retina (large arrowhead). The ventricle is shown by an arrow. (C) A coronal section of a P4 rat brain. The 2,8ST transcripts were observed in the cerebral cortex and CA1-4 region of the hippocampus (arrow). (D) A sagittal section of a P7 rat cerebellum. The 2,8ST was localized in the internal granular layer (large arrowhead), external granular layer (small arrowhead), and dentate nucleus (arrow). (E) A sagittal section of an adult rat cerebellum. The 2,8ST was localized in granular neurons (large arrowhead), Purkinje cells (small arrowhead), and dentate nucleus (arrow). Adult cerebral cortex. (F) A coronal section of an adult rat brain. The 2,8ST was expressed in the cerebral cortex, hippocampus [CA1-4 region and dentate gyrus (small arrowheads)], anterior nuclei of the thalamus (arrows) and reticular nucleus of the thalamus (large arrowhead). (G) A coronal section of an adult rat brain. The 2,8ST was expressed in the cerebral cortex, and was strongest in the cingulate gyrus (arrow), hippocampus [CA1-4 region (small arrowheads)] and dentate gyrus (large arrowhead).

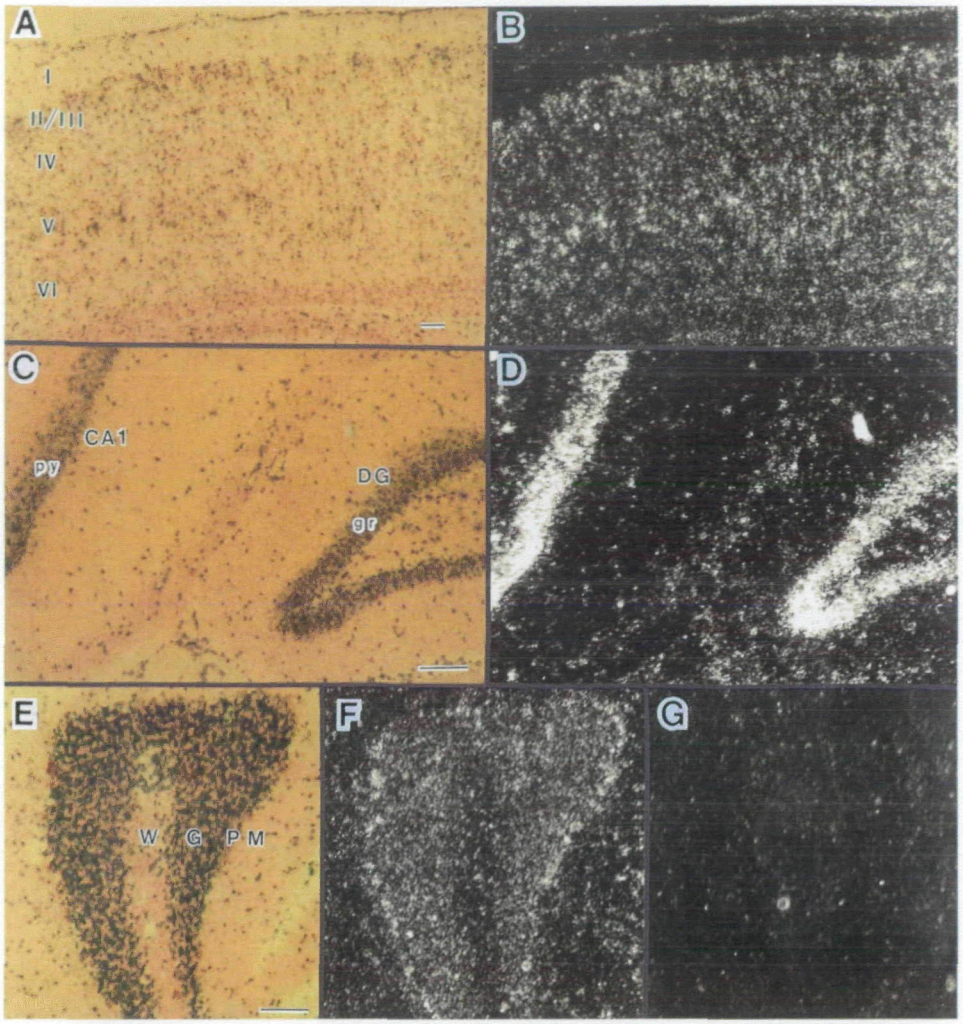

Fig. 6. **Expression of GD3 synthase mRNAs in the adult rat cerebral cortex, hippocampus, and cerebellum.** Bright field (A, C, E) and dark field (B, D, F, G) images of rat tissue sections hybridized with 2,8ST antisense (A, B, C, D, E, F) or sense (G) cRNA probe and counterstained with hematoxylin and eosin as described under "MATERIALS AND METHODS." (A), (B) A coronal section of the cerebral cortex. (C), (D) A coronal section of the hippocampal region. (E), (F) A sagittal section of cerebellum hybridized with 2,8ST antisense cRNA probe. (G) Adjacent section of (E) hybridized with 2,8ST sense cRNA probe. No signal was observed in (G). DG, dentate gyrus; CA1, CA1 region in the hippocampus; py, pyramidal cell layer of the CA1 region of the hippocampus; gr, granular cell layer of the dentate gyrus; W, white matter; G, granular layer; P, Purkinje cell layer; M, molecular layer. The bar represents 100 μm.

Northern Blot Analysis of 2,8ST mRNA Expression in Adult Rat Tissues—To analyze the tissue distribution of rat 2,8ST mRNA, Northern blots of poly(A)$^+$ RNA derived from various rat tissues were performed as described under "MATERIALS AND METHODS." As shown in Fig. 3, two kinds of transcripts (*ca.* 8.5 and 2.4 kb) were detected in the adult rat brain and spleen. A moderate level of 2,8ST expression was detected in the lung but only a low level in the liver and heart. In the testis, in addition to the 2.4-kb mRNA, shorter transcripts were detected.

Expression of 2,8ST mRNA in Developing and Adult Rat Brain—To determine whether 2,8ST participates in brain development, we compared the expression levels of 2,8ST at different embryonic stages. From E15 to E18, 2,8ST was exclusively expressed in the developing brain (Fig. 4A). The peak of the signal in the brain might be seen on E15. In the adult brain, the region-restricted expression of the 2,8ST was observed (Fig. 4B). The 2,8ST mRNA was expressed most strongly in the cerebellum and hippocampus. In the cerebral cortex, a relatively weak signal was detected (Fig. 4B).

In Situ Hybridization Analysis of 2,8ST mRNA in the Developing Rat Embryo and Brain—To study the cellular localization and pattern of 2,8ST mRNA expression in neural tissues, we carried out *in situ* hybridization analyses on embryonic and adult brain sections. In the sagittal section of E15, 2,8ST was expressed in the developing central nervous system and developing intestine (Fig. 5A). In the sagittal section of E15 (Fig. 5A) and coronal section of E18 (Fig. 5B), the expression level of 2,8ST was highest in the ventricular and subventricular zone of the rostral brain. In the coronal section of P4, strong signals were observed in the cerebral cortex and CA1-4 region of the hippocampus (Fig. 5C). In the adult, expression of 2,8ST declined and the 2,8ST transcript was detected at the highest levels in the cerebral cortex, hippocampus, thalamus and cerebellum (Fig. 5, E, F, and G). In the cerebral cortex, the laminar structure of cell distribution was observed (Fig. 5G). Although the 2,8ST mRNAs were present in cortical layers II-IV, strong signals were detected in the large pyramidal neurons in layers II/III and V (Fig. 6B). In the hippocampal formation, a high level of expression was detected in the pyramidal cell layers of the CA1-4 region and in the granule layer of the dentate gyrus (Fig. 5, G and D). In the thalamus, the expression level of 2,8ST was high in the anterior, reticular, dorsomedian, and dorsolateral nuclei (Fig. 5F). The 2,8ST expression pattern was examined during cerebellar development. From P7 to P20, expression of 2,8ST was seen in the cells of the external and internal granular layer (Fig. 5D). In the adult,

the signal was detected in the granular layer, Purkinje cell layer and neurons in the cerebellar nucleus (Figs. 5E and 6F).

DISCUSSION

Gangliosides are known to be synthesized by the action of specific glycosyltransferases in the Golgi apparatus through various biosynthetic pathways (a- or b-series) (*13*). 2,8ST acts at the first step of the b-series pathway and is thought to be important in the expression of the b-series gangliosides (*14*). In this study we have shown that the 2,8ST protein efficiently catalyzed the transfer of sialic acid to GM3 and to some extent to GD1a, GT1b, or GD3 (Table I). Although the role of this substrate specificity of the 2,8ST protein in ganglioside metabolism is unknown, it is possible that the single enzyme may catalyze both GD3 synthesis and GQ1b (or GT1a) biosynthesis *in vivo*. Recently, we were able to demonstrate that 2,8ST catalyzed the reaction GT1b→GQ1b, by establishing permanent cell lines transfected with human 2,8ST cDNA (*24*). Nakayama *et al.* also demonstrated that 2,8ST could catalyze the reaction GD3→GT3 (*25*). Because GQ1b possesses a potent neurite-promoting activity in neuroblastoma cells (26), and neural differentiation can be induced by the expression of 2,8ST cDNA (*27*), this enzyme may play a key role in neural differentiation.

The 2,8ST gene is highly expressed in rat brain, spleen, and testis, and exhibits low expression in liver and lung (Fig. 3). With regard to the tissue distribution of 2,8ST gene expression, however, remarkable contrasts are seen among mouse (*28*), human (*25*), and rat. In mouse, 2,8ST mRNA was abundantly expressed in the brain, thymus, and kidney, while only a low level of hybridization was detected in the spleen. In rat, however, strong signals were obtained in the brain and spleen as described in this study, while 2,8ST expression in the rat kidney was below the threshold of detectability by Northern blot hybridization analysis. Another major difference was found in rat testis: mRNA level in rat was quite high compared with those in human and mouse; and the mRNA in rat testis was smaller in size than those of other rat tissues. Differences in the tissue distribution of 2,8ST mRNA among mouse, rat, and human cannot be explained at present. Further work is required to analyze the genomic organization and the mechanism of the tissue-restricted expression of 2,8ST gene in addition to its enzymatic activity in each tissue.

These tissue-specific expressions of the 2,8ST gene may explain the difference in ganglioside profiles of each tissue (*14, 29*). Cell surface GD3 in T lymphocytes is involved in their activation (*30*). The preferential expression of the GD3 synthase mRNA in both spleen and brain suggests common functional mechanisms relevant to GD3 in the immune system and central nervous system.

In fetal rat brain (E14), GD3 constitutes ~50% of the total ganglioside content, whereas in adult brain, it accounts for only 0.6% (*4*). The decrease in GD3 content with development has been seen as a general phenomenon in the nervous system, occurring in fetal mouse and rat brain (*31*), neonatal peripheral nervous system in rat (*32*), embryonic chick optic lobe (*33*), and chick brain (*34-36*). Although the molecular mechanism of decreased expression of GD3 during development remains unclear, the level of 2,8ST activity should reflect these changes. In fact, 2,8ST activity is constant between E14 and E18 but decreases rapidly from E18 to birth (*4*). We demonstrated that 2,8ST, on the mRNA level, is expressed strongly in the early stage of brain development and its expression decreases in the course of brain development. In this context, our observations are consistent with earlier findings (*4*), suggesting that the transcriptional regulation of GD3 synthase gene expression occurs in part in this process. The similar developmental changes in 2,8ST mRNA expression were also reported in mouse (*28*).

Through the use of several monoclonal antibodies, GD3 has been shown to localize in the granular layer of the human cerebellum (*37*), the granular cells in the adult mouse brain (*6*), Purkinje cells in the developing rat brain (*38*), the granular layer and the white matter in the adult rat cerebellum (*8*), and the neuronal cells in the cerebral cortex, striatum, thalamus, hippocampus, and cerebellum in the adult rat brain (*9*). Goldman *et al.* (*5*) demonstrated that GD3-positive cells were present in areas known to contain immature neuroectodermal populations: the subventricular zone beneath the lateral ventricles, the external germinative layer of the cerebellar cortex and dentate gyrus of the hippocampus. The distributions of 2,8ST mRNA are very consistent with those of GD3, the reaction product of this enzyme.

In marked contrast to other CNS tissues, the retina contains an abundance of GD3. *In situ* hybridization studies indicate that the transcript of 2,8ST is clearly visualized in the E18 retina (Fig. 5B). This observation is consistent with high level expression of GD3 ganglioside and 2,8ST activity. Seyfried *et al.* (*39*) suggested that a large portion of the retinal GD3 may be localized in the giant Müller cells. On the other hand, GD3 was detected immunocytochemically in 60% of retinal interneurons (*40*). A possible explanation for this discrepancy is that an unknown mechanism of transfer of GD3 from the Müller cells to neuronal cells could operate in the retina. A recent study by Yamamoto *et al.* demonstrated that 2,8ST mRNA is expressed in the ganglion cell layer and neuroblastic cell layer in E18 retina and the inner segment of photoreceptor cells in adult retina (*41*). Further studies on the cellular localization of 2,8ST gene and GD3 expression in the retina will be needed to resolve this phenomenon.

REFERENCES

1. Ledeen, R.W. and Yu, R.K. (1982) Gangliosides: Structure, isolation, and analysis. *Methods Enzymol.* **83**, 139-191
2. Yu, R.K. (1994) Development regulation of ganglioside metabolism. *Prog. Brain Res.* **101**, 31-44
3. Rösner, H., Al-Aqtum, M., and Rahmann, H. (1992) Gangliosides and neuronal differentiation. *Neurochem. Int.* **20**, 339-351
4. Yu, R.K., Macala, L.J., Taki, T., Weinfield, H.M., and Yu, F.S. (1988) Developmental changes in ganglioside composition and synthesis in embryonic rat brain. *J. Neurochem.* **50**, 1825-1829
5. Goldman, J.E., Hirano, M., Yu, R.K., and Seyfried, T.N. (1984) GD3 ganglioside is a glycolipid characteristic of immature neuroectodermal cells. *J. Neuroimmunol.* **7**, 179-192
6. Schaal, H., Wille, C., and Wille, W. (1985) Changes of ganglioside pattern during cerebellar development of normal and staggerer mice. *J. Neurochem.* **45**, 544-551
7. Rösner, H. (1983) Developmental expression of gangliosides *in vivo* and *in vitro* in *Polysialic Acid* (Roth, J., Rutishauser, U., and Troy, F.A., eds.) pp. 279-297, Birkhäuser Verlag, Basel

8. Kotani, M., Kawashima, I., Ozawa, H., Ogura, K., Ishizuka, I., Terashima, T., and Tai, T. (1994) Immunohistochemical localization of minor gangliosides in the rat central nervous system. *Glycobiology* **4**, 855-865
9. Kawai, K., Mori, M., Watarai, S., and Yasuda, T. (1994) Immunohistochemical demonstration of ganglioside GD3 in the central nervous system. *Neurosci. Res.* **19**, 119-124
10. Kawai, K., Kuroda, S., Watarai, S., and Takahashi, H. (1994) Developmental profile of ganglioside GD3 in the central nervous system: An immunohistochemical study in the rat. *Neurosci. Res.* **19**, 77-82
11. Cheresh, D.A., Pierschbacher, M.D., Herzig, M.A., and Mujoo, K. (1986) Disialogangliosides GD2 and GD3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. *J. Cell Biol.* **102**, 688-696
12. Sariola, H., Aufderheide, E., Bernhard, H., Henke, F.S., Dippold, W., and Ekblom, P. (1988) Antibodies to cell surface ganglioside GD3 perturb inductive epithelial-mesenchymal interactions. *Cell* **54**, 235-245
13. van Echten, G. and Sandhoff, K. (1993) Ganglioside metabolism. Enzymology, topology, and regulation. *J. Biol. Chem.* **268**, 5341-5344
14. Schüz, H.R., Ullmer, E., Prinz, C., and Decker, K. (1989) The activity of GD3 synthase modulates the ganglioside pattern in rat liver. *Eur. J. Biochem.* **185**, 327-330
15. Nara, K., Watanabe, Y., Maruyama, K., Kasahara, K., Nagai, Y., and Sanai, Y. (1994) Expression cloning of a CMP-NeuAc: NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase (GD3 synthase) from human melanoma cell. *Proc. Natl. Acad. Sci. USA* **91**, 7952-7956
16. Sasaki, K., Kurata, K., Kojima, N., Kurosawa, N., Ohta, S., Hanai, N., Tsuji, S., and Nishi, T. (1994) Expression cloning of a GM3-specific α-2,8-sialyltransferase (GD3 synthase). *J. Biol. Chem.* **269**, 15950-15956
17. Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K.O., Shiku, H., and Furukawa, K. (1994) Isolation of GD3 synthase gene by expression cloning of GM3 α-2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **91**, 10455-10459
18. Matsuda, Y., Nara, K., Watanabe, Y., Saito, T., and Sanai, Y. (1996) Chromosome mapping of the GD3 synthase gene (SIAT8) in human and mouse. *Genomics* **32**, 137-139
19. Wen, D.X., Livingston, B.D., Medzihradszky, K.F., Kelm, S., Burlingame, A.L., and Paulson, J.C. (1992) Primary structure of Gal β1,3(4)GlcNAc α2,3-sialyltransferase determined by mass spectrometry sequence analysis and molecular cloning. Evidence for a protein motif in the sialyltransferase gene family. *J. Biol. Chem.* **267**, 21011-21019
20. Sanchez-Lopez, R., Nicholson, R., Gesnel, M.C., Matrisian, L.M., and Breathnach, R. (1988) Structure-function relationships in the collagenase family member transin. *J. Biol. Chem.* **263**, 11892-11899
21. Shirasawa, T., Sakamoto, K., Akashi, T., Takahashi, H., and Kawashima, A. (1992) Nucleotide sequence of rat elongation factor-1α cDNA. *Nucleic Acid Res.* **20**, 909
22. Kasahara, K., Guo, L., Nagai, Y., and Sanai, Y. (1994) Enzymatic assay of glycosphingolipid sialyltransferase using reverse-phase thin-layer chromatography. *Anal. Biochem.* **218**, 224-226
23. Simmons, D.M., Arriza, J.L., and Swanson, L.W. (1989) A complete protocol for *in situ* hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *J. Histotechnol.* **12**, 169-181
24. Nara, K., Watanabe, Y., Kawashima, I., Tai, T., Nagai, Y., and Sanai, Y. (1996) Acceptor substrate specificity of a cloned GD3 synthase that catalyzes the biosynthesis of both GD3 and GD1c/GT1a/GQ1b. *Eur. J. Biochem.* **238**, 647-652
25. Nakayama, J., Fukuda, M.N., Hirabayashi, Y., Kanamori, A., Sasaki, K., Nishi, T., and Fukuda, M. (1996) Expression cloning of a human GT3 synthase, GD3 and GT3 are synthesized by a single enzyme. *J. Biol. Chem.* **271**, 3684-3691
26. Tsuji, S., Arita, M., and Nagai, Y. (1983) GQ1b, a bioactive ganglioside that exhibits novel nerve growth factor (NGF)-like activities in the two neuroblastoma cell lines. *J. Biochem.* **94**, 303-306
27. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) Induction of cholinergic differentiation with neurite sprouting by *de novo* biosynthesis and expression of GD3 and b-series gangliosides in Neuro2a cells. *J. Biol. Chem.* **269**, 30451-30456
28. Yamamoto, A., Haraguchi, M., Yamashiro, S., Fukumoto, S., Furukawa, K., Takamiya, K., Atsuta, M., Shiku, H., and Furukawa, K. (1996) Heterogeneity in the expression pattern of two ganglioside synthase genes during mouse brain development. *J. Neurochem.* **66**, 26-34
29. Nakamura, K., Hashimoto, Y., Yamakawa, T., and Suzuki, A. (1988) Genetic polymorphism of ganglioside expression in mouse organs. *J. Biochem.* **103**, 201-208
30. Welte, K., Miller, G., Chapman, P.B., Yuasa, H., Natoli, E., Kunicka, J.E., Cordon, C.C., Buhrer, C., Old, L.J., and Houghton, A.N. (1987) Stimulation of T lymphocyte proliferation by monoclonal antibodies against GD3 ganglioside. *J. Immunol.* **139**, 1763-1771
31. Irwin, L.N., Michael, D.B., and Irwin, C.C. (1980) Ganglioside patterns of fetal rat and mouse brain. *J. Neurochem.* **34**, 1527-1530
32. Chou, K.H., Nolan, C.E., and Jungalwala, F.B. (1982) Composition and metabolism of gangliosides in rat peripheral nervous system during development. *J. Neurochem.* **39**, 1547-1558
33. Rösner, H. (1982) Ganglioside changes in the chicken optic lobes as biochemical indicators of brain development and maturation. *Brain Res.* **236**, 49-61
34. Dreyfus, H., Urban, P.F., Edel, H.S., and Mandel, P. (1975) Developmental patterns of gangliosides and of phospholipids in chick retina and brain. *J. Neurochem.* **25**, 245-250
35. Irwin, L.N. and Irwin, C.C. (1979) Phylogenetic and regional variations in brain gangliosides of tetrapods. *Comp. Biochem. Physiol. B: Comp. Biochem.* **64**, 121-123
36. Rösner, H. (1980) A new thin-layer chromatographic approach for separation of multisialogangliosides. Novel gangliosides fractions in the embryonic chicken brain. *Anal. Biochem.* **109**, 437-442
37. Graus, F., Cordon, C.C., Houghton, A.N., Melamed, M.R., and Old, L.J. (1984) Distribution of the ganglioside GD3 in the human nervous system detected by R24 mouse monoclonal antibody. *Brain Res.* **324**, 190-194
38. Reynolds, R. and Wilkin, G.P. (1988) Expression of GD3 ganglioside by developing rat cerebellar Purkinje cells in situ. *J. Neurosci. Res.* **20**, 311-319
39. Seyfried, T.M., Yu, R.K., Miyazawa, N., and Lai, Y.L. (1982) Retinal gangliosides in RCS mutant rats. *J. Neurochem.* **39**, 277-279
40. Daniotti, J.L., Landa, C.A., Rösner, H., and Maccioni, H.J.F. (1992) Adult rat retina interneurons synthesize GD3: GD3 expression by these cells is regulated by cell-cell interactions. *J. Neurochem.* **59**, 107-117
41. Yamamoto, A., Yamashiro, S., Fukumoto, S., Haraguchi, M., Atsuta, M., Shiku, H., and Furukawa, K. (1996) Site restricted and neuron dominant expression of α2,8sialyltransferase gene in the adult mouse brain and retina. *Glycoconjugate J.* **13**, 471-480
42. Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613-623