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Developmental Patterns of mST3GalV mRNA Expression in the Mouse: *In Situ* Hybridization using DIG-labeled RNA Probes

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mST3GalV synthesizes ganglioside GM3, the precursor for simple and complex a- and b- series gangliosides, and the expression and regulation of mST3GalV (CMP-NeuAc: lactosylceramide α2,3-sialyltransferase) activity is central to the production of almost all gangliosides, a class of glycosphingolipids implicated in variety of cellular processes such as transmembrane signaling, synaptic transmission, specialized membrane domain formation and cell-cell interactions. To understand the developmental expression of mST3GalV in mice, we investigated the spatial and temporal expression of mST3GalV mRNA during the mouse embryogenesis [embryonic (E) days; E9, E11, E13, E15] by *in situ* hybridization with digoxigenin-labeled RNA probes. All tissues from E9 and E11 were positive for mST3GalV mRNA. On E13, mST3GalV mRNA was expressed in various neural and non-neural tissues. In contrast to these, on E15, the telencephalon and liver produced a strong expression of mST3Gal V which was a quite similar to that of E13. In this stage, mST3GalV mRNA was also expressed in some non-neural tissues. These data indicate that mST3GalV is differently expressed at developmental stages of embryo, and this may be importantly related with regulation of organogenesis in mice.

Key words: mST3GalV, Embryogenesis

INTRODUCTION

Gangliosides are sialic acid (NeuAc)-containing glycosphingolipids found widely in the plasma membranes of all vertebrate tissues and are particularly abundant in the central nervous system (CNS) (Svennerholm, 1980; Ji *et al.*, 1999). They play important roles in a large variety of biological processes, such as cell-cell interaction, adhesion, cell differentiation, growth control and receptor function (Varki, 1993; Choo *et al.*, 1995). The gangliosides are synthesized *via* four primary biosynthetic pathways by a family of glycosyltransferases in the Golgi apparatus (Iber *et al.*, 1992). The diversity of ganglioside composition among vertebrates reflects the differential regulation of ganglioside biosynthesis through different pathways.

Among the gangliosides, GM3 has the simplest carbohydrate structure and is known to be involved in induction of HL-60 differentiation (Nojiri *et al.*, 1986), modulation of cell proliferation (Hakomori, 1990; Choo *et al.*, 1995, 1999), signal transduction (Hakomori *et al.*, 1998), maintenance of fibroblast morphology (Meivar-Levy *et al.*, 1997) and integrin-mediated cell adhesion (Kojima and Hakomori, 1996). GM3 synthesis is the first step for ganglioside biosynthesis and all ganglio-series gangliosides are synthesized from GM3 by linkage-specific glycosyltransferases (Iber *et al.*, 1992). GM3 is synthesized by CMP-NeuAc:lactosylceramide α2,3-sialyltransferase (GM3 synthase, EC 2.4.99.9) which catalyzes the transfer of NeuAc from CMP-NeuAc to the nonreducing terminal sugar of lactosylceramide.

mST3GalV is a key regulatory enzyme for ganglioside biosynthesis (Fishman and Brady, 1976; Iber *et al.*, 1992), because it catalyzes the first committed step in the

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synthesis of nearly all gangliosides. To clarify the molecular mechanism for ganglioside biosynthesis as well as to understand the biological roles of GM3 and other gangliosides, it is critical to isolate a cDNA clone of mST3GalV. Very recently, mST3Gal V cDNAs were cloned from mouse and human cDNA libraries and characterized (Ishii *et al.*, 1998; Kono *et al.*, 1998; Fukumoto *et al.*, 1999). The mST3GalV gene exhibited predominant expression in mouse brain and liver, which contain a large amount of ganglioside (Kono *et al.*, 1998; Stern *et al.*, 2000).

The expression of sialyltransferases is tissue-specific, cell-specific and highly regulated during differentiation and proliferation, like in the case of other glycosyltransferases (Paulson *et al.*, 1989; Paulson and Colley, 1989; Choo *et al.*, 1995; Ji *et al.*, 1999). Since the main regulation of sialylation of glycoconjugates occurs at the level of sialyltransferase expression, the comparison of enzyme expression in tissue at different stage of development is interest. In order to understand the developmental expression of mST3GalV, we have investigated the spatial and temporal expression of mST3GalV mRNA during the mouse embryogenesis by *in situ* hybridization. The present study describes *in situ* hybridization analysis of spatio-temporal expression of mST3GalV mRNA during mouse embryogenesis after implantation.

MATERIALS AND METHODS

Preparation of tissues

For histological observation, four prenatal [embryonic (E) days; 9, 11, 13, 15] B6C3F1 mice (Samyuk Laboratory Animals Inc., Korea) were used. To determine the age of embryos, the mean value of crown-rump (c-r) lengths of normal unfixed embryos in the same litter was calculated. Theiler's embryonic age scale and the corresponding mean c-r length is generally accepted method for determination of mouse embryonic age (Theiler, 1989; Kaufman, 1992). At least three embryos from different litters were studied at each embryonic age. Pregnant mice were kept in a constant dark-light cycle and allowed foods pellets and water ad libitum. Embryos were dissected and immersed immediately into freshly prepared phosphate-buffered saline (PBS) containing 4% paraformaldehyde (pH 7.0). After fixation for 24 h at 4°C, the embryos were washed in PBS, dehydrated through a graded series of ethanol washes, and then cleared with xylenes and embedded in paraffin. Sections were cut at a thickness of 7 and mounted onto slides pretreated with aminopropyltriethoxysilane for adherence of sections.

Preparation of probes

To obtain a probe for *in situ* hybridization assays, first strand cDNAs were synthesized from the poly $(A)^+$RNA of adult mouse brains using random primers and Superscript II RNase H^- reverse transcriptase (Gibco BRL, USA), and applied as templates for the PCR. The cDNA fragment of mST3GalV was amplified under the conditions described previously (Lee *et al.*, 1993) with a sense primer (5'-CGAATTCGCAAG-CGCTGTGTGGTTGTTGG-3', containing an *Eco*RI site) and an antisense primer (5'-CAAGCTTT GCCAGGCTGACTTCATCACAC-3', containing a *Hind*III site), for a region corresponding to amino acids 161-331 of the previously cloned mST3GalV sequence (Kono *et al.*, 1998). The PCR product (517 bp) was subcloned into the *Eco*RV site on pT7Blue cloning vector (Novagen, USA) and subsequently sequenced. The resulting cDNA sequences for mST3GalV were coding regions including sialyl-motifs L and S of mST3GalV. An *Eco*RI-*Hind*III fragment of the cDNA for mST3Gal V was then subcloned into the same sites on pGEM-3Z (Promega, USA) and was linearized with *Bam*HI or *Xba*I. Digoxigenin-labeled antisense and sense RNA probes were synthesized by using T7 and SP6 RNA polymerase in the presence of digoxigenin-labeled UTP, respectively (Boehringer Mannheim, Germany).

In situ hybridization

DIG-labeled RNA *in situ* hybridization was done according to standard protocols with slight modifications (Kurosawa *et al.*, 1997). The sections were deparaffinized by placing slides in three changes of xylene for 10 min each and hydrated through a graded series of ethanol. All sections were treated in 0.2 M HCl at room temperature for 10 min and washed in PBS (pH 7.4).

The hydrated slides were treated with predigested proteinase K (10 μg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), fixed with 4% paraformaldehyde and acetylated with acetic anhydride. After ethanol dehydration, the sections were air dried, and the tissue on the slides encircled with PAP-PEN (Daido Sangyo Co., LTD, Japan). 30 μl of hybridization solution (50% deionized formamide; 10 mM Tris-HCl, pH 7.6; 200 μl/ml yeast tRNA; 1 × Denhardt's solution; 10% dextransulfate; 600 mM NaCl; 0.25% SDS; 1 mM EDTA, pH 8.0) containing 0.5 ng/μl DIG-labeled RNA probe were placed on each section and the sections were placed without coverslipping in an air-tight, humidified chamber containing 50% formamide and 2 × standard saline citrate (SSC); the tissue sections were covered completely with the solution. The hybridization was carried out at 50°C overnight. After hybridization, all washes were performed at 50 consisting of two washes in 2 × SSC, two in 0.2 × SSC (each for 20 min).

Finally, immunohistochemical detection of DIG-labeled RNA probes was carried out in accordance with the instruction manual supplied by Boehringer Mannheim with some modifications: After washing the slides briefly (5 min) in buffer I (100 mM Tric-HCl, 150 mM NaCl, pH 7.5), the slides were incubated in buffer I with 10% normal bovine serum (Sigma), 0.2% Tween 20 for 1 h at room tempe-

rature. After 1:700 dilution of the anti-digoxigenin conjugate in buffer I containing 10% normal bovine serum, 50-100 µl of the antibody solution was applied to each tissue and incubated for 1 h at room temperature in a humidified chamber using buffer I. Thereafter, the sections were washed for 15 min in buffer I and for additional 3 min in buffer II (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). Approximately 50-100 µl of color developing solution [per ml: 4.2 µl 5-bromo-4-chloro-3-indolyl phos-phate (BCIP), 4.5 µl nitro blue tetrazolium (NBT)] was applied to each section, and slides were incubated at room temperature in the dark in a humidified chamber using buffer II. After 18 h, the reaction was stopped by washing the slides for 3 min in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, at room temperature. Finally, sections were mounted in mounting medium (10 mM Tris-HCl, 1 mM EDTA, 50% glycerine, pH 7.0).

RESULTS

By Northern blot analysis, in all the adult mouse tissues examined, brain and liver expressed mST3GalV gene most highly (Kono *et al.*, 1998). Regarding in this, to elucidate development-related expression of mST3GalV, we investigated the expression pattern of mST3GalV mRNA during mouse embryogenesis [embryonic (E) days: E9, E11, E13, E15] by *in situ* hybridization using DIG-labeled antisense RNA probe. In each embryonic day examined, the intensity of the hybridization signal, as evidenced by alkaline phosphatase-induced purple color, was much stronger in the section hybridized with the antisense probe (Fig. 1A-D) than in the control sections hybridized with sense probe (Fig. 2). The sense RNA probe gave back ground levels of labeling.

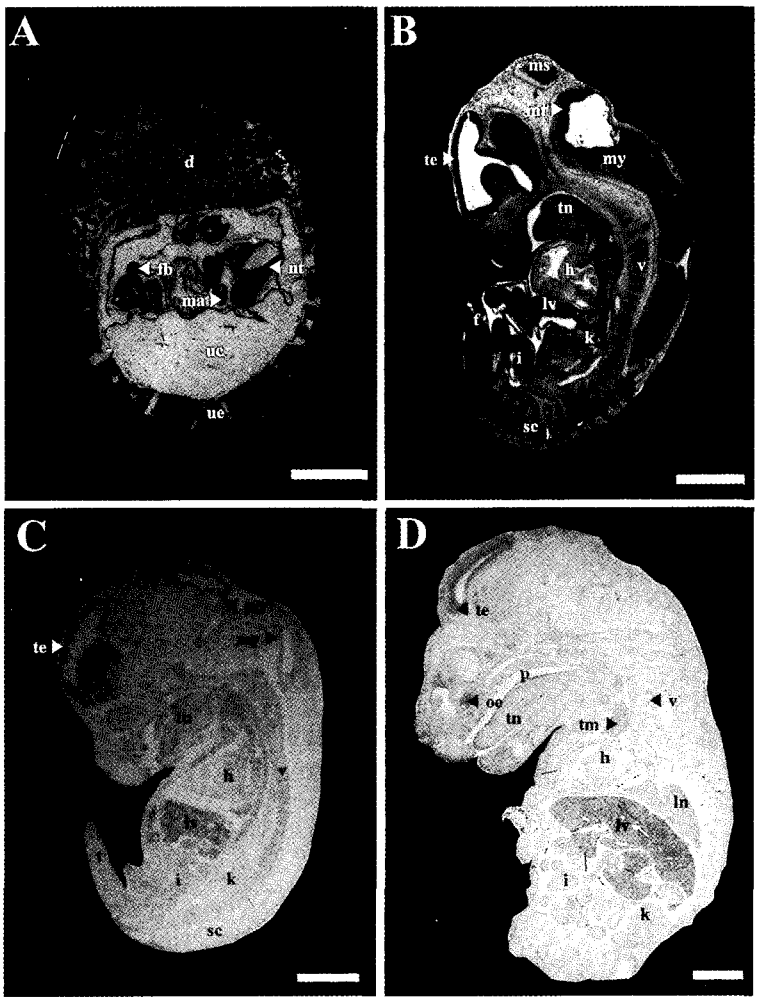

Fig. 1. Expression pattern of mST3GalV mRNA in mouse embryo. mST3GalV transcripts were detected by *in situ* hybridization with digoxigenin to sagittal sections of E9 (A), E11 (B), E13 (C) and E15 (D) mouse embryo. d, decidua; fb, fore limb; h, heart; i, intestine; k, kidney; ln, lung; lv, liver; ma, mandibular arch; ms, mesencephalon; mt, metencephalon; my, myelencephalon; nt, neural tube; oe, olfactory epithelium; p, pharynx; sc, spinal cord; t, tail; te, telencephalon; tm, thymus; tn, tongue; uc, uterine cavity; ue, uterine epithelium; v, vertebrate; ys, yolk sac. The size of the scale bar is 1 mm.

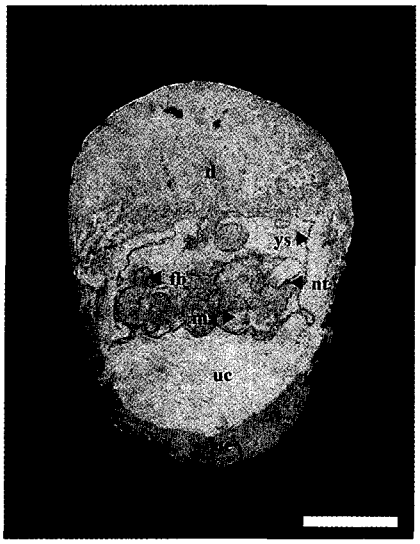

Fig. 2. Analysis of mST3GalV sense probe on E9. The specificity of the reaction was shown by the negative staining observed when sense probe was used. d, decidua; fb, fore limb; ma, mandibular arch; nt, neural tube; uc, uterine cavity; ue, uterine epithelium; ys, yolk sac. The size of the scale bar is 1 mm.

Table I. Summary of mST3GalV mRNA expression during mouse embryogenesis

Tissues	E11	E13	E15
Intestine	++	–	+
kideny	++	–	+
liver	++	+++	+++
lung	++	+	+
mesencephalon	++	++	–
metencephalon	++	+	–
mylenecephalon	++	+	–
olfactory epithelium	++	+	+
spinal cord	++	–	–
telencephalon	++	+++	+++
thymus	++	+	+
tongue	++	+	+
vertebrae	++	+	–

+++, very strong; ++, strong ; +, weak; –, negative.

During embryogenesis, on embryonic day 9 and 11, specific signal to mST3GalV mRNA was detected throughout tissues of all organs (Fig. 1A and B). On E13, specific signal was detected in neural tissues of CNS including telencephalon, mesencephalon, metencephalon, mylencephalon, vertebrae and non-neural tissues such as liver and heart (Fig. 1C). Interestingly, On E15, the strong signal for mST3GalV mRNA was detected only in the telencephalon and liver, although mRNA was also detected in most non-neural tissues, olfactory epithelium, thymus, tongue, lung, kidney and intestine (Fig. 1D). Table I summarizes the results of the temporal and spatial expression pattern of mST3GalV mRNA during embryogenesis.

DISCUSSION

Ganglioside GM3 is a major glycosphingolipid in the plasma membrane and is widely distributed in vertebrates. GM3 is known to play important roles in the modulation of cell growth through the modified signal transduction and cell differentiation. GM3 inhibits tyrosine phosphorylation of the epidermal growth factor (EGF) receptor and EGF-dependent cell growth independent of receptor-receptor interaction, whereas De-*N*-acetyl-GM3 enhances serine phosphorylation of EGF receptor and stimulates cell proliferation (Hakomori, 1990). GM3 induces monocytic differentiation of human myeloid and monocytoid leukemic cell lines such as HL-60 and U937 during macrophage-like cell differentiation (Nojiri *et al.*, 1986). The mST3Gal V synthesizes ganglioside GM3, the precursor for simple and complex gangliosides (Stern *et al.*, 2000).

Recently, mST3GalV has been expressed in various tissues of mice by Northern blot analysis (Kono *et al.*, 1998). The present study is, to our knowledge, the first *in situ* hybridization report showing the expression pattern of mST3GalV mRNA in various tissues during mouse embryogenesis. On embryonic days E9 and E11, mST3Gal V mRNA was highly expressed in tissues of all organs from embryos (Fig. 1A), suggesting that the dramatic increase of these mST3GalV mRNA consisted of differentiation and proliferation of various tissues during early organogenesis. On E13, the specific signal was expressed in various organs such as telencephalon, mesencephalon, metencephalon, myelencephalon, vertebrae, lung, thymus, liver, heart, olfactory epithelium and tongue, but not in intestine, kidney and spinal cord (Fig. 1C). These results led us to consider that mST3GalV might have an important role in the regulation of differentiation of CNS and liver, heart, lung, thymus, olfactory epithelium and tongue during embryogenesis. Ganglioside synthases have been found frequently in Golgi vesicles derived from rat liver and mouse primary cultured cerebellar neurons (Iber *et al.*, 1991; Iber *et al.*, 1992; Iber *et al.*, 1992; Pohlentz *et al.*, 1988; Pohlentz *et al.*, 1994). Indeed, on E15 mST3Gal V mRNA also was strongly expressed in the telencephalon of CNS and liver, suggesting that mST3GalV may be involved in the whole processes of development and differention of these tissues.

Taken together, the results obtained in this study may be a meaningful evidence, suggesting that tissue specificity of mST3GalV mRNA expression conserved among the different developmental stages. The results described here to mST3GalV mRNA are consistent with the possibility that GM3 itself might play important roles at diverse situ-

ations for the regulation of cell proliferation and differentiation, and will provide further information for understanding the cellular control of recognition events mediated by carbohydrate groups during embryogenesis. Especially, to further confirm the intracellular location of mST3GalV during neuronal differentiation, *in situ* hybridization analysis is required on the sagittal, coronal and horizontal section of the developing brain.

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ABBREVIATIONS

PCR, polymerase chain reaction,; PB, phosphate buffer; EDTA, ethylene diaminetetraaceticacid; DEPC, diethylpyrocarbonate; TNE, Tris-HCl, NaCl, EDTA; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 20 × SSC, standard saline citrate.

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