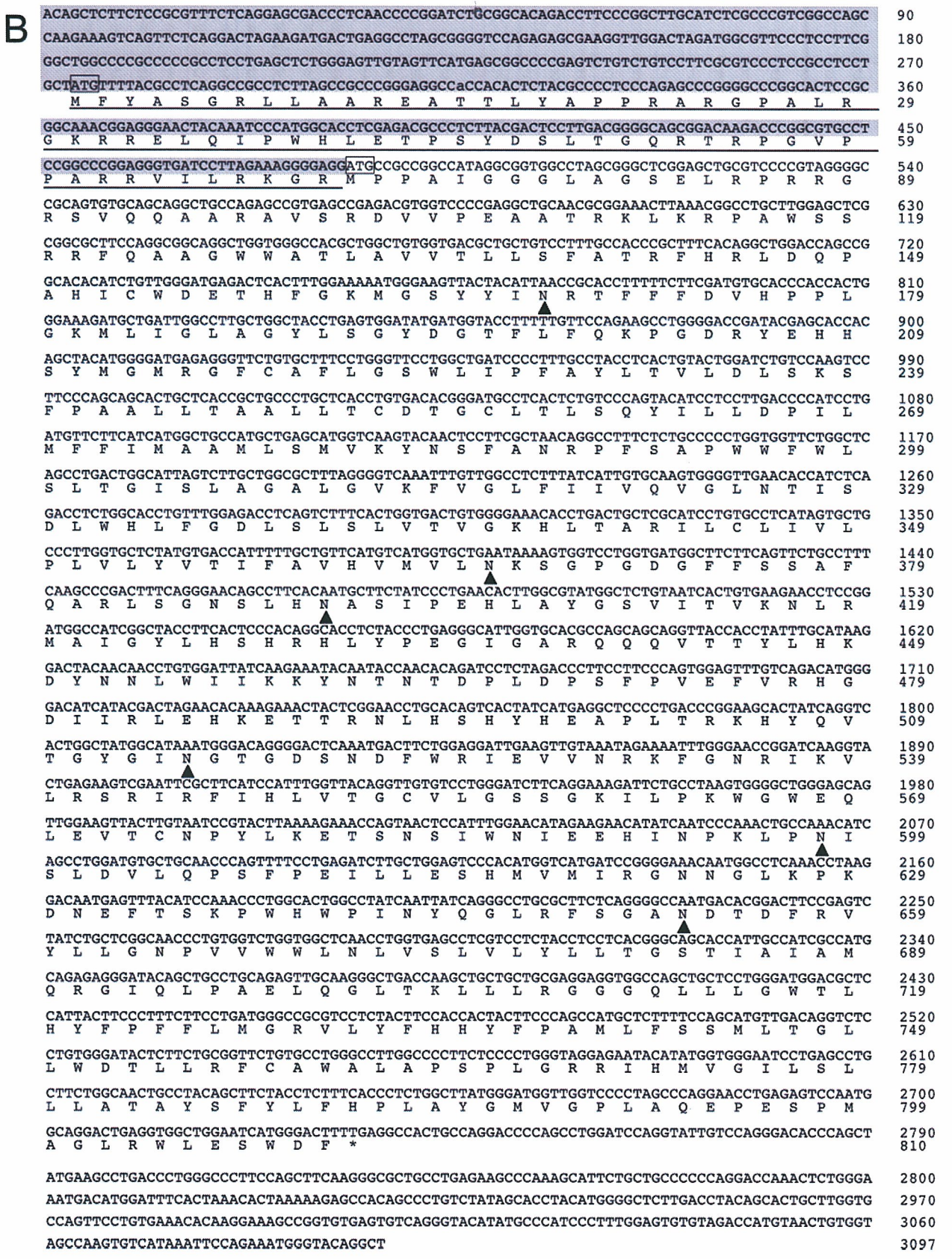

Fig. 1. continued

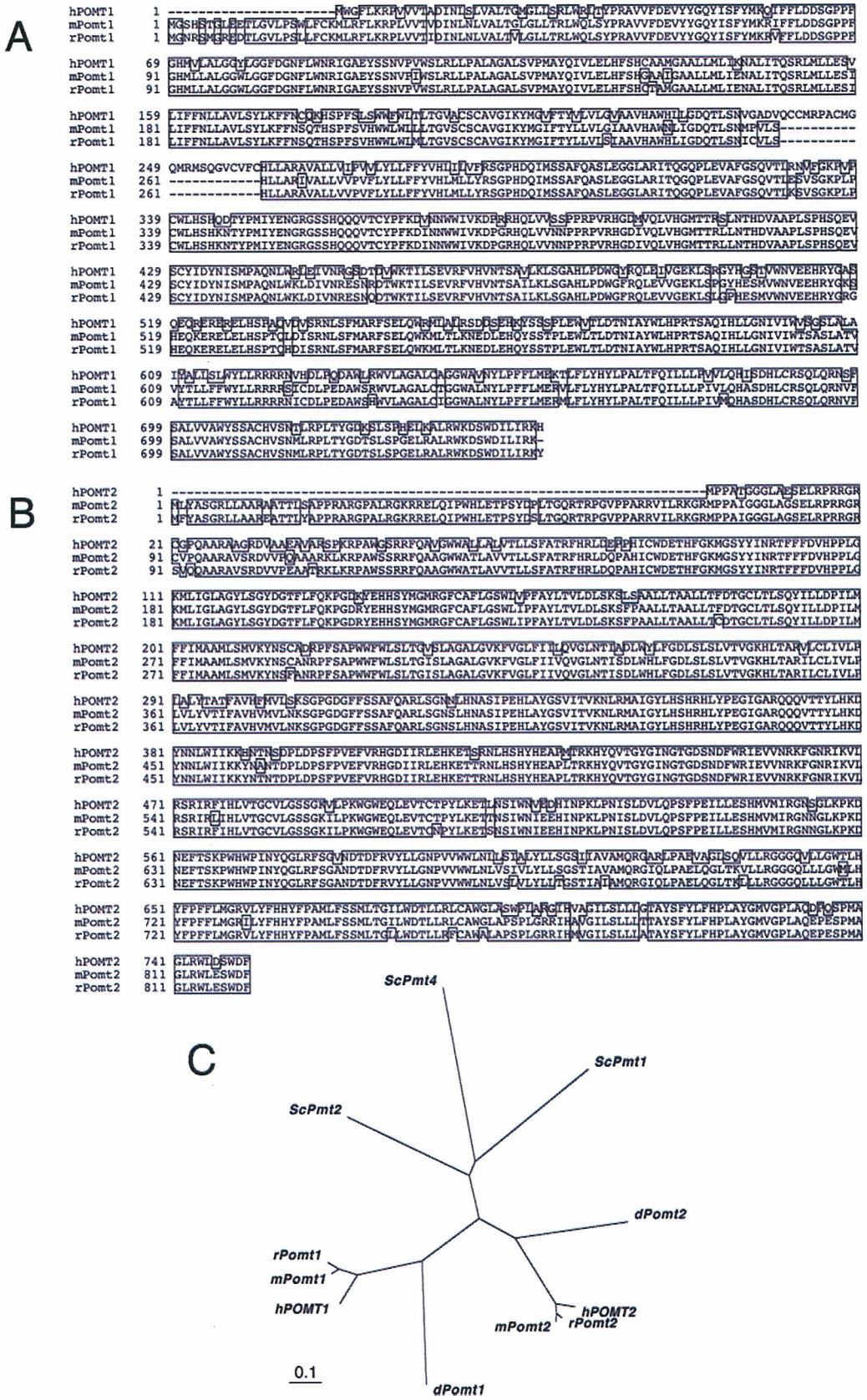

Fig. 2. Comparison of human, mouse, and rat POMTs. (**A** and **B**) ClustalW alignments of human, mouse and rat POMT1 and POMT2 amino acid sequences, respectively. Conserved amino acids are boxed. (**C**) ClustalW phylogenic tree of human, mouse, rat, and *Drosophila* POMTs and *Saccharomyces cerevisiae* Pmt1, Pmt2, and Pmt4. The branch lengths indicate amino acid substitutions per site. h, human; m, mouse; r, rat.

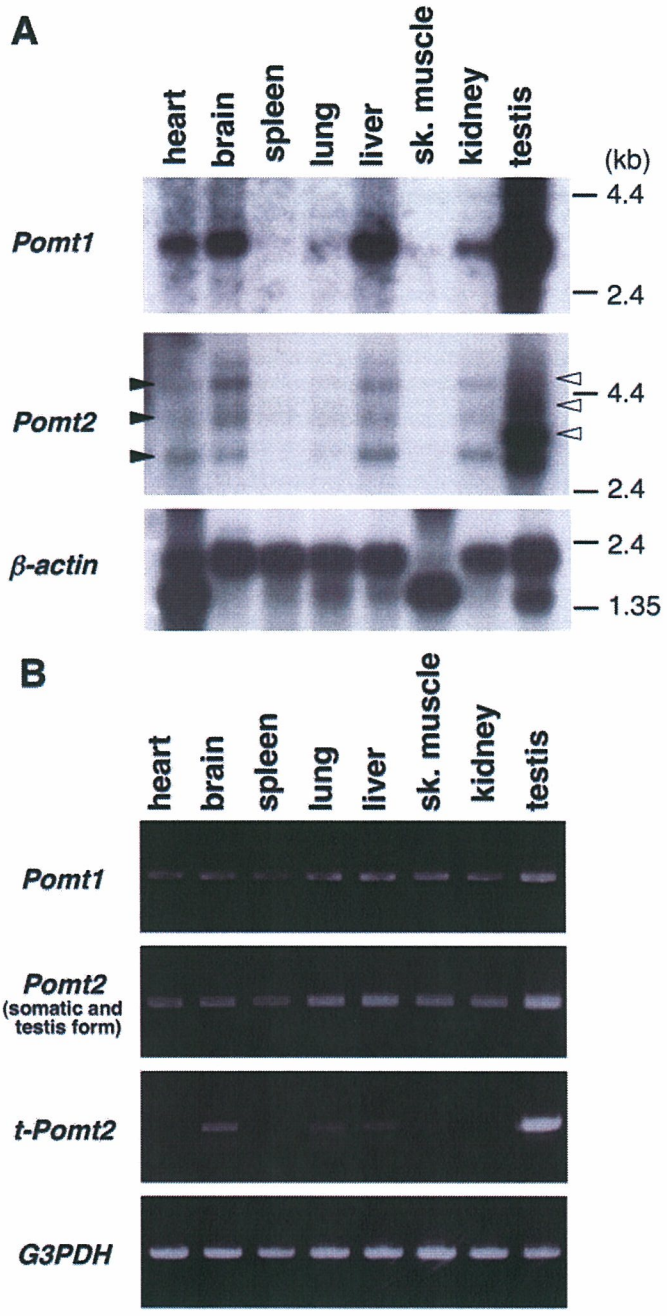

Fig. 3. Expression of rat *Pomt1* and *Pomt2* genes. (**A**) Northern blot analysis probed with rat *Pomt1* (upper panel), rat *Pomt2* (middle panel) and β-actin (lower panel). Each lane contains 2 μg poly(A)$^+$ RNA (Rat MTN blot). The sizes of RNA marker bands are indicated on the right. The basic transcript of *Pomt2* was around 2.7 kb, but because of alternative polyadenylation, 3.7 kb and 4.7 kb mRNAs of *Pomt2* were also detectable (closed triangles). In testis, the transcript sizes were slightly larger due to differential transcription initiation (open triangles). (**B**) RT–PCR analysis of rat *Pomt1* (top panel), rat *Pomt2* (second panel), testis form of rat *Pomt2* (third panel), and rat *G3PDH* (bottom panel) mRNAs. PCR products of *Pomt1* and *Pomt2* were detected in all tissues. Testis form of *Pomt2* mRNA was predominantly expressed in testis and slightly detected in brain, lung, and liver. sk. muscle, skeletal muscle.

therefore possible that mutations of other factors that regulate POMT activity may cause unidentified WWS, although further studies are needed to test this hypothesis.

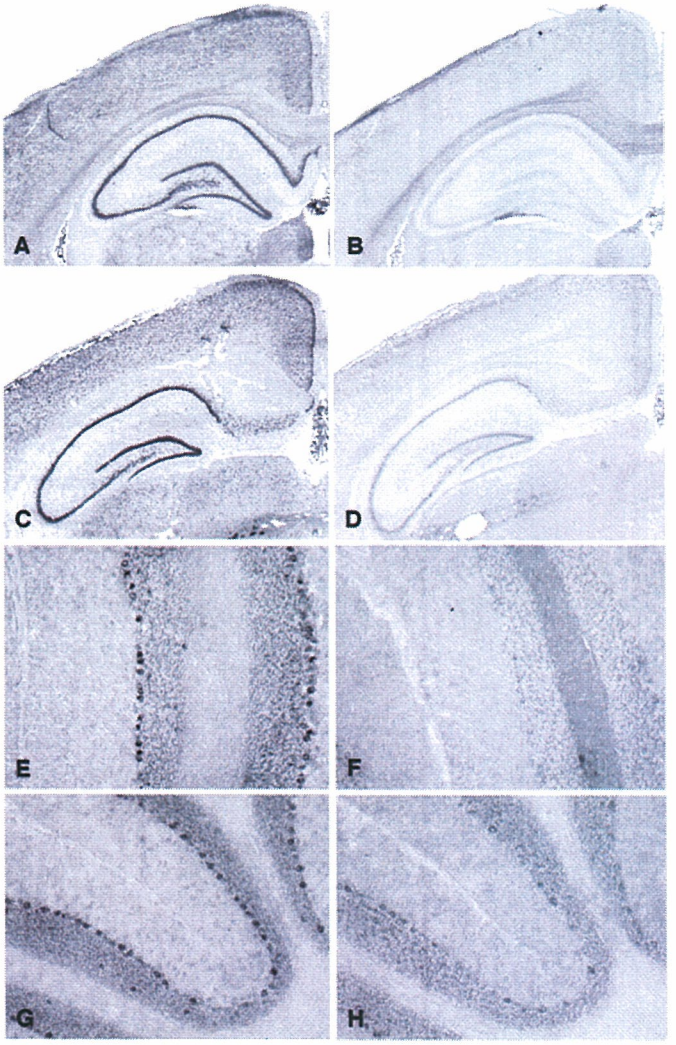

Fig. 4. Colocalization of rat *Pomt1* and *Pomt2* mRNA in adult rat brain. *In situ* hybridization histochemistry of adult rat cerebrum (**A–D**), and cerebellar cortex (**E–H**) shows the same mRNA expression pattern of rat *Pomt1* (**A** and **E**, antisense probe; **B** and **F**, sense probe) and *Pomt2* (**C** and **G**, antisense probe; **D** and **H**, sense probe). Both of the mRNAs are mainly expressed in cells in gray matter and strongly expressed in neurons of the dentate gyrus and CA1-CA3 region in hippocampus formation and Purkinje cells in cerebellar cortex.

Pomt2 was found to have two transcription-initiation sites, giving rise to the testis form (*t-Pomt2*) and the somatic form (*s-Pomt2*). Both t-Pomt2 and s-Pomt2 showed protein *O*-mannosyltransferase activity equally when coexpressed with rat Pomt1. In addition, human POMT1 and POMT2 could be exchanged for rat Pomt1 and Pomt2, without loss of activity. Human POMT1 and rat Pomt1 proteins share 96% similarity and 86% identity; human POMT2 and rat Pomt2 share 99% similarity and 90% identity. Such high similarity may explain the exchangeability of each component for enzymatic activity. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weights of t-Pomt2 and s-Pomt2 expressed in HEK293T cells were 85 and 75 kDa, respectively. The difference was due to the presence of an additional 70 amino acids in the *N*-terminal region of t-Pomt2. However, this difference did

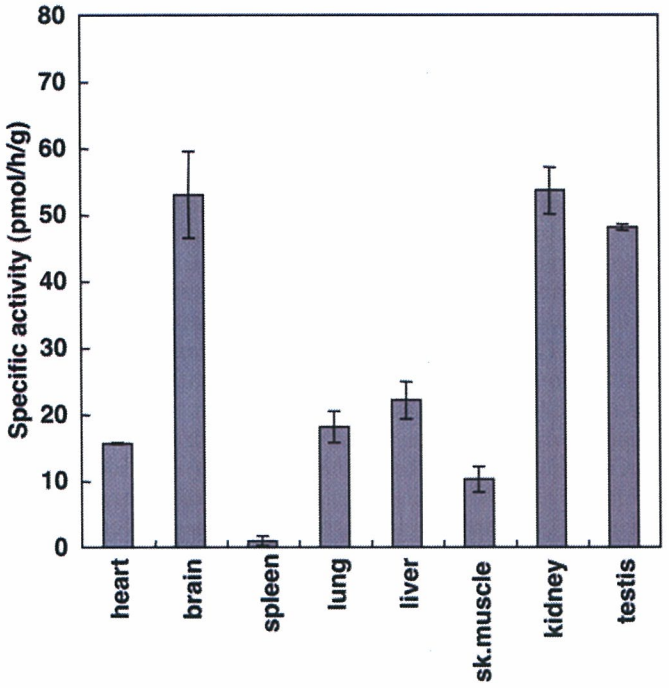

Fig. 5. Protein *O*-mannosyltransferase activity in rat tissues. Protein *O*-mannosyltransferase activity was measured in 0.5 mL Eppendorf tubes in a 20 μL reaction volume containing 20 mM Tris–HCl (pH 8.0), 100 nM of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol), 0.5% *n*-octyl-β-D-thioglucoside, 2 mM 2-mercaptethanol, 10 mM EDTA, 10 μg GST-αDG, and 80 μg of microsomal membrane fraction. The reaction was initiated by adding the protein extract and continued at 25°C for 1 h. After incubation, GST-αDG was separated by Glutathione-Sepharose 4B beads and then the incorporated [^{3}H]-mannose to GST-αDG was measured with a liquid scintillation counter. Average values of three independent experiments are shown. sk. muscle, skeletal muscle.

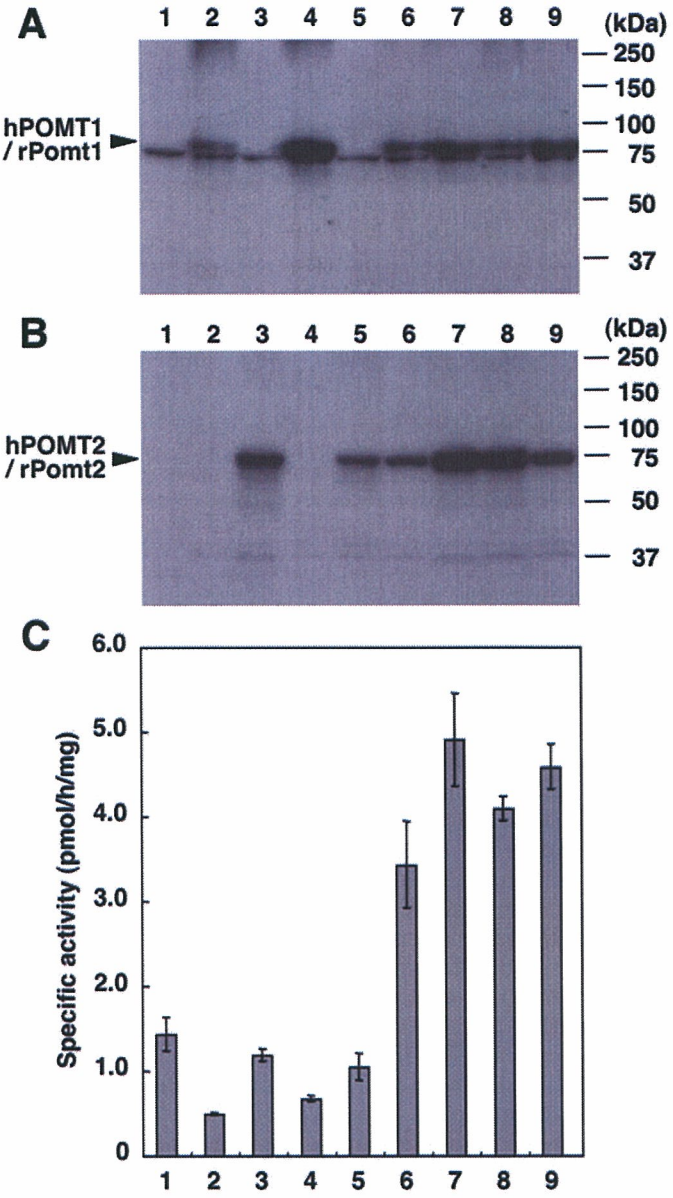

Fig. 6. Expression and activity of rat Pomt1 and s-Pomt2. (**A**, **B**) Western blot analyses of rat Pomt1, rat Pomt2, human POMT1, and human POMT2 expressed in HEK293T cells. The proteins (20 μg of microsomal membrane fraction) were subjected to SDS–PAGE (10% gel), and the separated proteins were transferred to a PVDF membrane. The PVDF membrane was stained with anti-POMT1 (**A**) or anti-POMT2 antibody (**B**). Molecular weight standards are shown on the right. (**C**) Protein *O*-mannosyltransferase activities in several combination conditions of rat and human POMTs. Protein *O*-mannosyltransferase activity was based on the rate of mannose transfer from Dol-P-Man to a GST-αDG. Lanes 1, cells transfected with vector alone; lanes 2, cells transfected with rat *Pomt1*; lanes 3, cells transfected with rat *s-Pomt2*; lanes 4, cells transfected with human *POMT1*; lanes 5, cells transfected with human *POMT2*; lanes 6, cells cotransfected with rat *Pomt1* and rat *s-Pomt2*; lanes 7, cells cotransfected with human *POMT1* and human *POMT2*; lanes 8, cells cotransfected with rat *Pomt1* and human *POMT2*; lanes 9, cells cotransfected with human *POMT1* and rat *s-Pomt2*. Average values of three independent experiments are shown.

not affect protein *O*-mannosyltransferase activity. A testis-specific form of Pomt2 translation was also observed in mouse (Willer *et al.*, 2002). In mice, the testis form of Pomt2 was localized to maturing spermatids and was distributed within the acrosome and the endoplasmic reticulum (ER). The authors of that study speculated that the ER-localized Pomt2 is involved in the synthesis of *O*-mannosyl glycans, and the acrosome-localized Pomt2 acts as a lectin that is involved in adhesive interactions of sperm and egg during fertilization (Willer *et al.*, 2002). In Figure 3A (middle panel), a band corresponding to the 2.7 kb mRNA in testis is thought to be the *s-Pomt2*. This conclusion is consistent with data from the mouse, insofar as mRNAs of both *s-Pomt2* and *t-Pomt2* were detected in mouse testis (Willer *et al.*, 2002). In Figure 7B, the band was detected around the migration position of s-Pomt2 at 75 kDa in the cells transfected with t-Pomt2 (lanes 2 and 4). Because the *t-Pomt2* cDNA has two ATG-start sites, it is likely that the 75-kDa band of t-Pomt2 is derived from the transcription starting at the second ATG-start site. The biological significance of the presence of different *Pomt2s* remains to be determined.

Why protein *O*-mannosyltransferase activity requires coexpression of Pomt1 and Pomt2 is unclear. One possibility is that Pomt1 is a catalytic molecule and Pomt2 is a regulatory molecule or *vice versa*. Another possibility is that assembly of Pomt1 and Pomt2 forms a catalytic domain. That is the reason why expression of Pomt1 or Pomt2 alone does not show any enzymatic activity. Recently, it has been reported that complex formation between a glycosyltransferase and

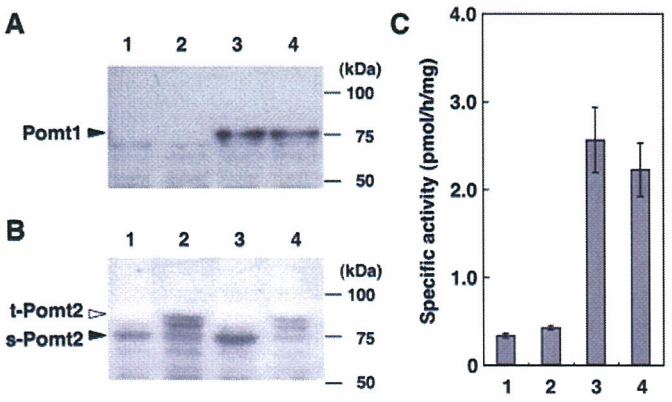

Fig. 7. Protein *O*-mannosyltransferase activity of testis form of rat Pomt2. (**A**, **B**) Western blot analyses of recombinant Pomt1 and Pomt2 detected by anti-POMT1 (**A**) and anti-POMT2 antibody (**B**). Molecular weight standards are shown on the right. (**C**) Protein *O*-mannosyltransferase activities of s-Pomt2 and t-Pomt2 coexpressed with Pomt1. Lanes 1, cells transfected with *s-Pomt2*; lanes 2, cells transfected with *t-Pomt2*; lanes 3, cells cotransfected with *Pomt1* and *s-Pomt2*; lanes 4, cells cotransfected with *Pomt1* and *t-Pomt2*. Average values of three independent experiments are shown.

its homologue changed its enzymatic character. Human chondroitin synthase exhibits glucuronyltransferase II and *N*-acetylgalactosaminyltransferase II activities but cannot polymerize the chondroitin chain *in vitro* (Kitagawa *et al.*, 2001). A recent study indicated that chondroitin-polymerizing activity requires the coexpression of chondroitin-polymerizing factor with chondroitin synthase (Kitagawa *et al.*, 2003). Although the amino acid sequence of chondroitin-polymerizing factor displayed 23% identity to that of chondroitin synthase, chondroitin-polymerizing factor did not show any enzymatic activity. Heparan sulfate polymerization is another case. Heparan sulfate polymerization *in vitro* requires both EXT1 and EXT2 that have *N*-acetylglucosaminyltransferase II and glucuronyltransferase II activities. A heterocomplex formation of EXT1 and EXT2 is required for chain elongation of heparan sulfate and to be present in the appropriate intracellular locations (McCormick *et al.*, 2000; Kim *et al.*, 2003). Further studies are needed to understand the regulation of protein *O*-mannosylation by two *Pomt* homologues.

Mutations in the human *POMT1* and *POMT2* genes give rise to WWS, a congenital muscular dystrophy with severe neuronal migration disorder (Beltran-Valero de Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). Elucidating the regulation of *O*-mannosylation in brain will help to understand the molecular pathology of WWS. To address the pathogenesis of WWS, it is also important to determine what proteins in addition to α-DG may be modified by *O*-mannosylation. The mannosyl-*O*-Ser/Thr linkage was first identified in chondroitin sulfate proteoglycans, neurocan, phosphacan, and phosphacan-keratan sulfate of brain (Finne *et al.*, 1979; Krusius *et al.*, 1986, 1987; Margolis *et al.*, 1996). The content of mannosyl-*O*-Ser/Thr linkage in these proteoglycans is regulated developmentally. On the basis of the yield of mannitol in hydrolysates of oligosaccharides after alkaline borohydride treatment of neurocan, the proportion of mannosyl-*O*-Ser/Thr linkage was calculated to increase from an insignificant level in one week postnatal rat brain to 15% of the total mannose in adult brain, whereas in the cases of phosphacan and phosphacan–keratan sulfate, the corresponding values were 26–31% at 7 days postnatal and 28–52% in adult brain (Rauch *et al.*, 1991). On the other hand, *N*-acetylgalactosamine (GalNAc)-linked oligosaccharides disappeared from phosphacan during the course of postnatal brain development, and these were replaced in adult brain by a significant proportion of oligosaccharides and keratan sulfate chains containing mannosyl-*O*-Ser/Thr linkages (Rauch *et al.*, 1991). If *O*-mannosylation and *O*-GalNAcylation occur on the same Ser/Thr residues of these proteoglycans, a developmental change of *O*-mannosylation may affect *O*-GalNAcylation, because *O*-mannosylation occurs in the ER and *O*-GalNAcylation takes place at a later processing step in the Golgi apparatus (Rottger *et al.*, 1998). Elucidating the regulation of *O*-mannosylation should therefore help in understanding the developmental roles of *O*-glycosylation in brain.

Materials and Methods

Cloning of rat Pomt1

Two degenerate oligonucleotide primers were designed on the basis of the amino acid sequences of *Saccharomyces cerevisiae* PMT1 corresponding to amino acids 486–496 and 652–662: 5′-cctcctcgagtggggitt(c/t)i(a/g)ica(a/g)caiga(a/g)gt-3′ (sense) and 5′-cgaactcgagigciggia(a/g)(a/g)ta(a/g)t(g/t)(a/g)t(a/g)ia(a/g)(a/g)aa-3′ (antisense) (XhoI sites are underscored). mRNA was isolated from adult Sprague–Dawley rat brain using the FastTrack kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's directions, and RT–PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer, Wellesley, MA). Amplified products were ligated into pBluescript SK(-) (Stratagene, La Jolla, CA) after XhoI digestion. One clone with a high-sequence identity to the *S. cerevisiae PMT*s and the *PMT* homologue of *Drosophila melanogaster* was obtained. Primers were designed on the basis of the sequence: 5′-ttcatggctctgccatagcggtg-3′ (antisense) and 5′-tggctggagccctgtgtattgg-3′ (sense). Using a combination of these primers and the Bluescript forward and reverse (BSK and BKS) primers to which we added six bases in each case, 5′ and 3′ portions of the clone were extended by PCR using a 6-week rat brain λZAPII cDNA library (Stratagene) as template. Amplified products were ligated directly into pGEM-7Z (Promega, Madison, WI). One clone was obtained for each side, and a sequence from 3′-extended one was utilized as a probe for cDNA library screening. The 854-bp cDNA fragment amplified by PCR was labeled by random priming with [α-^{32}P]dCTP, and using this radiolabeled probe, the 6-week rat brain λZAPII cDNA library was screened by plaque hybridization. After the second hybridization using the same probe, positive plaques were excised *in vivo* according to the manufacturer's instructions and sequenced.

To obtain further 5′-cDNA sequence, 5′-rapid amplification of cDNA ends (RACE) was carried out with a GeneAmp RNA PCR kit, terminal deoxynucleotidyl transferase (Promega) and *Taq* DNA polymerase (Perkin Elmer). The

specific antisense primers were 5′-catcgaatcctcctagccaacc-3′ comprising the nucleotides 377–356 for the reverse transcription and 5′-gtggtccactgtcatccaggaag-3′ comprising the nucleotides 326–304 for the nested PCR. The sense primers with linker were 5′-tggaagaattcgcggccgcagttttttttttttttt-3′ and 5′-tggaagaattcgcggccgcag-3′ (EcoRI sites are underscored). The amplified products were ligated into the pGEM-7Z vector and sequenced.

Cloning of rat Pomt2

Using a cDNA sequence of human *POMT2* as probe, we identified a sequence (accession number XM_345708.1) that appeared likely to encode a part of rat Pomt2 by BLAST search in the GenBank database. On the basis of this sequence, we obtained further 5′- and 3′-cDNA sequences of somatic form *Pomt2* (*s-Pomt2*) by RT–PCR from rat testis poly(A)$^+$ RNA (BD Biosciences, Franklin Lakes, NJ) using a SMART RACE cDNA Amplification Kit (BD Biosciences), according to the manufacturer's instructions. The specific primers for 5′-RACE and 3′-RACE were 5′-agccccacttaggcagaatc-3′ and 5′-agtctatctgctcggcaacc-3′, respectively. To obtain more of the 5′-cDNA sequence of the testis form *Pomt2* (*t-Pomt2*), RT–PCR was carried out with SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen) and KOD-Plus-DNA polymerase (Toyobo Co., Osaka, Japan) using 5′-acagctcttctccgcgtttc-3′ for the sense primer, based on the cDNA sequence of mouse *Pomt2* (NM_153415), and 5′-aaagtcgggcttgaaaggca-3′ for the antisense primer, based on rat *s-Pomt2* mentioned above. The amplified cDNAs were cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced.

Vector construction of rat Pomt1 and Pomt2

The *Pomt1* fragment in the pGEM-7Z vector was digested and then introduced into the EcoRI sites of the pcDNA3.1 vector (Invitrogen) to express Pomt1. To make expression plasmid vectors of *s-* and *t-Pomt2*, the cDNAs containing putative open reading frame (ORF) of *s-* and *t-Pomt2* were amplified by PCR using cloned cDNAs in pCR4Blunt-TOPO as template. Primers of *s-Pomt2* were 5′-ttaagcttgccaccatgc-cgccggccataggc-3′ and 5′-cactcgagtcaaaagtcccatgattcc-3′ (HindIII and XhoI sites are underscored). Primers of *t-Pomt2* were 5′-ctaagcttcctgctatgttttacgcctc-3′ and 5′-gctgtggtgctcg-tatcggt-3′ (HindIII site is underscored). The PCR product of *s-Pomt2* was cloned into the HindIII and XhoI sites of pcDNA3.1 vector (pcDNA3.1-*s-Pomt2*), and the PCR product of *t-Pomt2* was introduced into the HindIII and KpnI sites of pcDNA3.1-*s-Pomt2*. Human *POMT1* and *POMT2* cDNAs were cloned into pcDNA3.1 as described previously (Manya *et al.*, 2004).

Nucleotide sequence and protein sequence analyses

Protein sequences were aligned and placed in a phylogenetic tree with ClustalW (http://www.ddbj.nig.ac.jp/searches-e.html). Similarities and identities were analyzed using the GENETYX-Mac program (GENETYX Corp., Tokyo, Japan), based on Lipman-Pearson's method, and the gap was not counted.

Northern blot analysis

Northern blots of rat tissues (Rat MTN blot) were purchased from BD Biosciences. Probe DNA fragments for rat *Pomt1* and *Pomt2* were prepared by PCR, and β-actin was supplied with the Rat MTN blot. Primers for *Pomt1* were 5′-cccactgtactgccatgggg-3′ and 5′-ccatgtctcacaggcctg-3′. Primers for *Pomt2* were 5′-cctgggttcctggctgatc-3′ and 5′-cggattacaa gtaacttccaactgc-3′. Each probe was labeled with [α-^{32}P] dCTP using a Random Primer DNA labeling kit (Takara Bio Inc., Shiga, Japan). Blots were hybridized with a ^{32}P-labeled DNA probe in ExpressHyb Hybridization solution (BD Biosciences) at 68°C for 1 h, followed by washing according to the manufacturer's instruction.

RT–PCR analysis

First-strand cDNAs were synthesized from poly(A)$^+$ RNAs of rat tissues (Rat MTC Panel I; BD Biosciences) using SuperScript III RNase H$^-$ Reverse Transcriptase. PCR was carried out with KOD-Plus-DNA polymerase using the following primers: 5′-acagctcttctccgcgtttc-3′ and 5′-gtagccag-caaggccaatca-3′ for the testis-specific sequence of *Pomt2* (841 bp); 5′-gagacattgtacagctcgtt-3′ and 5′-ctggctgaagtcca-gatcac-3′ for *Pomt1* (618 bp); and 5′-taatcactgtgaagaacctc-3′ and 5′-gaattcgacttctcagtacc-3′ for *Pomt2* (399 bp). Primers for rat glyceraldehyde 3-phosphate dehydrogenase (Rat G3PDH Control Amplimer Set) were purchased from BD Biosciences. The cycling parameters for PCR were 94°C for 15 s, 60°C for 30 s, and 68°C for 1 min, and cycle numbers were 40 cycles for testis-specific sequence of *Pomt2* and 35 cycles for *Pomt1*, *Pomt2*, and *G3PDH*.

In situ *hybridization histochemistry*

An EcoRI-XbaI restriction fragment of *Pomt1* comprising 331 nucleotides (nucleotides 2424–2754) and an EcoRI-PstI restriction fragment of *Pomt2* comprising 463 nucleotides (nucleotides 1902–2364) were subcloned into the pGEM-3Z vector (Promega), and the vectors were linearized with EcoRI for antisense probes of *Pomt1* and *Pomt2*, XbaI for sense probe of *Pomt1*, or PstI for sense probe of Pomt2. For PstI digestion, the linearized template end was converted to a blunt end with T4 DNA polymerase (Promega). Digoxigenin-labeled RNA probes were synthesized with the Riboprobe System (Promega) and digoxigenin-11-dUTP (Roche Diagnostics, Tokyo, Japan). To detect the expression of *Pomt1* and *Pomt2* in brain, frozen sections of rat brain tissues were fixed with 3% paraformardehyde/10 mM phosphate-buffered saline (PBS) and then analyzed with an In situ Hybridization kit (NIPPON GENE, Tokyo, Japan) according to the manufacturer's directions except that the sections were washed at 48°C after hybridization.

Expression of POMTs and cell extract preparation

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, and 100 units/mL penicillin–50 mg/mL streptomycin at 37°C with 5% CO_2. The expression plasmids of pcDNA3.1-rat *Pomt1*, pcDNA3.1-rat *s-Pomt2*, pcDNA3.1-rat *t-Pomt2*, pcDNA3.1-human *POMT1-myc*,

and pcDNA3.1-human *POMT2* were transfected into HEK293T cells using LipofectAMIN PLUS reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured for 3 days in complete medium, harvested and homogenized. The cells were homogenized in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, with a protease inhibitor cocktail (3 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM benzamidine–HCl, and 1 mM PMSF). After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultra centrifugation at 100,000 × *g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay (PIERCE, Rockford, IL).

Preparation of rat tissues

Heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis were obtained from 4-month-old Wistar rats. Tissue samples were homogenized with nine volumes (weight/volume) of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 250 mM sucrose. After centrifugation at 900 × *g* for 10 min, the supernatant was subjected to ultra centrifugation at 100,000 × *g* for 1 h. The precipitates were used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology. All efforts were made to minimize the number of animals used and their suffering.

Western blot analysis

Rabbit antibodies specific to POMT1 and POMT2 were described previously (Manya *et al.*, 2004). The microsomal fractions (20 μg) were separated by SDS–PAGE (10% gel), and proteins were transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with each antibody, and treated with anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ). Proteins bound to antibody were visualized with an ECL kit (Amersham Biosciences). As reported previously (Manya *et al.*, 2004), anti-POMT1 and anti-POMT2 polyclonal antibodies did not detect endogenous POMT1 and POMT2, respectively. Each antibody is specific for the respective recombinant protein.

Assay for protein O-mannosyltransferase activity

Protein *O*-mannosyltransferase activity was based on the amount of [^{3}H]-mannose transferred from Dol-P-Man to a glutathione-S-transferase fusion α-DG (GST-αDG) as described previously (Manya *et al.*, 2004). Briefly, the reaction mixture contained 20 mM Tris–HCl (pH 8.0), 100 nM of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemicals Inc., St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 10 μg GST-αDG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μL total volume. After 1 h incubation at 25°C, the reaction was stopped by adding 150 μL PBS containing 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan), and the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed, mixed with 400 μL of PBS containing 1% Triton X-100 and 10 μL of Glutathione-Sepharose 4B beads (Amersham Biosciences), rotated at 4°C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured with a liquid scintillation counter.

Acknowledgments

This work was supported by Research Grants for Nervous and Mental Disorders (17A-10) and Research on Psychiatric and Neurological Diseases and Mental Health from MHLW, a Grant-in-Aid for Scientific Research on Priority Area (14082209) from MEXT, JSPS Core-to-Core Program, and NIH grant NS-13876.

Conflict of interest statement

None declared.

Abbreviations

α-DG, α-dystroglycan; Dol-P-Man, dolichyl phosphate mannose; ER, endoplasmic reticulum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GalNAc, *N*-acetylgalactosamine; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; WWS, Walker–Warburg syndrome.

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