Glycoconjugate Journal (1995) **12**: 824–828

Cloning and expression of a porcine UDP-GalNAc: polypeptide *N*-acetylgalactosaminyl transferase

ARUTO YOSHIDA, TOMOKA HARA, HIROSHI IKENAGA and MAKOTO TAKEUCHI

Central Laboratories for Key Technology, Kirin Brewery Co., Ltd, Fukuura, Kanazawa-ku, Yokohama 236, Japan

Received 7 April 1995, revised 2 June 1995

By employing a bovine UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyl transferase (O-GalNAc transferase) cDNA as a probe, we isolated four overlapping cDNAs from a porcine lung cDNA library. Both the nucleotide sequence of the porcine cDNA and the predicted primary structure of the protein (559 amino acids) proved to be very similar to those of the bovine enzyme (95% and 99% identity, respectively). Transient expression of the clone in COS-7 cells, followed by enzymatic activity assays, demonstrated that this cDNA sequence encodes a porcine O-GalNAc transferase. The intracellular O-GalNAc transferase activity was increased approximately 100-fold by transfecting cells with the porcine cDNA.

Keywords: UDP-*N*-acetylgalactosamine; polypeptide *N*-acetyl galactosaminyl transferase, glycosyltransferase, O-linked sugars, mucin, porcine

Abbreviations: O-GalNAc transferase, UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyl-transferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GnT-III, UDP-*N*-acetylglucosamine: β-mannoside β-1,4 *N*-acetylglucosaminyltransferase III.

Introduction

There are a number of reports indicating functional roles of mucin type sugar chains on glycoproteins [1] e.g. as carriers of blood-type antigen in self non-self recognition, as ligands for selectins in recruiting lymphocytes at inflammatory sites [2], and as ligands for cell to cell interaction in metastasis [3]. Several bioactive soluble proteins, like cytokines, also have mucin type sugar chains. It has been suggested that mucin type sugar chains have a functional role as an extension of the protein portion [4], but which may be less remarkable than that of Asn-linked sugar chains [5].

UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyl transferase (O-GalNAc transferase) initiates the biosynthesis of mucin type sugar chains on glycoproteins. This enzyme was first purified by Sugiura *et al.* in 1982 from the ascitic tumour cell AH66 [6]. Then, Takeuchi *et al.* [7], Elhammer *et al.* [8] and Wang *et al.* [9] purified the enzyme from bovine mammary gland, bovine colostrum, and porcine submaxillary gland, respectively. Recently, two groups have isolated the bovine O-GalNAc transferase gene [10, 11], and several groups have described the substrate specificity of the O-GalNAc transferase [12–14], making that enzyme a major topic in the glycobiology field.

We have described a function of mucin type sugar chains in casein micelle formation [15], and several functions of Asn-linked sugar chains in the bioactivity of human erythropoietin [16, 17]. It would be of great interest to determine the functions of sugar chains for other proteins. This requires detailed information about peptide sequences which affect glycosylation. However, to design and produce mucin type glycoproteins, we must first obtain significant quantities of O-GalNAc transferase. In this paper, molecular cloning of porcine lung O-GalNAc transferase cDNA and its expression in COS-7 cells are described.

Materials and methods

PCR amplification of bovine O-GalNAc cDNA

Bovine small intestine cDNA (Clontech) was used as a template to generate bovine O-GalNAc transferase by using the PCR reaction. The cDNA segment was amplified by using the sense primer 5′-TTCTGCAGCCATGAGAAAATTTGCATACTGC-3′ and the antisense primer 5′-ATGGTACCTCAGAATATTTCTGGAAGGGTGA-3′ [10]. PCR was carried out for 35 cycles (1 min at 94 °C, 1 min at 45 °C, and 2 min 72 °C). Amplification was performed using Perfect Match™ polymerase enhancer (Stratagene) and Taq polymerase (Perkin-Elmer) in the supplied buffer. After amplification, the cDNA was cloned into pUC18 using SureClone Ligation Kit (Pharmacia).

Screening of porcine cDNA library

A porcine lung cDNA library (Clontech) was probed with the N-terminal part of the bovine O-GalANc transferase gene at a reduced hybridization stringency. The probe was approximately 1.2 kb long and was labelled by using the random primer labelling kit Ver. 2 (Takara Shuzo). Hybridization was carried out in 5 × SSPE/5 × Denhardt's solution/0.5% SDS at 55 °C. The filters were washed in 2 × SSPE/0.1% SDS at 55 °C [18].

DNA sequencing analysis

DNA sequencing was carried out using the AmpliTaq DyePrimer Cycle sequencing kit (Applied Biosystems) and an Applied Biosystems model 373A sequencer.

Transient expression of bovine or porcine O-GalNAc transferase in COS-7 cells

The porcine and bovine cDNAs encoding the putative open reading frame of O-GalNAc transferase were ligated into the plasmid expression vector pSVL (Pharmacia). COS-7 cells (RIKEN Cell Bank, Tsukuba Science City, Japan) were transfected with the pSVL alone or with pSVL containing porcine or bovine cDNA by electroporation. Approximately 5×10^6 cells in 0.8 ml of PBS(−) (Nissui Pharmaceutical) were transferred to a 0.4 cm cuvette and mixed with 10 μg of plasmid DNA. Electroporation was performed at room temperature at 1600 V and 25 μF by using a Gene Pulser apparatus (Bio-Rad). The cells were then transferred at 90 mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After a 67 h incubation at 37 °C, the cells were rinsed with PBS(−), harvested by scraping, washed again with PBS(−), and centrifuged to pellet the cells. After the PBS(−) had been aspirated, the cell pellet was resuspended in 50 mM imidazole-HCl(pH 7.2), 10 mM $MnCl_2$, 0.5% Triton X-100 and disrupted by sonication. The lysates were centrifuged and the supernatant was assayed for O-GalNAc transferase activity.

The plasmid pCH110 (Pharmacia), containing a β-galactosidase gene, was transfected to measure the transfection efficiency, as described above. The cells were fixed in 0.25% glutaraldehyde and stained in 0.05% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ at 37 °C for several hours.

O-GalNAc transferase enzymatic assays

Enzymatic assays for O-GalNAc transferase were performed as described by Elhammer and Kornfeld [19].

Results and discussion

A porcine lung cDNA library was screened using the bovine O-GalNAc transferase cDNA as a hybridization probe. Four overlapping O-GalNAc transferase cDNA phage clones, λPLOT-1, λPLOT-2, λPLOT-3, and λPLOT-4, were isolated after screening approximately 8.0×10^5 recombinant phages. Restriction digestion and nucleotide sequence analysis of these clones revealed that they were overlapping and covered a total length of approximately 2.8 kb (Fig. 1).

The sequence data obtained from four λ clones were used to construct a full-length cDNA, whose complete sequence is shown in Fig. 2. The final construct contains a 1677 bp open reading frame and 5′ and 3′ untranslated regions of 604 bp and 517 bp, respectively. The putative initiation site is the ATG at position 605, which is preceded by a relatively long 5′ untranslated region, and is nine bases downstream of the TAA stop codon at 596. A polyadenylation signal, having the consensus sequence AATAAA, is located at position 2722 in the 3′ untranslated region. The open reading frame encodes a 559 amino acid protein with a calculated molecular mass of 64 115 Da. Hydrophobicity analysis [20] of the amino acid sequence indicated a putative signal-anchor domain near the N-terminus, amino acids 9–26, as expected for a type II membrane protein similar to other lumenal Golgi glycosyltransferases [21]. There are three potential N-glycosylation sites having the consensus sequence N-X-S/T.

The nucleotide sequence and the predicted amino acid sequence of the porcine O-GalNAc transferase were compared with those of the bovine enzyme. Both sequences were found to be very similar, with 95% and 99% identity, respectively [10]. Unlike other glycosyltransferase families that generally have species variations in the cytoplasmic tails and/or the stem regions, all four amino acid substitutions for the two O-GalNAc trans-

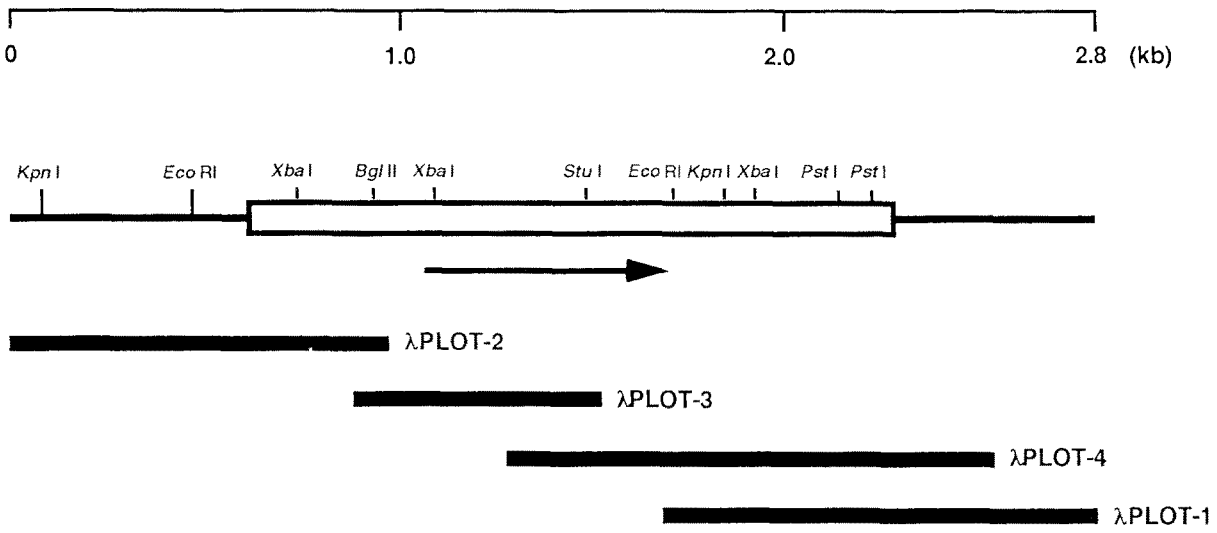

Figure 1. Restriction map of the porcine O-GalNAc transferase cDNA clones. The protein coding region of the O-GalNAc transferase and the noncoding regions are represented by an open box and thin lines, respectively. The arrow indicates the direction of transcription. Thick lines indicate the insert regions of phage clones.

ferases occurred in the catalytic domain [21]. These amino acid substitutions may cause subtle differences between the kinetic parameters of the bovine and porcine enzymes. Investigation of the significance of these differences may further our understanding of the O-GalNAc transferase aiding future protein engineering studies.

To determine if the open reading frame encodes a functional O-GalNAc transferase, the putative coding region was subcloned into the mammalian expression vector pSVL and tested in transfection experiments. Plasmids, containing the porcine or bovine cDNA, were transfected into COS-7 cells. Identical cultures of cells were transfected with the vector alone as a control. The cells were harvested after 67 h and assayed for O-GalNAc transferase activity using apomucin as an acceptor. O-GalNAc transferase activity of the cells transfected with the porcine cDNA was five-fold higher than the cells that received vector alone (Fig. 3), indicating that this cDNA sequence encodes a porcine O-GalNAc transferase. By taking into consideration the transfection efficiency, determined by transfection with pCH110, the intracellular O-GalNAc transferase activity per cell was estimated to be increased about 100-fold. In the case of bovine cDNA, the increase of intracellular O-GalNAc transferase activity was estimated to be about 180-fold. These expression levels of O-GalNAc transferase are relatively low. Transient expression levels in COS cells generally increase several thousand-fold; Nishikawa *et al.* reported that GnT-III activity of COS-1 cells transfected with rat GnT-III cDNA was increased approximately 500- to 3600-fold regardless of transfection efficiency [22]. Whether a specific control mechanism suppresses O-GalNAc transferase activity in COS-7 cells remains to be determined.

The purification of O-GalNAc transferase from porcine submaxillary glands has been described by Wang *et al.* [9] who by using N-glycanase digestion, have shown that the O-GalNAc transferase is a glycoprotein with N-linked oligosaccharides. This observation is consistent with the existence of three potential N-glycosylation sites in the amino acid sequence. Translation of the open reading frame encoded by the O-GalNAc transferase cDNA predicts a protein of about 64 kDa. This is larger than the size observed for affinity purified porcine submaxillary gland O-GalNAc transferase, which appears as a band of 60 kDa on SDS-PAGE after N-glycanase digestion. This difference may be caused by proteolysis of the submaxillary enzyme during purification. A similar difference between calculated and observed molecular mass has been detected in rat liver β-galactoside α2,6-sialyltransferase, suggesting the proteolytic removal of the N-terminal membrane domain [23]. The cleavage of the intact enzyme to soluble, lower molecular mass forms may reflect a normal proteolytic event that allows the enzyme to be released from cells, since soluble O-GalNAc transferase has been detected in bovine colostrum [8].

In conclusion, we have isolated a cDNA containing the entire O-GalNAc transferase coding sequence that expresses a catalytically active enzyme after transient transfection into COS-7 cells. Stable transfectants of several cell lines are now being prepared to be used in experiments designed to test the biological influence of O-GalNAc transferase in cells.

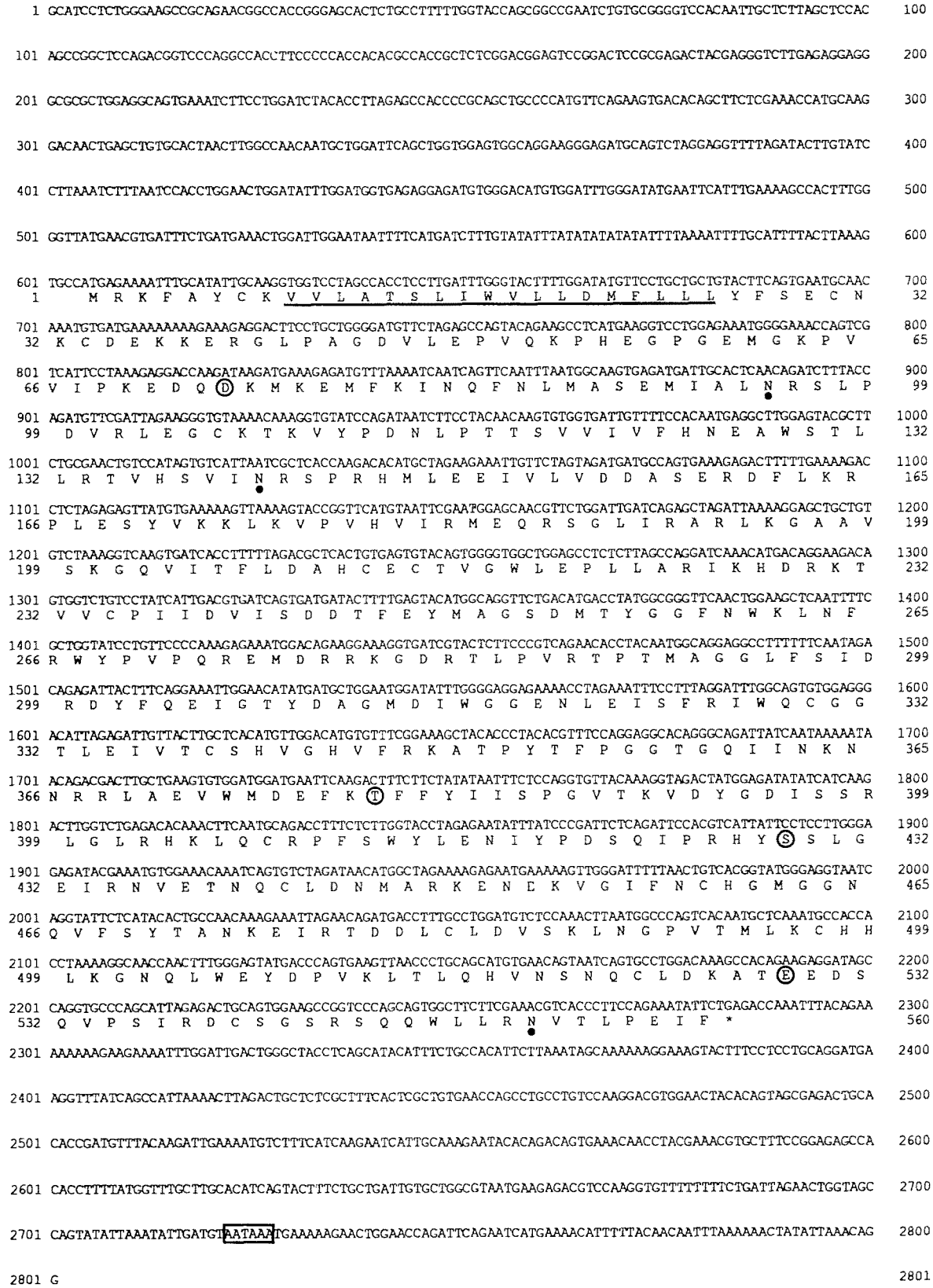

Figure 2. Nucleotide sequence and deduced amino acid sequence of O-GalNAc transferase cDNA from porcine lung. The deduced amino acid sequence of O-GalNAc transferase is given below its nucleotide sequence. The putative signal-anchor domain is underlined. The closed circles under amino acid residues indicate potential N-glycosylation sites (Asn-X-Ser/Thr). Circled amino acid residues represent non conserved residues, when compared with the bovine sequence. The putative polyadenylation signal AATAAA is boxed.

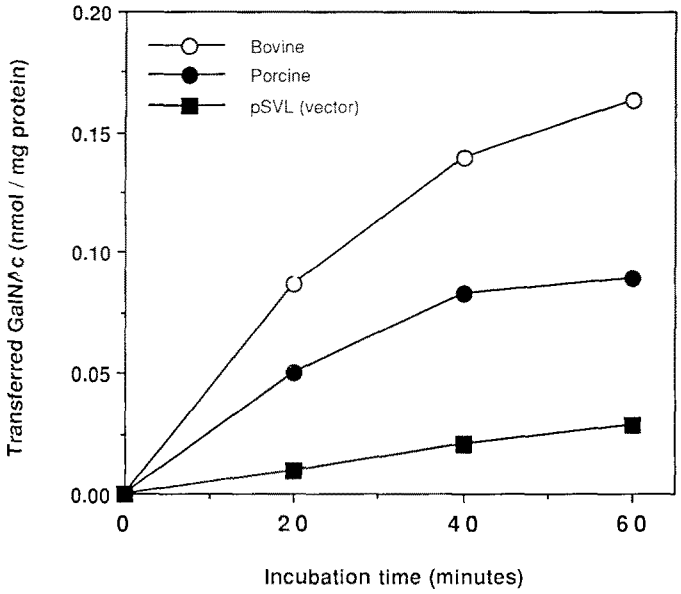

Figure 3. O-GalNAc transferase assay of COS-7 cells transiently expressing O-GalNAc transferase cDNA from porcine lung or bovine small intestine. Cell lysates were assayed as described in Materials and methods.

Acknowledgements

This work was performed as a part of the Research and Development Projects of Industrial Science and Technology Frontier Program supported by NEDO (New Energy and Industrial Technology Development Organization). We thank Dr Stefano Ferri for critical reading of the manuscript.

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