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Analyses of chicken sialyltransferases related to *O*-glycosylation

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The chicken β-galactoside α2,3-sialyltransferase 1, 2, and 5 (ST3Gal1, 2, and 5) genes were cloned, and their enzymes were expressed in 293FT cells. ST3Gal1 and 2 exhibited enzymatic activities toward galactose-β1,3-*N*-acetylgalactosamine and galactose-β1,3-*N*-acetylglucosamine. ST3Gal5 only exhibited activity toward lactosylceramide. ST3Gal1 and 2 and previously cloned ST3Gal3 and 6 transferred CMP-sialic acid to asialofetuin. Reverse-transcription-quantitative PCR indicated that ST3Gal1 was expressed at higher levels in the trachea, lung, spleen, and magnum, and the strong expression of ST3Gal5 was observed in the spleen, magnum, and small and large intestines. ST3Gal1, 5, and 6 were also expressed in the tubular gland cells of the magnum, which secretes egg-white proteins. ST3Gal1, 5, and 6 were expressed in the egg chorioallantoic membrane, in which influenza viruses are propagated for the production of vaccines.

[**Key words:** Sialyltransferase; Chicken; *O*-Glycosylation; Transgenic chicken; Ganglioside]

Glycosylation plays important roles in eukaryotes, not only in the stability of proteins *in vivo*, but also in cell–cell communication and the functions of intracellular molecules such as transcription factors (1–3). *N*-linked and *O*-linked glycosylation are well-known sugar modifications to proteins, and the importance of *N*-glycosylation has been established (4).

O-linked glycosylation occurs through serine or threonine (Ser/Thr) residues. The most common types of *O*-linked glycans are mucin-type glycans, which have an initial *N*-acetylgalactosamine (GalNAc) residue linked to Ser/Thr. Other *O*-linked glycans contain *N*-acetylglucosamine (GlcNAc), xylose, galactose, fucose, or mannose as the initial sugar that binds to Ser/Thr residues (5). Accumulating evidence has indicated that mucin-type *O*-linked glycans play important roles in protein stability, processing, and function. Rifai et al. (6) demonstrated that the clearance of recombinant IgA2 was faster than that of IgA1. The main difference between the IgA1 and IgA2 subclasses is a 13-amino acid sequence in the IgA1 hinge region containing 3 to 5 *O*-linked glycan moieties that is absent in IgA2. Additionally, B cell-activating receptor 3 (BR3)-Fc has multiple *O*-linked glycosylation sites with the degree of sialylation affecting the clearance of a protein. The main reason for the rapid clearance of asialoglycoproteins appears to be the uptake of asialo-Gal or -GalNAc by lectin receptors in the liver (7).

Many therapeutics produced in the egg white are glycoproteins. The optimization of protein glycoforms is an essential technology because their biological activities occasionally depend on glycosylation. Extensive efforts have been devoted to the design and optimization of glycoforms. Important success has been achieved with designed *N*-linked glycosylation in higher eukaryotes such as plants, insects, and Chinese hamster ovary cells (8). We previously established transgenic chickens that produced useful proteins (9–12), and found that target proteins produced in the egg white lacked terminal sialic acid and galactose in their *N*-glycans (13,14). We recently succeeded in achieving the galactosylation of *N*-glycans in genetically manipulated chickens (15,16). In contrast, limited progress has been made with *O*-linked glycosylation because its regulation is complex and also poorly understood (17).

In an attempt to elucidate the molecular bases underlying the regulation of *O*-linked glycosylation in chickens, particularly sialylation, we cloned chicken sialyltransferases (STs). Three chicken STs were expressed in 293FT cells, and their enzymatic activities were identified with several substrates. We also analyzed the substrate specificities of previously reported chicken STs (18). Furthermore, the distribution of STs in chicken organs was measured by reverse-transcription-quantitative PCR (RT-qPCR).

MATERIALS AND METHODS

Materials Galactose-β1,4-*N*-acetylglucosamine (Galβ1,4GlcNAc), GM1a, and asialofetuin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Galactose-β1,3-*N*-acetylgalactosamine (Galβ1,3GalNAc) was purchased from Toronto Research Chemicals (Ontario, Canada). Galactose-β1,3-*N*-acetylglucosamine (Galβ1,3GlcNAc) was from Dextra Laboratories Ltd. (Reading, UK). Lactosylceramide was from Nagara Science Co., Ltd. (Gifu, Japan). These materials were used as sugar acceptors for the measurement of ST activity.

Cloning of ST genes Chicken β-galactoside α2,3-sialyltransferase (ST3Gal) DNAs were amplified by PCR from chicken blastodermal or brain cDNA as a template with the following primers; ST3Gal1, Dir: AAAGAATTCACCATGG TCACCGTCAGGAAAAGG and Rev: AAACTCGAGTCTGCCCTTGAAAAATTTTAT; ST3Gal2, Dir: AAAGGATCCACCATGAAGTGCTCGCTGCGCGTC and Rev: AAA CTCGAGATTGCCCCGGTACACCTCAAT; ST3Gal5, Dir: AAAGGATCCACCATGAGA AGACCAATCTGGTTT and Rev: AAACTCGAGGCTGTCTTTGTTGCAGAATTC (the

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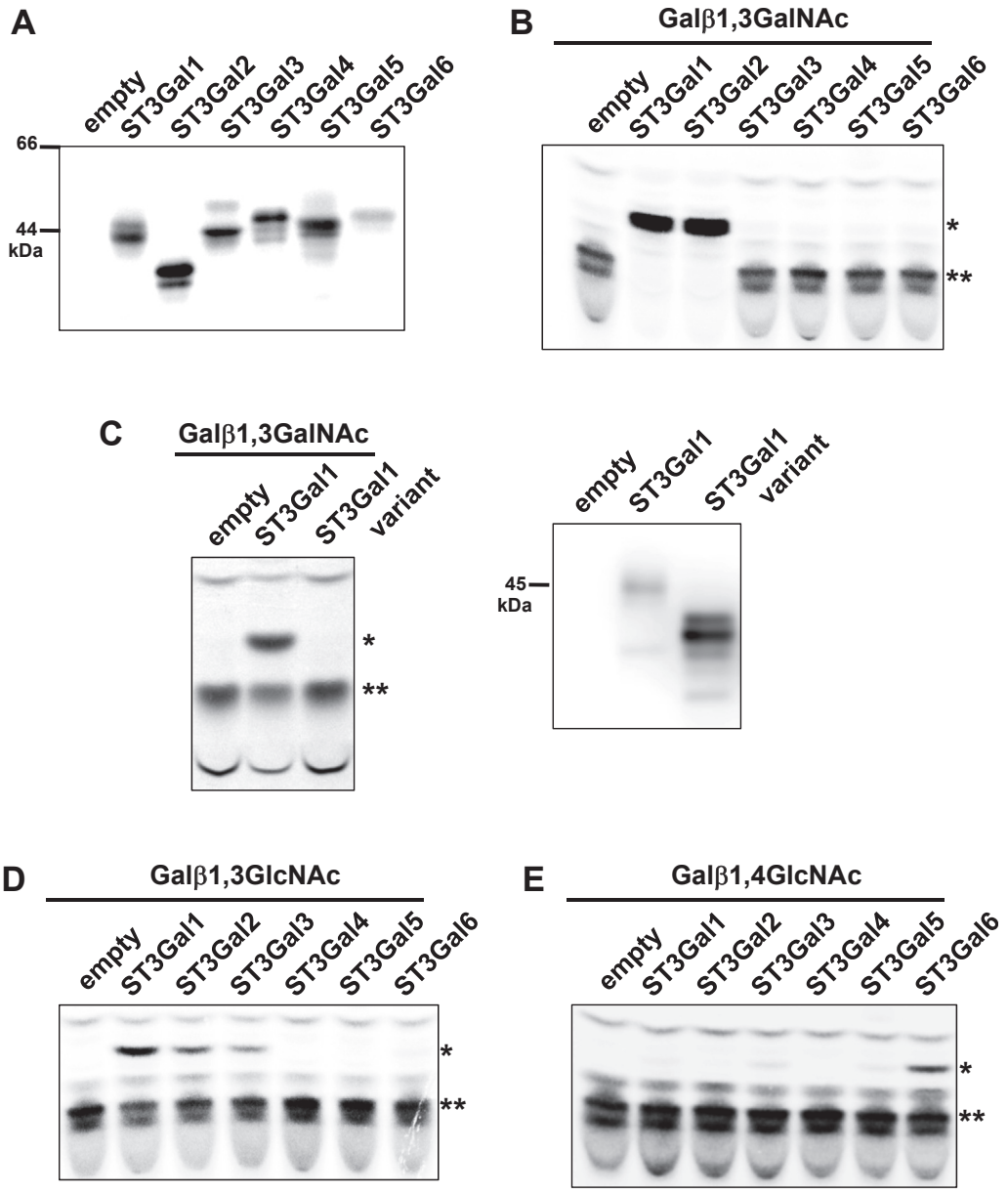

FIG. 1. *In vitro* ST activities of putative chicken STs expressed in 293FT cells. (A) Expressed STs were detected by Western blotting using an anti-His antibody. These samples were used for the enzymatic assay shown in Figs. 1B, D, E, 2A and B. (B) Recombinant chicken STs were incubated with Galβ1,3GalNAc and CMP-[^{14}C]-sialic acid as the acceptor and donor, respectively. Asterisk, NeuAcα2,3Galβ1,4GalNAc; double asterisk, CMP-sialic acid. Empty, negative control with an empty vector. (C) Enzymatic activity of deleted chicken ST3Gal1. Expression levels were also shown on the right. Assay conditions were the same as those in Fig. 1B. Asterisk, NeuAcα2,3Galβ1,4GalNAc; double asterisk, CMP-sialic acid. (D) Enzymatic activity toward Galβ1,3GlcNAc. Asterisk, NeuAcα2,3Galβ1,3GlcNAc; double asterisk, CMP-sialic acid. (E) Enzymatic activity toward Galβ1,4GlcNAc. Asterisk, NeuAcα2,3Galβ1,4GlcNAc; double asterisk, CMP-sialic acid. The typical results of three independent experiments are shown.

*Eco*RI, *Xho*I, and *Bam*HI sites are underlined). The amplified DNAs were cloned into pcDNA4/TO/Myc-HisA (Invitrogen, Carlsbad, CA, USA). Primers were designed following the putative DNA sequence data of chicken ST3Gals: ST3Gal1, NM_205217; ST3Gal2, NM_204480; and ST3Gal5, NM_001001192. We also cloned mouse ST3Gal5 as a control. DNA was amplified by PCR from the cDNA of 3T3 mouse fibroblast cells as a template with the following primers; Dir: AAAGAATTCACCATGCACACAGAGGCGGTGGGC and Rev: AAACTCGAGGTGGATGCCGCCGCTGAGGTC (the *Eco*RI and *Xho*I sites are underlined, respectively). Primers were designed following the putative DNA sequence data of mouse ST3Gal5 (NM_001035228).

Measurement of enzymatic activity 293FT cells were maintained in DMEM high glucose medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS), penicillin, and streptomycin. These cells (5×10^5 cells per 35-mm culture dish were seeded one day before) were transfected with 2.5 μg each of the expression vectors of various chicken STs (pcDNA4/TO/myc-HisA/ckST) using Lipofectamine 2000 (Invitrogen) as recommended by the supplier. Two days after being transfected, cells were harvested and suspended in phosphate-buffered saline (PBS) containing 1% Triton X-100. After sonication, cell debris was removed by centrifugation at 10,000 rpm at 4°C for 5 min, and crude cell extracts were obtained. ST activity was measured in 50 mM HEPES-NaOH (pH 6.9), 1 mg/ml of the sugar acceptor, 2.5 μCi/ml ^{14}C-labeled CMP-sialic acid (cytidine 5′-monophosphate sialic acid [sialic-6-^{14}C], American Radiolabeled Chemicals, St. Louis, MO, USA), 1 mM $MgCl_2$, 0.5% Triton X-100, and a suitable amount of the cell lysate in a total volume of 20 μl. After the overnight reaction at 37°C, 5 μl of the samples was spotted onto HPTLC silica gel 60 (10 cm × 10 cm, Merck Millipore, Darmstadt, Germany), which was developed three times with 1-butanol: ethanol: H_2O = 5:3:2 for sugar substrates, and chloroform: methanol: 0.25% KClaq = 60:35:8 for glycolipids, respectively. The plate was exposed to an X-ray film for 2 days at −80°C or an imaging plate at room temperature overnight. The expression levels of recombinant chicken STs were detected by Western blotting with a mouse anti-His antibody (MBL, Nagoya, Japan) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In order to determine enzymatic activity toward glycoprotein, 25 μg asialofetuin was incubated with the crude cell lysate at 37°C overnight in 50 mM HEPES-NaOH (pH 6.9), 0.5% Triton-X 100, and ^{14}C-

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