**A cDNA that encodes UDP-N-acetyl-D-glucosamine (GlcNAc): GlcNAcβ1-6(GlcNAcβ1-2)-Manα1-R[GlcnAc to Man]β1,4N-acetylglucosaminyltransferase VI**

From the Departments of 3Biochemistry and 4Ophthalmology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, the 5Synthetic Cellular Chemistry Laboratory and 6Division of Biomolecular Characterization, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan, and the 7Department of Molecular Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

Yoshihiro Sakamoto§§, Tomohiko Taguchi∥∥, Koichi Honke§, Hiroaki Korekaneg†, Hitoshi Watanabe†, Yasuo Tano†, Naoshi Dohmae†, Koji Takioi†, Akira Horii††, and Naoyuki Taniguchi∥∥∥ ||||

A cDNA that encodes UDP-N-acetyl-D-glucosamine (GlcNAc): GlcNAcβ1-6(GlcNAcβ1-2)-Manα1-R[GlcnAc to Man]β1,4N-acetylglucosaminyltransferase VI (GnT VI), which is responsible for the formation of pentaantennary asparagine-linked oligosaccharides (N-glycans), has been cloned from a hen oviduct cDNA library based on the partial amino acid sequences of the purified enzyme. The isolated cDNA clone contained an open reading frame encoding 464 amino acids, including all of the peptides that were sequenced. The deduced amino acid sequence predicts a type II transmembrane topology and contains two potential N-glycosylation sites. The primary structure was found to be significantly similar to human GnT IV-homologue, the gene for which was known. GnT VI catalyzes the transfer of GlcNAc to position 4 of the Manα1,6 arm of the core structure of N-glycan, forming the most highly branched pentaantennary glycans with a bisecting GlcNAc. These glycans have been found in hen ovomucoid (17, 19) and fish egg glycoprotein (19) and GnT VI activity has been demonstrated in hen oviduct (20, 21) and fish ovary (22).

In order to establish the identity of GnT-VI and obtain insight into the function of pentaantennary N-glycans, molecular cloning of this enzyme is essential. Very recently, this enzyme has been purified to apparent homogeneity from hen oviduct (23) making use of a specific assay method (21). In this report, we describe the molecular cloning of cDNA encoding chicken GnT VI on the basis of the partial amino acid sequence of the purified enzyme. The similarities in the primary structures of GnT VI and IV are presented.

**EXPERIMENTAL PROCEDURES**

**Amino Acid Sequencing of Peptides Derived from Purified GnT VI**—GnT VI was purified from hen oviduct as described previously (23). Approximately 2 μg of the purified GnT VI was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions followed by staining with Coomassie Brilliant Blue R-250. The band of 61 kDa was excised and treated with 0.1 μg of *Achromobacter protease I* (lysoylendopeptidase) (24) (a gift from Dr. T. Masaki, Ibaraki University, Ibaraki, Japan) at 37 °C for 12 h in 0.1 M Tris-HCl (pH 9.0) containing 0.1% SDS and 1 mM EDTA. The peptides generated were extracted from the gel and separated on columns of DEAE-5PW (1 × 20 mm; TSK-DEAE, Toyobo Co., Ltd., Osaka, Japan) and CAPCELL PAK C18 UG120 (1 × 20 mm; Tosoh, Tokyo, Japan) connected in series with a model 1100 (Hewlett Packard) liquid chromatography system. The peptides were eluted at a flow rate of 35 μl/min using a linear gradient of 0–60% solvent B in 96 min, where solvents A and B were 0.09% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile, respectively. Selected peptides were subjected to Edman degradation using a model Procise 494 cLC sequencer (PerkinElmer Life Sciences) and to matrix-assisted laser desorption ionization time of flight mass spectrometry on a Reflex MALD-TOF (Bruker-Franzen Analytik, Bremen, Germany) in linear mode using 2-mercaptobenzothiazole (25) as a matrix.

**Oligonucleotides and Polymerase Chain Reaction**—Hen oviduct was kindly provided by Oriental Yeast Corp. (Tokyo, Japan). Hen oviduct total RNA was extracted with TRIzol reagent (Life Technologies, Inc.). Based on the amino acid sequence of peptides derived from the purified GnT VI (Table I), degenerate oligonucleotides of both sense and antisense strands were synthesized with deoxynosinol substitution as indicated in Table II. These oligonucleotides served as primers for...
RT-PCR analysis using total RNA from hen oviduct. A reverse transcriptase reaction was performed at 42 °C for 1 h using 50 pmol of random hexamers as primer, 2 μg of total RNA, 0.5 μM each dNTP, 20 μM Tris-HCl (pH 8.4), 50 μM KCl, 5 μM MgCl2, 10 μM dithiothreitol, and 200 units of Moloney murine leukemia virus reverse transcriptase (SuperScript II RT, Life Technologies, Inc.) in a final volume of 20 μl. The reaction mixture contained 2-μl aliquots of the reverse transcriptase reaction solution, 100 pmol of each primer, 0.2 μM of each dNTP, 50 μM KCl, 10 μM Tris-HCl (pH 8.3), 1.5 μM MgCl2, and 1.25 units Taq polymerase in a final volume of 50 μl. The reaction mixtures were subjected to 50 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 10 s. After polyacrylamide gel electrophoresis of the PCR products, DNA fragments were excised and subcloned into pT7Blue vector (Novagen, WI).

Based on the sequence of the RT-PCR products using primer sets consisting of 91S2 and 91A1 or of 89S2 and 89A1, oligonucleotides of both sense (5′-TCTCCATTGTCTTCCAC-3′, termed 91S1, or 5′-TGTCATCAGTGCGACGA-3′, termed 89S1 and antisense (5′-TGTTGAGACAATGGGAGA-3′, termed 91A1, or 5′-TGCTCGACCTGATGACA-3′, termed 89A1) strands were synthesized. RT-PCR products using primer set 89S1 and 91A1 or primer set 91S1 and 91A1 were subcloned into pT7Blue vector.

Preparation of Hen Oviduct cDNA Library—Total RNA was extracted from a hen oviduct as described above. Poly(A)^+ RNA was further purified with oligo(dT)-Latex (Oligotex-dT30(Super), Roche Molecular Biochemicals). Double stranded cDNA was synthesized using a cDNA synthesis kit (ZAP-cDNA synthesis kit, Stratagene). Briefly, the first cDNA strand was synthesized by reverse transcription of the total RNA with the primer including oligo(dT) and XhoI restriction enzyme recognition site. After synthesis of the second strand, the double stranded cDNA was ligated with an XhoI-digested Uni-ZAP XR vector and subcloned in vitro (Gigapack III Gold packaging extract, Stratagene). Isolation of cDNA Clones from a Hen Oviduct cDNA Library.—Approximately 3.9 × 10^8 recombinant phages were screened by plaque hybridization with a digoxigenin-labeled DNA probe that had been synthesized using a digoxigenin PCR probe synthesis kit (Roche Molecular Biochemicals) with primer sets of 89S2 and 91A1. Hybridization was carried out at 42 °C overnight with a nylon membrane (Roche Molecular Biochemicals) with primer sets of 89S2 and 91A1. The subcloned DNAs were sequenced by the dideoxy chain termination method using Taq DNA polymerase (Big Dye Taq) and Xenol restriction enzyme digestion. DNA Sequencing.—The subcloned DNAs were sequenced by the dyeoxy chain termination method using Taq DNA polymerase (Big Dye Terminator cycle sequencing kit, PerkinElmer Life Sciences) with a DNA sequencer (Applied Biosystems model 377).

Expression of GnT VI cDNA in COS-1 Cells—pBS-GnT VI was digested with EcoRI and XhoI, and the inserted DNA was ligated between the EcoRI and XhoI sites of the expression vector pSVK5 (Amersham Pharmacia Biotech) (termed pSV-GnT VI). pBS-human GnT IV-homologue (pBS-hGnT IVh) (27) was digested with XbaI and XhoI, and the inserted DNA was ligated between the XbaI and XhoI sites of pSVK5 (pSV-hGnT IVhR). Because pSV-hGnT IVhR contained an oppositely directed hGnT IVh against the SV40 promoter, it was digested with SstI and religated to obtain a clone containing an appropriate-directed hGnT IVh (pSV-hGnT IVh). COS-1 cells (1 × 10^6) precultured for a day in a 100-mm-diameter dish were transfected by lipofection with 2 μg of plasmid DNA, 8 μl of PLUS reagent, and 12 μl of LipofectAMINE (Life Technologies, Inc.). After 72 h, the cells were washed twice with 2 ml of cold phosphate-buffered saline and then harvested with 0.1 ml of phosphate-buffered saline using a silicon scraper. The cells were sonicated in ice and assayed for various GlcNAc transferase activities and protein concentration (BCA protein assay kit, Pierce) using bovine serum albumin as a standard. GnT III, IV, V, and VI activities were assayed as described previously (21, 26).

Northern Blot Analysis—Four μg of poly(A)^+ RNA from hen oviduct was denatured in 50% (v/v) formamide, 17.5% (v/v) formaldehyde, 20 mM MOPS (pH 7.0) at 65 °C, electrophoresed in a 1% agarose gel containing 6% formaldehyde, and then transferred to a nylon membrane (Roche Molecular Biochemicals). A digoxigenin-labeled DNA probe was synthesized using a digoxigenin PCR probe synthesis kit (Roche Molecular Biochemicals) with 5′-ATGCGGTGCTTCCGGCGA-3′ and 5′-GGTCCGGCTGCTCAAAGA-3′ as primers and pBS-GnT VI as template. The membrane was hybridized with the DNA probe at 50 °C. A digoxigenin-labeled RNA molecular weight marker II (Roche Molecular Biochemicals) was used as a size marker. Other methods were the same as those used for the plaque hybridization.

RESULTS

Amino Acid Sequence Determination and Preparation of cDNA Probe by Polymerase Chain Reaction—To determine a partial amino acid sequence, purified GnT VI was digested with lysylendopeptidase, and the resultant peptides were isolated on a reversed-phase high performance liquid chromatography.
Amino acid sequences determined for five peptides are shown in Table I. P93 was the fragment of P89 completely digested with lysylendopeptidase. Based on the amino acid sequences of P89 and P91, we synthesized degenerate oligonucleotides for sense and antisense primers (Table II). In order to reduce the primer combinations, deoxyinosine was substituted in positions where the codon degeneracy exceeded 2, and for each serine, arginine, or leucine residue, we prepared primer combinations of two codon types as shown in Table II. Oligonucleotides 91S1, 91S2, and 91A1–91A4 were synthesized on the basis of the amino-terminal and carboxyl-terminal sequences of P91. Eight possible pairs of sense and antisense primers were designed for each primer combination (Table II).

Fig. 1. Nucleotide and deduced amino acid sequences of chicken GnT VI and hydropathy plot of the protein. A, the predicted amino acid sequence is indicated by the single letter amino acid code below the nucleotide sequence. The positions of the five peptide sequences obtained by digestion of the purified GnT VI are underlined with a single continuous line. Asterisks indicate potential N-glycosylation sites. The putative transmembrane hydrophobic domain is underlined with a double continuous line.

B, the hydropathy plot was calculated by the method of Kyte and Doolittle (34) with a window of 11 amino acids.
primers were first employed in RT-PCR analysis using total RNA from hen oviduct as the template. The pair of 91S2 and 91A1 primer sets produced a cDNA fragment of 80 bp, corresponding to the length estimated from the amino acid sequence of P91. Similarly, oligonucleotides 89S1, 89S2, and 89A1–89A4 were synthesized on the basis of the peptide sequence of P89. Eight possible pairs of sense and antisense primers were used in RT-PCR analysis, and the pair of 89S2 and 89A1 primer sets produced a cDNA fragment of 71 bp, corresponding to the length estimated from the amino acid sequence of P89. When the fragments were subcloned and sequenced, the deduced amino acid sequences coincided with that of P91 and P89. Then, the sense (91SP and 89SP) and antisense (91AP and 89AP) primers based on nucleotide sequences of subcloned PCR fragments were synthesized. When we performed RT-PCR using 89SP and 91AP as a primer, a product of 850 bp was amplified. Sequencing analysis revealed that the 850-bp fragment included the sequence encoding P25, suggesting that this fragment was a part of the target gene.

**Screening of Hen Oviduct cDNA Library**—

A ZAP hen oviduct cDNA library was constructed from poly(A)+ RNA isolated from the oviduct. The library was screened by colony hybridization using cDNA clones as probes.

**Fig. 2.** Alignment of the predicted amino acid sequences of chicken GnT VI (galGnT-VI), human GnT IV-homologue (hGnT-IVh), bovine GnT IV (bosGnT-IV), human GnT IVa (hGnT-IVa), and human GnT IVb (hGnT-IVb). Sequence alignment was carried out with the Clustal W program, version 1.7. Asterisks, colons, and periods below the sequences indicate positions where all sequences have identical, strongly related, and weakly related residues, respectively.
duct cDNA library was screened using a digoxigenin-labeled DNA probe synthesized from the 850-bp cDNA fragment described above. A positive clone was isolated from $3.9 \times 10^5$ plaques, and in vitro excision of pBluescript phagemid was carried out to yield a plasmid pBS-GnT VI. The nucleotide sequence of its cDNA insert (2.1 kilobases) was determined (Fig. 1).

**cDNA and Predicted Protein Sequence of the GnT VI**—As shown in Fig. 1A, the cDNA consisted of 2046 nucleotides with a putative initiator codon at 219 and a TGA stop codon at 2161, having an open reading frame encoding 464 amino acid residues with a molecular mass of 52,817 Da. The deduced amino acid sequence included all of the peptide residues obtained from the digest of the purified protein. Because there is only one in-frame ATG codon upstream from the sequence of the P93 peptide, which is the most upstream peptide obtained, in the open reading frame, we identified the initiation codon a priori. A putative polyadenylation signal was located at nucleotide 2026 followed by poly(A) tracts. The 2065-bp cDNA length is highly consistent with the mRNA size observed in hen oviduct (Fig. 3), indicating that the cDNA is nearly full-length. The deduced amino acid sequence contained two potential N-linked glycosylation sites (Fig. 1). If the enzyme protein possesses two N-linked oligosaccharide chains, the molecular mass will agree with that of the purified protein, which is 60 kDa, observed on SDS-polyacrylamide gel electrophoresis under reducing conditions (23). A hydropathy plot analysis revealed one prominent hydrophobic segment with cationic borders in the amino-terminal region (Fig. 1B), predicting that this protein has type II transmembrane topology, as has been the case for most glycosyltransferases cloned to date.

A homology search of the predicted protein revealed a significant homology with human GnT IV-homologue (27), bovine GnT IV (11), human GnT IVa (12), and human GnT IVb (28) (Fig. 2). The human GnT IV-homologue gene was identified in the region commonly deleted in pancreatic cancer, but its function is unknown (27). GnT VI is more closely related to human GnT IV-homologue (approximately 50% identity) than to bovine GnT IV, human GnT IVa, or human GnT IVb (25% identity) (Fig. 2). This finding prompted us to examine whether GnT IV-homologue is a human counterpart of chicken GnT VI.

**Overexpression of Chicken GnT VI and Human GnT IV-homologue cDNAs in COS-1 Cells**—To confirm that the isolated cDNA clone actually encodes GnT VI and to investigate whether the human GnT IV-homologue has GnT VI activity, the cloned cDNA and human GnT IV-homologue cDNA were inserted into a mammalian expression vector, pSVK3, and overexpressed in COS-1 cells. As shown in Table III, COS-1 cells transfected with the pSV-GnT VI showed GnT VI activity, whereas those transfected with pSV-hGnT IVh or pSVK3 alone had no GnT VI activity. Neither pSV-GnT VI-transfected nor pSV-hGnT IVh-transfected cells showed GnT III and V activities (data not shown). These results indicate that the cloned cDNA encodes GnT VI, whereas the human GnT IV-homolog does not.

**Expression of the GnT VI Gene in Various Chicken Tissues**—Northern blot of poly(A)+ RNA from hen oviduct was hybridized with a digoxigenin-labeled DNA probe made from the GnT VI cDNA. As shown in Fig. 3, a transcript of 2.1 kilobases was observed in hen oviduct. Because we could detect GnT VI transcripts only when we used 4 μg of poly(A)+ RNA from hen oviduct, the amount of GnT VI mRNA in the tissues seemed to be very small. Therefore, we examined expression of the GnT VI gene in various chicken tissues by RT-PCR analysis. To avoid artifacts during these analyses, we performed PCR toward three parts of GnT VI cDNA and compared the results.

| Transfected plasmid | GnT VI activity | GnT IV activity |
|---------------------|----------------|----------------|
| No plasmid          | ND*            | ND*            |
| Mock control plasmid| ND             | ND             |
| pSV-GnT VI          | 26.8           | ND             |
| pSV-hGnT IVh        | ND             | ND             |

* ND, not detected.

**FIG. 3.** Expression of the GnT VI gene in various chicken tissues. a, Northern blot of GnT VI transcripts. Four μg of poly(A)+ RNA from hen oviduct was electrophoresed, blotted, and hybridized with a digoxigenin-labeled (+) strand RNA probe made from the full-length chicken GnT VI cDNA. The positions of size markers are shown at the left. The arrow indicates the position of GnT VI mRNA. b, RT-PCR analysis of GnT VI mRNAs. Five μg of total RNA from indicated organs was reverse-transcribed with random hexamers as a primer. The resulting cDNAs were separately amplified by PCR using three sets of primers: 5'-ACCGTCAACCGACTGGA-3' (nucleotides 1137–1153 in Fig. 1A) and 5'-GGTGCCTGACTGTTCTGC-3' (nucleotides 1594–1610) (row A); 5'-ATCCGGTGTCTCCCGGA-3' (nucleotides 219–235) and 5'-TCGCCAGTGAACCA-3' (nucleotides 490–506) (row B); and 5'-AGCCGAAGGCTCTCCAACG-3' (nucleotides 1041–1057) (row C). The PCR products were separately electrophoresed and visualized with ethidium bromide. The observed PCR products showed the predicted sizes of 474 (row A), 288 (row B), and 340 bp (row C). Lane 1, oviduct; lane 2, heart; lane 3, spleen; lane 4, brain; lane 5, lung; lane 6, colon; lane 7, liver.

All the data consistently showed that GnT VI mRNA was relatively highly expressed in oviduct, spleen, lung, and colon.

**DISCUSSION**

We have cloned a cDNA that encodes chicken GnT VI. Several lines of evidence indicate that the cloned cDNA corresponds to the GnT-VI previously purified from hen oviduct (23): (a) the predicted sequence of the protein contains all five peptides obtained from the purified enzyme protein; (b) when the cDNA was introduced into the eukaryotic expression vector and transfected into COS-1 cells, the enzyme activity was highly expressed; and (c) the characteristics of the predicted protein are consistent with those of the purified protein in terms of molecular mass and membrane localization.

Among N-acetylgalactosaminyltransferases acting on the branch formation of the N-glycan core structure, GnTs I–V have been already cloned (4, 5, 7, 8, 11, 15, 16). No homologies have been found in primary structures of those GnTs except that GnT IV has isoenzymes (28). In the present study, we found that GnT VI has a significant sequence similarity to human GnT IV-homologue (hGnT IVh) (27) and to human and bovine GnT IVs (11, 12, 28). hGnT IVh appears to be more closely related to GnT VI than to GnT IV. Because it has not been determined whether hGnT IVh is a functional GlcNAc transferase (27), we overexpressed hGnT IVh cDNA in COS-1 cells and measured various GlcNAc transferase activities. Recombinant hGnT IVh showed neither GnT IV nor GnT VI.
activity. In addition, hGnT IVh had no GnT III and GnT V activity, and its function remains unknown. It appears reasonable that GnT IV and VI have a similarity in primary structure, because both generate a β1,4-linked GlcNAc on a Man residue. Because the recombinant GnT VI had no GnT IV activity (present study) and GnT IV had no GnT VI activity (28), the non-homologous domains could be important for the recognition of acceptor structures. In any event, we have detected a diverged β-4 GlcNAc transferase family. It is interesting to determine whether there are other such members. It is also of interest that only GnT III has a unique evolutionary origin among Man β-4 GlcNAc transferases.

The GnT VI gene was relatively highly expressed in hen oviduct, spleen, lung, and colon. GnT VI activity in chicken tissues was, however, reported to be high in hen oviduct, low but significant in liver and colon, and absent in heart and spleen (20). Although the reason for the discrepancy between the amount of mRNA and enzymatic activity is unknown, translational regulation and posttranslational modification may be responsible.

Pentaantennary N-glycans have not as yet been found in mammalian tissues. This is consistent with the lack of GnT VI activity in several mammal species (20). To determine whether such pentaantennary structures are really absent in mammals, more sensitive and specific assay methods (e.g. immunochemical ones) should be developed. Studies on the presence of a GnT VI gene and its expression in mammals could prove useful. Multiantennary structures generated by GnT IV and GnT V have been associated with phenotypic changes in malignant transformation (29, 30). Bulky multiantennary N-linked glycan chains are also thought to be involved in communication between cells in conditions relating to fertility (31), receptor function (32), and immune responses (33). Our isolation of the cDNA of GnT VI enabled us to investigate the expression of this gene in various tissues of diverse species. Such studies and experimental remodeling of GnT VI expression may clarify the physiological role of highly branched N-glycans in various biological processes.

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