J. Biochem. **136**, 189–198 (2004)
DOI: 10.1093/jb/mvh106

Developmental Expression of a Sialyltransferase Responsible for Sialylation of Cortical Alveolus Glycoprotein during Oogenesis in Rainbow Trout (*Oncorhynchus mykiss*)

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Received April 16, 2004; accepted May 19, 2004

Polysialoglycoprotein (PSGP) is a major cortical alveolus glycoprotein of rainbow trout eggs that is characterized by the attachment of a polysialic acid structure to its *O*-glycan chains. It has been demonstrated that the polysialic acid structure is synthesized by at least three sialyltransferases mostly localized in cortical alveoli. Here we have cloned a cDNA encoding the sialyltransferase, designated rtST6GalNAc, responsible for the transfer of the first sialic acid residue onto the *O*-glycan chain of PSGP. This enzyme belongs to the vertebrate ST6GalNAc II family, and is strongly expressed in ovaries. Of those *O*-glycoproteins tested as substrates, asialo-PSGP is the best substrate. These results indicate that rtST6GalNAc is the enzyme responsible for the sialylation of PSGP during oogenesis. Furthermore, the rtST6GalNAc mRNA is expressed throughout oogenesis, is down-regulated at the late yolk vesicle stage (May), and then up-regulated during vitellogenesis (until August). This developmental profile is highly similar to that of STL2, a cortical alveolus lectin, while it is quite different from that of PSGP, which is extensively expressed at the yolk vesicle stage and down-regulated at later stages. Thus, not all cortical alveolus components are transcribed concomitantly. This is the first description of a developmental change in the transcription of a glycosyltransferase during oogenesis.

Key words: cortical alveolus, developmental biology, glycosyltransferase, oocyte development, ovary.

Cortical alveoli are secretory vesicles derived from the Golgi apparatus, and aligned in the peripheral cytoplasm of mature fish eggs (*1*, *2*). On fertilization, cortical alveoli fuse with the plasma membrane and discharge their contents into the perivitelline space. The discharged contents are known to play important roles in fertilization and subsequent early development, such as the formation of the fertilization envelope, enlargement of the perivitelline space, late polyspermy block process, and protection against microorganisms (*3–5*). Cortical alveoli contain heavily glycosylated glycoproteins, designated hyosophorin, as a major glycoprotein component (*6*, *7*).

In salmonid fish, hyosophorin is characterized by the presence of a homopolymer of sialic acid (Sia), polysialic acid, and named polysialoglycoprotein (PSGP). PSGP has a high sialic acid content (more than 60% by weight) that forms a polysialic acid structure on *O*-glycan chains of PSGP (*8*). Because of the polysialyl structure, PSGP has polyanionic properties, and, accordingly, cortical alveoli of mature oocytes show metachromasis upon staining with cationic dyes such as toluidine blue (*6*). The biosynthesis of PSGP starts at earlier stages of oogenesis (yolk vesicle stage; April) when cortical alveoli appear in the cytoplasm of oocytes (*9*). PSGP at the earlier stages contains exclusively a disialyl group, Siaα2→8Sia. In June and later, elongation of the disialyl structure on PSGP occurs to form oligo- and polysialic acid structures with chain lengths of up to 25 sialic acid residues (*9*). The sialylation of PSGP is known to be catalyzed by at least three sialyltransferases: a GalNAc α2,6-sialyltransferase (ST6GalNAc), a Sia α2,8-sialyltransferase, and an α2,8-polysialyltransferase. The ST6GalNAc catalyzes the incorporation of a Sia residue onto the C-6 position of the proximal GalNAc residue. The Sia α2,8-sialyltransferase transfers the second Sia residue to the C-8 position of the first Sia residue. This enzyme initiates α2,8-sialylation and catalyzes the formation of disialyl groups. Finally, the α2,8-polysialyltransferase forms α2,8-linked polysialic acid chains on *O*-glycans of PSGP. The ST6GalNAc activity is expressed throughout oogenesis from April to September, while the expression of the α2,8-polysialyltransferase activity starts in June, two months after that of ST6GalNAc (*9*). Interestingly, these sialyltransferase activities are detected in cortical alveoli as is their substrate PSGP (*9*), although it is generally recognized that sialyltransferases are localized in the Golgi apparatus (*10*). The localization of the sialyltransferases in secretory vesicles, *i.e.*, cortical alveoli, is unusual and it remains unknown when and how the sialyltransferases are expressed and sorted into the cortical alveoli. In addition, no examination of the developmental expression of the mRNAs for glycosyltransferases during oogenesis has

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been reported in any animal species, although it is well known that glycosylation of oocyte proteins is important not only for oogenesis, but also for preparation for fertilization and early development.

Thus, to understand how the expression of glycosyltransferases is developmentally and functionally regulated during oogenesis, we have identified a cDNA encoding the cortical alveolus-localized ST6GalNAc. We demonstrate that this enzyme is responsible for the sialylation of PSGP from rainbow trout ovary, and examine its developmental expression during oogenesis in comparison with other oocyte proteins.

MATERIALS AND METHODS

Rainbow Trout—Male and female rainbow trout were kindly provided by the Shiga Prefectural Samegai Trout Farm. Trout ovaries were excised from females in each month during oogenesis. Various tissues were excised from males and females in May (5 mo prior to ovulation). The excised tissues were frozen immediately in liquid nitrogen and kept at –80°C until use.

Materials—Fetuin, bovine submaxillary mucin (BSM) and sialidase from *Vibrio cholerae* were purchased from Sigma (St. Louis, MO, USA). Transferrin was purchased from Wako (Tokyo, Japan). Colominic acid and sialidase from *Arthrobactor ureafaciens* were obtained from Nacalai Co. (Kyoto, Japan). CMP-[^{14}C]NeuAc was obtained from Amarsham Biosciences (Piscataway, NJ, USA). Sialidase from Newcastle disease Virus (NDV), GM1 and GD1a were purchased from Calbiochem Co. (La Jolla, CA, USA). A pPROTA vector was kindly provided by Dr. John Lau (Roswell Park Cancer Inst., USA). Restriction enzymes and *Taq* polymerase were purchased from TaKaRa (Osaka, Japan). COS-1 cells were kindly provided by Dr. Karen J. Colley (University of Illinois at Chicago, College of Medicine, USA). Glycan-conjugated polyacrylamides (GalNAcα-PAA; Galβ1,3 GalNAcα-PAA; NeuAcα2,3 Galβ1,3 GalNAcα-PAA) were obtained from Glycotech Co. (Rockville, MD, USA). Synthetic oligonecleotide primers were obtained from Rikaken (Nagoya, Japan).

Polymerase Chain Reaction (PCR) to Clone a cDNA Fragment Encoding Rainbow Trout α2,6-Sialyltransferase—Degenerated primers were designed based on the sequence information for sialyl motifs L and S of chicken ST6GalNAc II (accession number X77775) and mouse ST6GalNAc II (accession number X93999). Total RNA was extracted from rainbow trout ovary by TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The single strand cDNAs were synthesized with random primers from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). To isolate the cDNA fragment encoding α2,6-sialyltransferase, PCR was performed with a sense primer, 5′-TGTGCCGTGGTGGG(CT)AA(CT)GG-3′, and an antisense primer, 5′-GCACTGACCTGGTC(AG)CAGGT-3′. The cDNA was amplified through 30 cycles of a step program (94°C, 30 s; 54°C, 1 min; 72°C, 2 min). The amplified products were analyzed by 1.0% agarose gel electrophoresis and subcloned into pGEM T-Easy vector (Promega, Madison, WI). DNA sequences were determined by the deoxynucleotide chain termination method. Amplification of the 5′- and 3′-ends of rainbow trout α2,6-sialyltransferase (rtST6GalNAc) cDNA was carried out as described previously (*11*).

Preparation of Recombinant rtST6GalNAc—A truncated form of rtST6GalNAc lacking of the first 32 amino acids of the open reading frame was prepared by PCR amplification. The primers used were the 5′-primer, 5′- ACCTGGAATTCGGACATCCTGAACCTGACA-3′ (underline shows asynthetic *Eco*RI site, nucleotides 99–118), and the 3′-primer, 5′-TAGTTCGAATTCTCACTGGCGCATGTAGAG-3′ (underline shows a synthetic *Eco*R I site, complementary to nucleotides 1324–1341). The amplified fragments were subcloned into the *Eco*RV site of pTargeT vector (Promega) and sequenced. The inserted fragments were cut out by digestion with *Eco*RI, and then inserted into the *Eco*RI site of pPROTA vector (*12*). The orientation of insertintion was confirmed by DNA sequencing. The resulting plasmid was designated pPROTA rtST6GalNAc, and encodes the fusion protein of the signal peptide sequence of transin, a collagenase family gene, the IgG binding domain of protein A and the truncated form of rtST6GalNAc. COS-1 cells on 90-mm plates (6×10^5 cells) were transiently transfected with 20 μg of pPROTA rtST6GalNAc using the lipofectin reagent (Invitrogen). Forty-eight hours after transfection, the culture medium was collected and the protein A-rtST6GalNAc fusion protein secreted in the culture medium was absorbed to IgG-Sepharose (Amersham Biosciences, 20 μl of 50% slurry resin/10 ml of culture medium). The absorbed fusion protein was used as an enzyme source for sialyltransferase activity assay.

Preparation of Asialo-Glycoproteins and Partial Hydrolysate of GD1a—For the preparation of asialo-fetuin and asialo-BSM, 1 mg of fetuin and 0.2 mg of BSM were treated with 5 milliunits of *A. ureafaciens* sialidase and 5 milliunits of *V. cholerae* sialidase in 150 μl of 50 mM acetate buffer (pH 5.5) for 20 h at 37°C. To remove the released free sialic acid, incubated mixtures were filtered through Microcon YM-10 (Millipore, Bedford, MA, USA). The desialylated glycoproteins were subjected to thin layer chromatography to confirm that the sialidase treatment was complete as described previously (*13*). Asialo-PSGP was prepared from PSGP as described previously (*13*). For partial hydrolysis of GD1a, 40 μg of GD1a was hydrolyzed in 0.1 N trifluoroacetic acid at 70°C for 1 h. The hydrolysate was purified using C18 Sep-Pak cartridge (Waters, Milford, MA, USA). The purified products were analyzed by high performance thin layer chromatography (HPTLC) as described previously (*14*).

Assay for Sialyltransferase Activity and Product Analysis—Enzyme activity was measured at 25°C for 3 h in 10 μl of reaction mixture containing the recombinant rtST6GalNAc, 50 mM MES buffer (pH 6.0), 10 mM $MnCl_2$, 0.5 % Triton CF-54, 10 μM CMP-[^{14}C]NeuAc (1.07 kBq), and 1 mg/ml acceptor substrate except BSM and asialo-BSM (0.2 mg/ml). The reaction was terminated by adding EDTA (final concentration 50 mM). For glycoprotein acceptors GalNAcα-PAA, Galβ1,3 GalNAcα-PAA, and NeuAcα2,3 Galβ1,3 GalNAcα-PAA, a 5-μl aliquot was spotted on Whatman 3 MM paper, and developed in ethanol/1 M ammonium acetate, pH 7.5 (7:3, v/v). After air-drying, the amount of incorporated [^{14}C]NeuAc remaining at the origin was determined by a BAS 2000 imaging analyzer (Fuji Film, Tokyo, Japan). For glycoli-

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pid acceptors, the reaction mixtures were applied to a C18 Sep-Pak cartridge. The purified products were spotted on HPTLC plates and developed in chloroform/methanol/0.2% $CaCl_2$, (55:45:10, v/v/v). ^{14}C-sialylated glycolipids were visualized and quantified with a BAS 2000 imaging analyzer.

[^{14}C]NeuAc-incorporated asialo-PSGP obtained by the above described paper chromatography was extracted with water, and treated in 20 μl of 50 mM acetate buffer (pH 5.5) with 1 milliunit of *A. ureafaciens* sialidase (specific for α2,3-, α2,6- and α2,8-linked sialic acid) or 1 milliunit of NDV sialidase (specific for α2,3- and α2,8-linked sialic acid), or without sialidase at 37°C for 20 h. After sialidase treatment, the product was chromatographed on Whatman 3 MM paper. As control experiments to ensure that the sialidase treatments went well, fetuin (for α2,3-linked sialic acid residues), transferrin (for α2,6-linked sialic acid residues), and colominic acid (for α2,8-linked sialic acid residues) were also treated with the sialidases under the same conditions, and analyzed by HPTLC as described above.

Northern Blot Analysis—Total RNA was isolated from various tissues of rainbow trout with TRIZOL reagent (Invitrogen). Poly(A)$^+$ RNAs were purified from total RNA with oligotex dT 30 (TaKaRa). One microgram of poly(A)$^+$ RNAs was separated in a 1.0% agarose gel containing 5% formaldehyde, and then transferred to a nylon membrane (Hybond N$^+$, Amersham Biosciences). Digoxigenin (DIG)-labeled rtST6GalNAc cRNA (nucleotide –241–1,609) was used as a probe. Hybridization was carried out at 65°C for 14 h in 5 % blocking buffer (Roch, Mannheim, Germany), 5× SSC, 1.5% SDS, 50% formamide, and 0.1 mg/ml tRNA from Baker's yeast. The membrane was washed with a series of solutions, 2× SSC/0.1 % SDS (at room temperature for 5 min, twice) and 0.1× SSC/0.1% SDS (at 65°C for 15 min, twice). The hybridized probe was detected with a DIG Nucleic Acid Detection Kit (Roch) according to the manufacturer's instructions. The membrane was deprobed at 80°C for 30 min three times with stripping buffer containing 50 mM Tris-HCl (pH 8.0), 60% formamide and 1% SDS. Then, the membrane was hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cRNA (accession number AF027130, nucleotide 25–1,062). Detection was carried out as described above.

The Gene Expression of Oocyte Proteins during Oogenesis—The following oligonucleotide primers for rainbow trout proteins were used: rtST6GalNAc (accession number AB097943, nucleotides 393–1162) 5′-GGCATCTGGTACACACAGGA-3′ and 5′-ACATAGCGTGTCTCCTCAGA-3′; PSGP (accession number J04051, nucleotides 46–330), 5′-AGAGAATTGCTGCTCGTTGTG-3′ and 5′-GAGCTTCTCCTCAGAGGAGAG-3′; STL2 (accession number AB039023, nucleotides 11–580), 5′-TCACACTGACTGGGTTCACC-3′ and 5′-GGACGTCACACTGGATCTTA-3′; SOX24 (accession number AB010741, nucleotides 294–844), 5′-ACAGCAACCGGTCACATCAA-3′ and 5′-AGACTCTGCAGTGGAGCTCA-3′; cathepsin D (accession number U90321, nucleotides 113–768), 5′-GAGGAAGTTCCGTTCCATCA-3′ and 5′-AGTACTTCGGGTCAGTTCCT-3′; vitellogenin receptor (VTGR) (accession number AJ003117, nucleotides 289–914) 5′-TCTGCTGAACAATGGAGGCT-3′ and 5′-AGAGTGGATCAACAGCGACA-3′; and GAPDH (accession number AF027130, nucleotides 25–1062) 5′-CAACCCAATCAACAGCAACT-3′ and 5′-AGCCGGACAGTTACTCCTTA-3′. Total RNAs were prepared from the ovaries of three rainbow trout in each month (April to September) using TRIZOL reagent. The single strand cDNA was synthesized as described above and used as a template for PCR. Both sense and antisense primers (5 pmol each) were added to 25 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, dNTP mixture (0.2 mM each), and 625 milliunits of rTaq polymerase (TaKaRa). PCR, 25–28 cycles involving a three-step incubation (94°C, 30 s; 54°C, 1 min; 72°C, 2 min) was carried out. The cycles were set in such a way that the amount of amplified DNA did not reach a plateau. Aliquots of PCR product were separated in a 1.0% agarose gel; signal intensity was estimated using an ATTO Lane & Spot Analyzer (ATTO, Tokyo, Japan).

RESULTS

Isolation of Rainbow Trout GalNAc: α2,6-Sialyltransferase (rtST6GalNAc) cDNA—To isolate the cDNA fragment of a GalNAc α2,6-sialyltransferase responsible for the synthesis of the polysialic acid on rainbow trout polysialoglycoprotein (PSGP), we designed degenerate primers for the sialyl motifs L and S, conserved sequences among vertebrate ST6GalNAc sialyltransferases (*15*, *16*). A 515 bp cDNA fragment was amplified using rainbow trout ovary cDNA as a template for a PCR reaction that showed high homology to vertebrate ST6GalNAc II cDNA sequences. Additional 3′- and 5′-RACE reactions were performed to isolate a full-length cDNA clone designated rtST6GalNAc. The cDNA sequence of rtST6GalNAc has two in-frame ATG codons. We assigned the downstream ATG codon (nucleotide 1, Fig. 1) as the initiation codon because the downstream ATG codon agrees with Kozak's rule better than the upstream one (nucleotide –9, Fig. 1) (*7*). A polyadenylation signal (AATAAA) is located 26 nucleotides upstream from the poly A sequence (*18*). Thus, the cDNA clone contains an open reading frame of 1,341 bp, and encodes a protein of 446 amino acids with a predicted molecular mass of approximately 51 kDa. The deduced amino acid sequence has a type II membrane topology with an NH_2-terminal cytoplasmic tail of 6 amino acids, a transmembrane domain of 25 amino acids, a stem domain and a COOH-terminal catalytic domain, as is the case with all other glycosyltransferases cloned to date (*19*).

Comparison of the primary structure of the catalytic domain of rtST6GalNAc with other members of the ST6GalNAc gene family shows significant similarity in three sialyl motifs L, S, and VS, which are highly conserved among all vertebrate sialyltransferases (*15*, *16*, *19*, *20*). In particular, rtST6GalNAc has higher homology to ST6GalNAc I (80–75%, 70–65%, and 54–38% to sialyl motifs L, S, and VS, respectively) and ST6GalNAc II (80–70%, 87%, and 54–46% to sialyl motif L, S, and VS, respectively) than other ST6GalNAc genes (50–32%, 39–30%, and 15% to sialyl motif L, S, and VS, respectively). In the region (amino acids 273–335) flanked by sialyl motifs L and S, rtST6GalNAc is more similar to ST6GalNAc II as compared with ST6GalNAc I. The

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-274                                                    GCTCCTACTATGCAGATAGATAACTGTCACTAAA -241
-240 GAAGACTCGTCACAGACTGTCATGTGGTAAGAGAGGCCCCAGAGTAGTGTGCACCTCTGT -181
-180 GCACTCATTTTACAGTCAACTCCAGCCCTCGTCTCTTTCCTCGTTGCTCCCCTGAAAACC -121
-120 CGAACAACCTCCTCAGAAGGACATTGGTGACTCCCCTCTACTCCCCCACATTCCTCCTCA  -61
 -60 TTGGAGGGTAGAGGGCACACACACAGCCGGCTGCTCAGGCTCAGGTCGACGATGACGAGC   -1
   1 ATGGGGCTCCAGAGGAGGCTATGGCTGTGCTTCCTGGGTGTCAGCTTCCTGCCAGTCATC   60
   1  M  G  L  Q  R  R  L  W  L  C  F  L  G  V  S  F  L  P  V  I   20
  61 TATATCATCTATTTGAACCTTGAGATCACCTGGCGCATGGACATCCTGAACCTGACACAA  120
  21  Y  I  I  Y  L  N  L  E  I  T  W  R  M  D  I  L  N  L  T  Q   40
 121 TTCCCTGGCATCTGGTACACACAGGAACCCAACAGGTTACATGACCCTGAAGAACCTCTT  180
  41  F  P  G  I  W  Y  T  Q  E  P  N  R  L  H  D  P  E  E  P  L   60
 181 GTTGTCAATACGCTGTCAGATAATAGTAGTATCGCACCACCAACACCTTTAAGTTTAAAG  240
  61  V  V  N  T  L  S  D  N  S  S  I  A  P  P  T  P  L  S  L  K   80
 241 GTGAAGTCCACATCCCCTGCCAGAGTCAGACCGTCAACAGAACCTGGAGCCAGGACTACC  300
  81  V  K  S  T  S  P  A  R  V  R  P  S  T  E  P  G  A  R  T  T  100
 301 AAGGGCTCTGATCTTAAAGGTGCTCTCCTCAAGTCTGCCAAACCAACAGAGCCACCCTTC  360
 101  K  G  S  D  L  K  G  A  L  L  K  S  A  K  P  T  E  P  P  F  120
 361 ATTGGCGACAGCTACTACAGTGAAGATGCCCCACCTCAAACAGACTGTCCAGACAGTATT  420
 121  I  G  D  S  Y  Y  S  E  D  A  P  P  Q  T  D  C  P  D  S  I  140
 421 CGGAGCAGGGTGTCCCAGACAGACTTTGGGGTACGGTATCTGGGCAGGATCCCTGTGCTT  480
 141  R  S  R  V  S  Q  T  D  F  G  V  R  Y  L  G  R  I  P  V  L  160
 481 CAGTGGGCCAAGCATGCCACTCCAGAACAGTACCAGCGCCTCCGATTGTATCCTGGGACT  540
 161  Q  W  A  K  H  A  T  P  E  Q  Y  Q  R  L  R  L  Y  P  G  T  180
 541 CATGGCTGGGCAAACCTCGACTTTAACACTCTGGTGTCGACCCTATCTGTTCTCAACACG  600
 181  H  G  W  A  N  L  D  F  N  T  L  V  S  T  L  S  V  L  N  T  200
 601 TCAGCCAACTGGCACATGTTTGATGACTGGGATCGTCGTAGCAACGGCTCACGGTGTGTC  660
 201  S  A  N  W  H  M  F  D  D  W  D  R  R  S  N  G  S  R  C  V  220
 661 CGCTGTGCTGTCGTGGGCAACGGAGGGATTCTGAATGAGTCAAAGAAAGGCCATGAGATT  720
 221  R  C  A  V  V  G  N  G  G  I  L  N  E  S  K  K  G  H  E  I  240
 721 GACCAGCATGACTACGTATTCAGGACCAACGGAGCTGTCATTAAAGGCTTTGAGCAGGAT  780
 241  D  Q  H  D  Y  V  F  R  T  N  G  A  V  I  K  G  F  E  Q  D  260
 781 GTGGGCTCCAGAACATCCTTCTACACGTTCTCCACCAACACACTCCGTAACTCCATGAGG  840
 261  V  G  S  R  T  S  F  Y  T  F  S  T  N  T  L  R  N  S  M  R  280
 841 AGCTACGCCGGAGCCGGCTACAGAGGACCACCGCTCTCTGAGGAGACACGCTATGTCTTC  900
 281  S  Y  A  G  A  G  Y  R  G  P  P  L  S  E  E  T  R  Y  V  F  300
 901 CTGCCAGACCATGACAGGGATTATCTGCTGATGAAAGCAGGTGCCACACACACTCCAGTA  960
 301  L  P  D  H  D  R  D  Y  L  L  M  K  A  G  A  T  H  T  P  V  320
 961 GAGAGAGGACCAGAAAGGAGCAAAACACCTCCCACATACTTTGGAGGAAATGTGACCGTA 1020
 321  E  R  G  P  E  R  S  K  T  P  P  T  Y  F  G  G  N  V  T  V  340
1021 GAAAAGTTGAAGATGTACCATCCAGATTTCATACGCTACATCAGAAACAGGTTCTTCCGG 1080
 341  E  K  L  K  M  Y  H  P  D  F  I  R  Y  I  R  N  R  F  F  R  360
1081 GCCCACGCCATGCAGACCAAATACAAGGACATCTACCGTCCATCAACAGGTGCTGTGATG 1140
 361  A  H  A  M  Q  T  K  Y  K  D  I  Y  R  P  S  T  G  A  V  M  380
1141 CTGCTGGCTGCGATACACACCTGTGACCAGGTGAGCGCGTATGGCTTCATGACTCCAGAC 1200
 381  L  L  A  A  I  H  T  C  D  Q  V  S  A  Y  G  F  M  T  P  D  400
1201 TATGTGAAATACTCTGACCACTACTTTGACCACCAATATCACCCAGTGGGTTTCTTCATT 1260
 401  Y  V  K  Y  S  D  H  Y  F  D  H  Q  Y  H  P  V  G  F  F  I  420
1261 AACCATGACATGAGGATGGAGATGACACTGTGGCAACAACTCCACCGTGCAGGCCTCATC 1320
 421  N  H  D  M  R  M  E  M  T  L  W  Q  Q  L  H  R  A  G  L  I  440
1321 ACTCTCTACATGCGCCAGTGAACTCAGGAACTAACTCACTCAAGGTTGCTACGGCAACGA 1380
 441  T  L  Y  M  R  Q  *                                          446
1381 CCACCTCCCGTAGGATTAAAAACTAGTCAAACTGTATATTTATATATTATTGAACAAAAA 1440
1441 AATATAAATTCAACATGGTGTTGGTTTTATGAGCTGAAATAAAAGATCCCAGAAATGTTC 1500
1501 CGTATGCAC                                                     1508
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Fig. 1. **Nucleotide and the deduced amino acid sequences of rainbow trout ST6GalNAc.** The nucleotide sequence (accession number AB097943) and the deduced amino acid sequence are numbered from the initiation codon and initiation methionine, respectively. The putative transmembrane domain is double underlined. Sialyl motifs L, S and VS are underlined. A polyadenylation signal (AATAAA) is located at nucleotides 1478–1483.

length of this region in ST6GalNAc I is shorter by 2 amino acid residues than those of rtST6GalNAc or ST6GalNAc II. Taken together, these results suggest that rtST6GalNAc is a rainbow trout ST6GalNAc II.

Substrate Specificity of rtST6GalNAc—To analyze the acceptor substrate specificity of rtST6GalNAc, a recombinant rtST6GalNAc was expressed by transfecting COS-1 cells with a pPROTA rtST6GalNAc plasmid. As summarized in Table 1, the recombinant rtST6GalNAc showed sialyltransferase activity toward asialo-PSGP, fetuin, asialo-fetuin and three synthetic glycopolymers tested. However, the recombinant enzyme showed no activity toward glycolipids. The enzyme preferred fetuin to asialo-fetuin as a substrate, and showed no activity toward asialo-BSM. About 78% of *O*-glycan chains in fetuin contain a NeuAcα2,3 Galβ1,3 GalNAcα sequence (*21*), and the *O*-glycan chain present in asialo-fetuin is exclusively Galβ1,3 GalNAcα. Asialo-BSM contains GalNAcα as a dominant glycan structure (*22*). These results suggest that NeuAcα2,3 Galβ1,3 GalNAcα is a better substrate than Galβ1,3 GalNAcα or GalNAcα. Consistent with these results, the enzyme showed much stronger

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Table 1. **Substrate specificity of rtST6GalNAc.**

Acceptor substrate[a]	Relative enzyme activity[b] (%)
Fetuin	100
Asialo-fetuin	27
BSM	0
Asialo-BSM	0
Asialo-PSGP	513
GalNAcα-PAA	22
Galβ1,3GalNAcα-PAA	18
NeuAcα2,3Galβ1,3GalNAcα-PAA	203
GD1a	0
GM1	0
GM1b[c]	0

[a]The concentrations of all acceptor substrates were set to 1 mg/ml, except those of BSM and asialo-BSM, which were set to 0.2 mg/ml. [b]Relative enzyme activity is reported as a percentage of the activity observed using fetuin as a substrate (0.124 pmol/h). The same amount of enzyme was used for all substrates. [c]GM1 is a contaminant in this GM1b preparation.

activity toward NeuAcα2,3 Galβ1,3 GalNAcα-PAA than Galβ1,3 GalNAcα-PAA or GalNAcα-PAA. Notably, asialo-PSGP was a much better substrate for rtST6GalNAc than fetuin or NeuAcα2,3 Galβ1,3 GalNAcα-PAA (Table 1). Asialo-PSGP from rainbow trout contains a Galβ1,3 GalNAcα sequence and extended forms of its core structure, such as GalNAcβ1,3 Galβ1,4 Galβ1,3 GalNAcα, in molar proportions of 36% and 48%, respectively (*13*, *23*). The latter extended forms of the core structure may be better substrates for rtST6GalNAc. Since asialo-PSGP is the best acceptor substrate, it is suggested that rtST6GalNAc is involved in the synthesis of the first sialyl residue in the polysialyl chains of PSGP.

In order to determine the linkage of the Sia residue formed by rtST6GalNAc, linkage-specific sialidase treatments were carried out (Fig. 2). [^{14}C]NeuAc incorporated

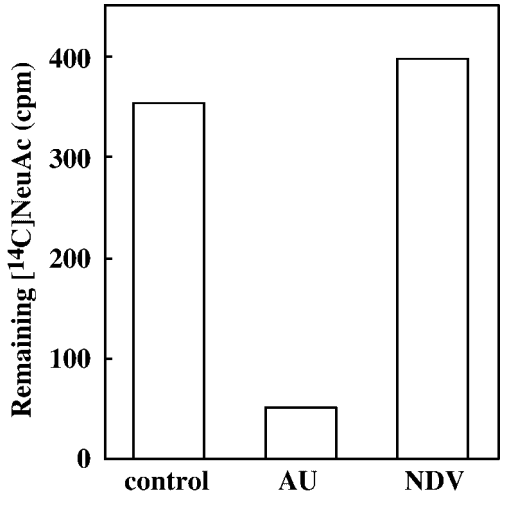

Fig. 2. **Determination of the linkage specificity of rtST6GalNAc.** [^{14}C]NeuAc-incorporated asialo-PSGP by the recombinant rtST6GalNAc was subjected to linkage-specific sialidase treatments: control, incubation without sialidases; AU, *A. urefaciens* sialidase (1 milliunit), which cleaves α2,3-, α2,6- and α2,8-linkages of sialic acid; NDV, NDV sialidase (1 milliunit), which is specific for α2,3- and α2,8-linkages of sialic acid.

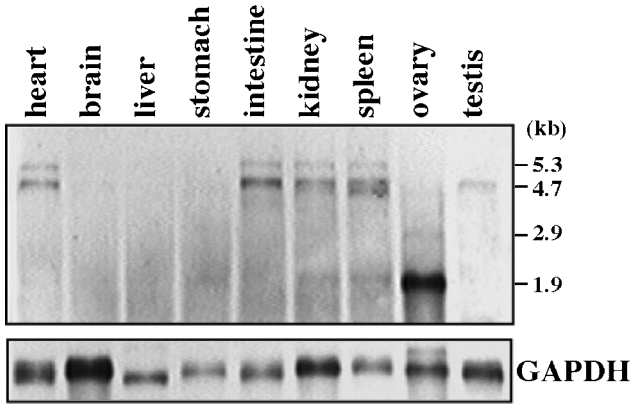

Fig. 3. **Northern blot analysis of the expression of rtST6GalNAc mRNA in various tissues of rainbow trout.** Poly(A)$^+$ RNA samples from various tissues of rainbow trout 5 mo prior to ovulation (May) were separated in a 1% agarose gel and blotted onto a nylon membrane. The membrane was hybridized with DIG-labeled rtST6GalNAc full-length cRNA as a probe, and visualized as described in "MATERIALS AND METHODS." The same membrane was also reprobed with DIG-labeled GAPDH cRNA

into asialo-PSGP was not removed by NDV sialidase, which catalyzes the hydrolysis of α2,3- and α2,8-linked Sia residues. A similar result was obtained for [^{14}C]NeuAc-incorporated GalNAc-PAA (data not shown). These results indicate that rtST6GalNAc transfers Sia residues onto the C-6 position of GalNAc residues.

Expression of the rtST6GalNAc Gene in Various Tissues of Rainbow Trout—To determine the size of the rtST6GalNAc mRNA and its distribution in various rainbow trout tissues, Northern blot analysis was carried out using 1 μg of poly(A)$^+$ RNA prepared from tissues obtained in May when the glycan chains on PSGP mainly contain the disialyl unit. As shown in Fig. 3, four mRNA species (5.3 kb, 4.7 kb, 2.9 kb and 1.9 kb) were detected. In heart, intestine, kidney, spleen and testis, the 4.7 kb transcript was strongly expressed together with a weaker expression of the 5.3 kb transcript. The 1.9 kb transcript was abundantly expressed only in ovary. In addition, the 2.9 kb transcript was faintly detectable only in ovary. The species present in ovary did not change during oogenesis (April to September, data not shown). These results indicate that the rtST6GalNAc gene is transcriptionally regulated in the ovary. Because of the length of the cDNA (1,783 bp), the cloned rtST6GalNAc cDNA may correspond to the 1.9 kb transcript that is strongly expressed in ovary.

Correlation of the β-Actin mRNA Expression Level with Oocyte Maturation—Oogenesis in rainbow trout occurs over a 6-month period from April to October. The formation of cortical alveoli starts in the early oogenesis stages in April, and yolk materials accumulate from June. The diameter of the oocyte increases following the accumulation of the yolk materials with ovulation of mature oocytes finally taking place in October. The diameter of oocytes is often used as a marker of oogenesis (*24–26*). However, it is often difficult in practice to measure the size of every oocyte used for biochemical analyses. Therefore, we tried to introduce a biochemical marker for oogenesis. It is assumed that the size of an oocyte is corre-

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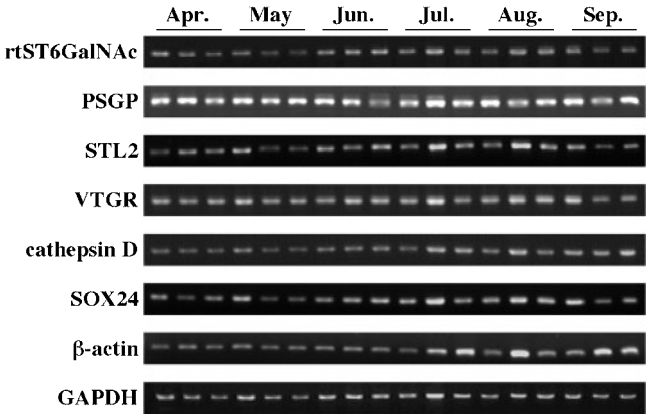

Fig. 4. **RT-PCR analysis of rtST6GalNAc mRNA expression during oogenesis.** The cDNA was prepared from ovaries of each of three animals harvested at each month from April to September, and used as a template in the subsequent semi-quantitative PCRs. The cDNA fragments were amplified through the following numbers of cycles of a step program (94°C, 30 s; 54°C, 1 min; 72°C, 2 min): 28–30 cycles for rtST6GalNAc; 27–30 cycles for PSGP, STL2, rtSOX2, cathepsin D, and VTGR; 20–22 cycles for β-actin; 25–27 cycles for GAPDH. The products were densitometrically analyzed in 1% agarose gels containing ethydium bromide. The amplification of the cDNA fragments was not saturated at the following cycle numbers, and the products of those cycles are shown in the figure: 28 cycles for rtST6GalNAc; 27 cycles for PSGP, STL2, rtSOX2, cathepsin D, and VTGR; 20 cycles for β-actin; 25 cycles for GAPDH. Photographs were taken under UV light.

lated with the amount of cytoskeletal protein, while enzymes involved in oocyte viability are constantly expressed independent of the increase in diameter or accumulation of yolk materials. We thus examined the expression levels of β-actin and GAPDH mRNAs by the RT-PCR method (Fig. 4) and measured the diameter of the oocytes. As shown in Fig. 5A, the relative ratio of β-actin to GAPDH mRNA expression is positively correlated with the diameter of the oocyte. Therefore, the relative expression level of β-actin mRNA can be a marker for oogenesis in rainbow trout ovary. In Fig. 5B, the relative expression level of β-actin mRNA is plotted as a function of the month when the oocyte was harvested. The observed changes in the β-actin mRNA from April to September coincide well with previously described morphological changes of oogenesis (*27*, *28*). Accordingly, we describe the developmental change of expression of various mRNAs as a function of month of rainbow trout oocyte harvest.

Developmental Expression of rtST6GalNAc mRNA during Oogenesis—The expression level of rtST6GalNAc mRNA (Fig. 4) relative to that of GAPDH during oogenesis is shown in Fig. 6A. The ratio of the expression of the rtST6GalNAc and GAPDH mRNAs in each month is represented as a value relative to the ratio in April, set to 1.0. The expression level of rtST6GalNAc mRNA decreased in May, then increased gradually until August, and decreased again in September. However, α2,6-sialyltransferase activity in the rainbow trout ovary during oogenesis increased constantly until September as measured using asialo-PSGP as a substrate (*9*). Thus, there is a discrepancy between the developmental expression of the mRNA for the enzyme and the developmental changes in the enzyme activity.

Developmental Expression of mRNAs for Other Proteins during Oogenesis—The developmental expression of mRNAs for glycosyltransferases during oogenesis has not been examined so far. It is generally recognized that glycosyltransferases are localized in the Golgi apparatus (*10*). It would be interesting to know whether or not the developmental expression of rtST6GalNAc mRNA during oogenesis follows the pattern typical of Golgi-localized proteins. Therefore, the expression profiles of mRNAs for other known proteins in rainbow trout oocytes were also examined (Fig. 4).

(a) PSGP: PSGP is a major glycoprotein of cortical alveoli in mature oocytes (*6*), and an acceptor substrate for rtST6GalNAc as described above. The expression of PSGP mRNA decreased in June and toward September, then gradually increased back to the expression level of April (Fig. 6B). The PSGP polypeptide is actively synthesized in April and May. This is consistent with the fact that cortical alveoli start forming and increase in number during these months (*1*). The expression profile of the mRNA for PSGP was different from that of the mRNA for rtST6GalNAc.

(b) STL2: Like PSGP, STL2 is a rhamnose-binding lectin (*29*) localized in cortical alveoli. The expression of its mRNA decreased in May, then increased to the highest level in August, and dropped again in September (Fig. 6C). This expression profile differs from that of PSGP, although both proteins are localized in cortical alveoli. This suggests that the kinetics of biosynthesis of cortical alveolus components during oogenesis vary depending on

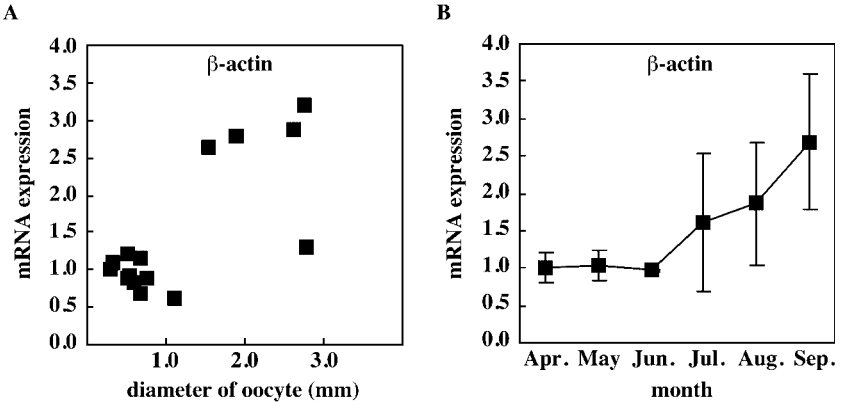

Fig. 5. **Correlation between rainbow trout β-actin mRNA expression and the increase in the diameter of oocytes during oogenesis.** A: Plots of β-actin mRNA expression against the average diameter of the same oocyte. Expression of β-actin mRNA is normalized against GAPDH mRNA. B: Developmental expression of β-actin mRNA normalized against GAPDH mRNA at each month during oogenesis. The values in A and B are relative, normalized ratios with the normalized ratio in April is set to 1.0, and shown as mean ± SEM. The standard error bar indicates the variability between values for three different animals.

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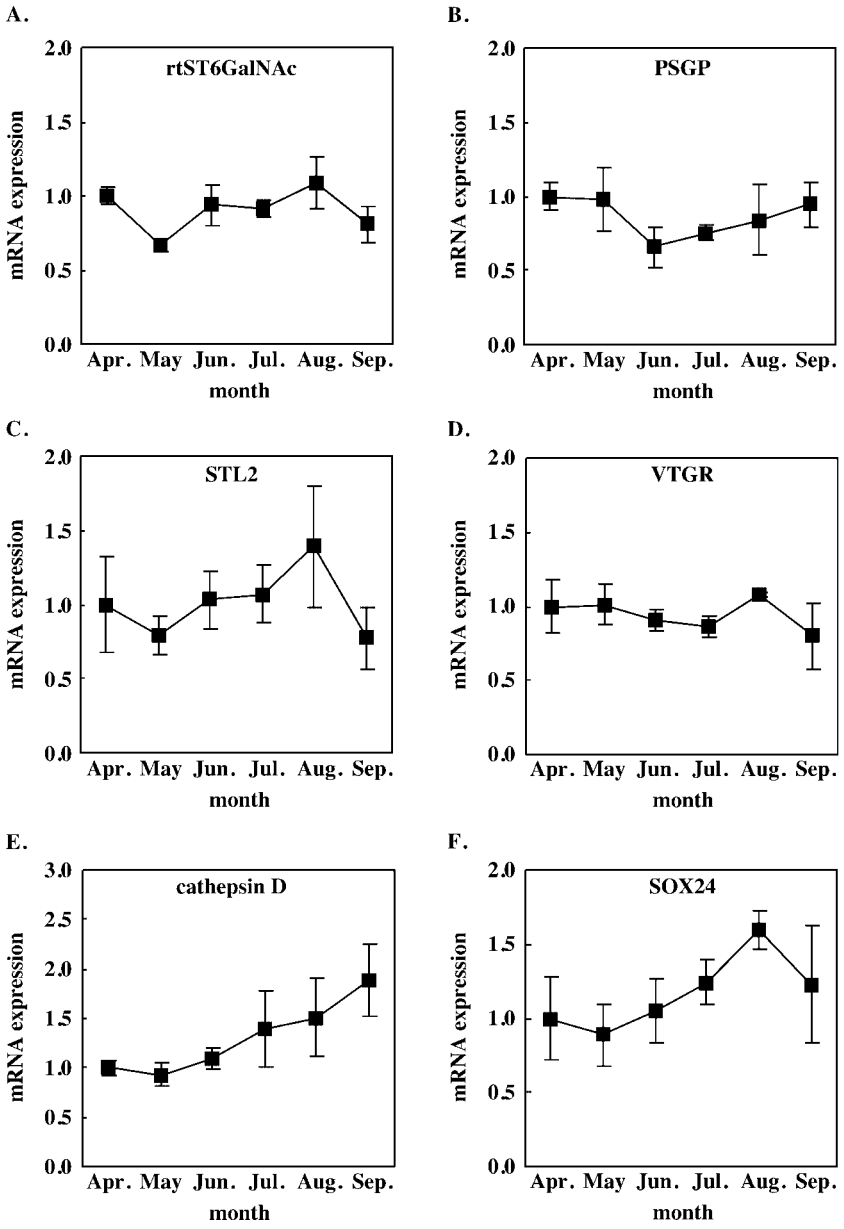

Fig. 6. **Develomental expression of mRNAs for oocyte proteins during oogenesis.** The quantity of each PCR product was determined by the densitometric method. Expression of each oocyte protein mRNA was normalized against that of GAPDH mRNA. The value for each month is expressed as the relative, normalized ratio of each protein mRNA in April, set at 1.0, and shown as means ± SEM. The standard error bar indicates the variability between values for three different animals. A, rtST6GalNAc; B, PSGP; C, STL2; D, VTGR; E, cathepsin D; and F, SOX24.

the component. It should be noted that the expression profile of the STL2 mRNA is similar to that of the rtST6GalNAc mRNA. Taken together, the down-regulation of mRNA expression in May appears to be unique to rtST6GalNAc and STL2.

(c) VTGR: Yolk formation in oocytes is an important process in oviparous animals (*2*), and includes vitellogenin accumulation and processing for storage. VTGR is localized in the plasma membrane of oocytes and mediates the uptake of vitellogenin, which is synthesized in and secreted from the liver into the blood stream (*2*). The expression levels of VTGR mRNA remained unchanged during oogenesis (Fig. 6D). This expression profile may be explained by the fact that VTGR is recycled (*30*). The expression profile of the VTGR mRNA is different from that of the rtST6GalNAc mRNA.

(d) Cathepsin D: Cathepsin D is a lysosomal endoprotease and plays an important role in the initial proteolytic processing of vitellogenin (*31*). As shown in Fig. 6E, the expression of cathepsin D mRNA starts increasing in July and has doubled by September. The time course of this increase is consistent with that of the accumulation of vitellogenin (*26*, *27*). Like VTGR, the expression profile of cathepsin D mRNA is different from that of rtST6GalNAc mRNA. The expression profiles of the mRNAs for VTGR and cathepsin D are also different from each other. This difference may be related to the fact that VTGR is recycled, while cathepsin D has to be co-localized in vast amounts with its substrate, vitellogenin, in endosomes. Notably, the timing of the increase of cathepsin D mRNA agrees with that of β-actin mRNA, suggesting that yolk formation is accompanied by cytoskeletal change.

(e) Sox24: Sox24 is a member of the SRY-type HMG box protein family (*32*), and is a cytosolic protein. The expression of Sox24 reached a maximum level (about 1.5 times

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the initial level) in August, and decreased in September (Fig. 6F). This expression profile is also different from that of rtST6GalNAc.

DISCUSSION

In this study, we focused on the expression of ST6GalNAc, which is responsible for the incorporation of the first sialic acid residue into the *O*-glycan chain of PSGP and is a constitutively expressed enzyme during oogenesis. We isolated a cDNA for GalNAc α2,6-sialyltransferase (rtST6GalNAc) from rainbow trout ovary. This is the first example of a sialyltransferase cDNA cloned in fish. The cDNA for rtST6GalNAc encodes an amino acid sequence that shares the common features of vertebrate sialyltransferases: it is a type II transmembrane protein, consisting of short N-terminal cytoplasmic tail, putative transmembrane domain, stem domain and C-terminal catalytic domain (*19*). It contains three conserved sequences, sialyl motifs L, S, and VS (*15*, *16*, *20*). Based on sequence homology to other members of the ST6GalNAc family, rtST6GalNAc is most likely to be the rainbow trout ST6GalNAc II. Previous results showed that most of the enzyme activity of rtST6GalNAc is soluble, not membrane-bound (*9*). Therefore, rtST6GalNAc is expressed as a type II membrane protein, and appears to be integrated into secretory vesicles (cortical alveoli) as a soluble form via proteolytic cleavage.

We have demonstrated that the recombinant rtST6GalNAc has GalNAc α2,6-sialyltransferase activity. Substrate specificity analysis shows that rtST6GalNAc prefers the NeuAcα2,3 Galβ1,3 GalNAcα to the Galβ1,3 GalNAcα or GalNAcα structures as a substrate. Notably, of glycoproteins used in this study, asialo-PSGP is most effectively sialylated by rtST6GalNAc. Asialo-PSGP is also an endogenous substrate. Thus, rtST6GalNAc is a good candidate for the sialyltransferase that is involved in the first sialylation step in the synthesis of the polysialic acid structure on *O*-glycan chains of PSGP.

Northern blot analysis has shown that rtST6GalNAc is expressed in various tissues, including intestine, kidney, spleen, heart, and testis, as well as ovary. The 1.9 kb transcript is ovary-specific and strongly expressed in ovary, while the 4.7 kb transcript is abundantly expressed in other tissues. Therefore, it is possible that the 4.7 kb transcript is responsible for the expression of a housekeeping ST6GalNAc that catalyzes the synthesis of mucin-like glycoproteins in these tissues. On the other hand, the 1.9 kb transcript may be involved in the sialylation of oocyte-specific glycoproteins during oogenesis. Again, PSGP is a candidate substrate that is sialylated by rtST6GalNAc, because PSGP is expressed only in the ovary as a major *O*-linked glycoprotein in cortical alveoli (*33*).

The developmental expression of mRNA for rtST6GalNAc is largely constitutive throughout oogenesis; the expression is down-regulated in May and then up-regulated until August. On the other hand, it is known that the α2,6-sialyltransferase activity gradually increases during oogenesis when the activity is assayed using asialo-PSGP as a substrate (*9*). Thus, the expression of rtST6GalNAc at the transcriptional level does not coincide with the observed increase in α2,6-sialyltransferase activity. This may be explained by assuming that not only PSGP, but also many other *O*-glycoproteins that are even more highly expressed throughout oogenesis, are substrates for rtST6GalNAc, resulting in a constant expression of the enzyme. The difference between the mRNA and enzyme activity patterns may also be due to posttranscriptional regulation of the enzyme activity in oocytes, either at the translational or posttranslational level. It is therefore interesting to note that maternal mRNAs in oocytes and embryos are often translationally regulated during oogenesis as well as during early development (*34*). Alternatively, rtST6GalNAc might be stored in oocytes, as is the case of cortical alveolus components and yolk materials. The accumulation of the enzyme in cortical alveoli is highly possible, because the enzyme activity is actually shown to be present exclusively in cortical alveoli of rainbow trout (*9*). The formation and accumulation of cortical alveoli have already started in April and continue until September, immediately before ovulation (*1*, *28*). Therefore, the enzyme should be stored once accumulated in cortical alveoli. It should be noted that STL2, a cortical alveolus lectin, shows very similar mRNA expression profiles to rtST6GalNAc, while it shows completely different profiles from other oocyte components such as plasma membranous (VTGR), lysosomal (cathepsin D), or cytosolic (SOX24) components. This suggests that rtST6GalNAc may be expressed and integrated into cortical alveoli in a similar way to STL2 during oogenesis.

Unlike rtST6GalNAc and STL2, however, another cortical alveolus component, PSGP, shows a different mRNA expression profile. The mRNA for PSGP is expressed in April and May at earlier stages of oogenesis and down-regulated at later stages. Based on previous morphological observations (*28*), April and May correspond to the appearance and growth of yolk vesicles, a precursor of cortical alveoli, in the cytoplasm. At later stages, yolk materials start entering the cytoplasm of oocytes and yolk vesicles gradually migrate to the periphery of the cytoplasm under the plasma membrane, where they are identified as cortical alveoli. This appears to be consistent with the fact that the expression of the PSGP mRNA is relatively restricted in the earlier stages of oogenesis, and the synthesis of PSGP appears to be down-regulated when yolk vesicles migrate to the periphery of oocytes. Once PSGP is translated, the sialylation of PSGP continues during the course of oogenesis after June. Sialylation usually occurs in the Golgi apparatus (*10*); however, previous results (*9*) suggest that sialylation of PSGP occurs in cortical alveoli and their precursor, yolk vesicles, during migration to the periphery of the cytoplasm, as well as in the Golgi apparatus. In this regard, it has been reported that tritium-labeled glucose is incorporated into cortical alveoli during oogenesis, and that this incorporation is not restricted to the yolk vesicle stage (*35*). This indicates that the glycosylation reaction may occur not only in the Golgi apparatus, but also at least partly in cortical alveoli in fish. In addition, the co-localization of PSGP and rtST6GalNAc in the same cortical alveoli may result in complete sialylation of a large number of *O*-glycan chains of PSGP. In fact, all *O*-glycan chains of PSGP are known to be completely sialylated without exception (*13*), which may be evidence for the long-term co-localiza-

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tion of the enzyme and substrate during oogenesis. In contrast, most mucins usually contain both sialylated and unsialylated *O*-glycan chains, even when they are massively synthesized, glycosylated in the Golgi apparatus, and secreted (*22*, *36*).

Finally, this is the first examination of the developmental expression of an mRNA for a glycosyltransferase during oogenesis. Glycosylation events during oogenesis are complex because glycosylation occurs not only on a series of proteins necessary for oogenesis, but also on proteins synthesized and stored in oocytes until fertilization to play important roles in sperm-egg interaction as well as in early development. In summary, this and previous studies suggest that the sialyltransferase that is involved in the sialylation of cortical alveolus components, *i.e.*, PSGP, possesses unique features. Most rtST6GalNAc molecules are expressed as a membrane-bound form in the Golgi, and appear to be transported into cortical alveoli by processing as a soluble form. The cortical alveolus rtST6GalNAc may be involved in the sialylation of coexisting PSGP throughout oogenesis to result in complete sialylation of PSGP. It will be interesting to see whether the unusual features of rtST6GalNAc are common to other glycosyltransferases involved in the glycosylation of cortical alveolus components. Furthermore, it will be important to determine whether the unusual features of rtST6GalNAc are common to other animals; such a study is currently underway in our laboratory using medaka fish as a model system.

We are indebted to Dr. Tsukasa Matsuda for valuable discussion and encouragement all through this work. We thank Dr. Brett E. Close and Dr. Karen J. Colley for their kind assistance wih the transfection experiments. We also thank Dr. Shuichi Tsuji for his advice and kind encouragement in the beginning of this study. This work was supported in part by Grants-in-Aid for CREST of the Japan Science and Technology Corporation, for the 21st Century COE Program, and for Scientific Research (C) (15570096).

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