# β1-4N-Acetylgalactosaminyltransferase can synthesize both asialo-glycosphingolipid $G_{M2}$ and glycosphingolipid $G_{M2}$ *in vitro* and *in vivo*: isolation and characterization of a β1-4N-acetylgalactosaminyltransferase cDNA clone from rat ascites hepatoma cell line AH7974F

Kazuya I.-P. Jwa HIDARI,* Shinichi ICHIKAWA,* Koichi FURUKAWA,† Masahiko YAMASAKI‡ and Yoshio HIRABAYASHI*§

*Laboratory for Glyco Cell Biology, Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), 2-1, Hirosawa, Wako-shi, Saitama 351-01, †the Department of Oncology, Nagasaki University School of Medicine, Sakamoto, Nagasaki 852, and ‡Section No. 2, Development Center, Konica Corporation, 1, Sakura-cho, Hino-shi, Tokyo 191, Japan

We have cloned a cDNA encoding β1-4N-acetylgalactosaminyltransferase (EC 2.4.1.92) (GalNAc-T) from rat ascites hepatoma of the free-cell type AH7974F. The cell line only expressed asialo-series glycosphingolipids (GSLs) including asialo-$G_{M2}$ [Taki, T., Hirabayashi, Y., Ishiwata, Y., Matsumoto, M., and Kojima, K. (1979) Biochim. Biophys. Acta **572**, 113–120]. The cDNA, pGNA56, was isolated by screening AH7974F cDNA library in λgt10 with a probe. The probe was obtained from AH7974F cDNA by PCR using primers with the nucleotide sequence of the human GalNAc-T cDNA. The amino acid sequence deduced from the nucleotide sequence of pGNA56 exhibited 88% similarity to the human GalNAc-T sequence. The enzyme was a typical type II membrane protein, which consisted of a short N-terminal residue, a transmembrane region, and a long C-terminal residue, including the catalytic domain. The substrate specificity of rat GalNAc-T was determined using homogenates from cells into which the cDNA clone was transfected. The enzyme catalysed not only the formation of $G_{M2}$ and $G_{D2}$ from $G_{M3}$ and $G_{D3}$ respectively, but also asialo-$G_{M2}$ from CDH. It also acted on GSL substrates, including $G_{M1b}$, sialylparagloboside and $G_{D1\alpha}$. On the other hand, the enzyme did not transfer GalNAc to soluble substrates such as glycoproteins and oligosaccharide. The GSL compositional and immunocytochemical analyses of stable transfectants obtained by transfection of the cDNA showed simultaneous expression of asialo-$G_{M2}$ and $G_{M2}$ on the plasma membrane. Therefore, we concluded that the formation of asialo-$G_{M2}$, $G_{M2}$ and $G_{D2}$ was catalysed by the single GalNAc-T. Northern-blot hybridization showed that the GalNAc-T mRNA was strongly expressed in rat brain, testis, and spleen. The gene was also expressed in rat normal liver to a lesser extent. We found the GSLs in asialo- and α-pathways such as asialo-$G_{M1}$ and $G_{D1\alpha}$ in the rat tissues by using a sensitive t.l.c.–immunostaining method. These observations also supported our conclusion that the single GalNAc-T synthesizes asialo-$G_{M2}$, $G_{M2}$ and $G_{D2}$ *in vivo*.

## INTRODUCTION

It is well known that glycosphingolipids (GSLs) are ubiquitous components of the eukaryotic plasma membrane. More than 300 molecular species of GSL have been identified on the basis of the diversity of their carbohydrate structures. They have been reported as tumour antigens and regional and temporal markers in early embryogenesis, and are suggested to play essential roles in cellular phenomena such as cell–cell interaction, differentiation, and signal transduction through receptors (for reviews, see [2–5]).

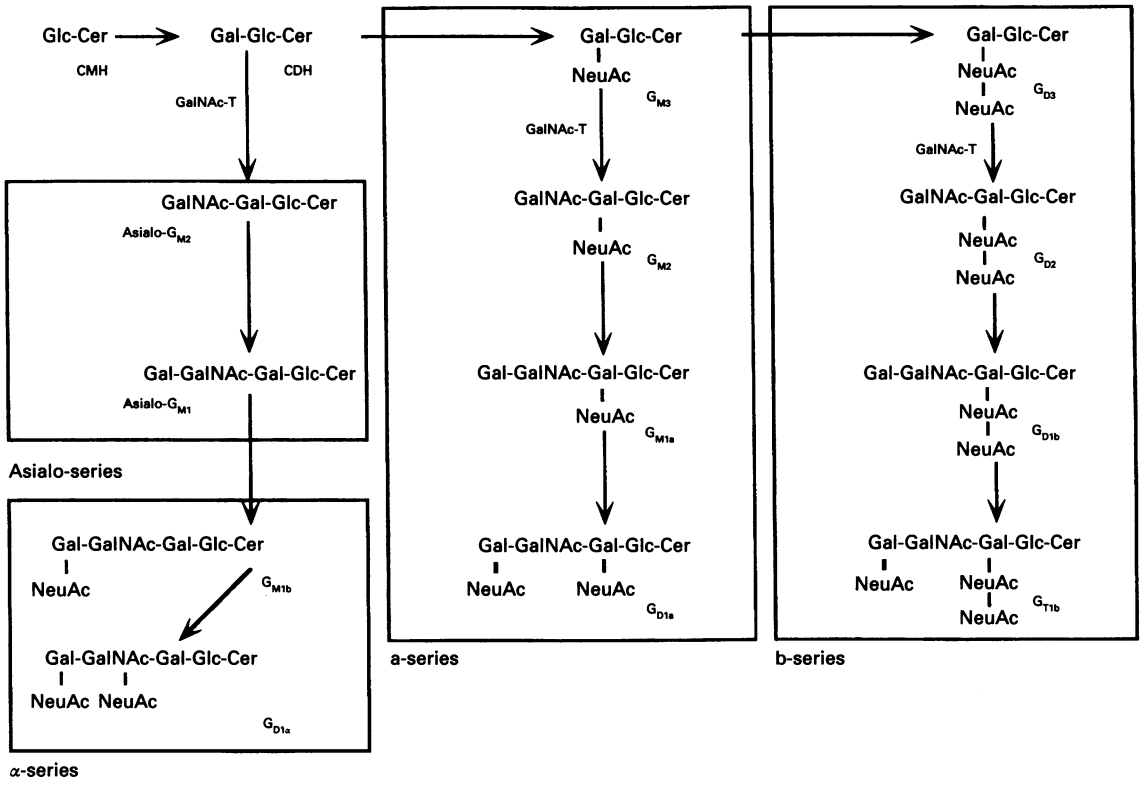
However, the molecular basis for their structural diversity and the mechanisms for their diverse biological functions have remained elusive. To elucidate these questions, it is essential to isolate genes encoding glycosyltransferases and to characterize them. Recently several groups have isolated cDNA clones encoding glycosyltransferases such as sialyltransferases, fucosyltransferases, and galactosyltransferases. These glycosyltransferases were more reactive toward glycoprotein substrates than glycolipid substrates (for a review, see [6]). Nagata et al. [7] isolated a new gene encoding β1-4N-acetylgalactosaminyltransferase (GalNAc-T) from a human melanoma cell line using expression cloning. The enzyme efficiently catalysed the formation of $G_{M2}$ and $G_{D2}$ from $G_{M3}$ and $G_{D3}$ as respective precursors (Scheme 1) (see the abbreviations footnote for ganglioside definitions). However, asialo-$G_{M2}$ formation was not observed, suggesting that asialo-$G_{M2}$ is synthesized by a different GalNAc-T. On the other hand, kinetic and competitive experiments have shown that asialo-$G_{M2}$, $G_{M2}$ and $G_{D2}$ were synthesized by a single GalNAc-T in rat liver (Scheme 1) [8].

Asialo-series GSLs and α-series gangliosides are sequentially formed from asialo-$G_{M2}$ (Scheme 1) [9] (classification of GSLs and gangliosides such as asialo-, a-, b- and α-series is based on the nomenclature of Pohlentz et al. [9] and Taki et al. [10]). These GSLs and gangliosides have been reported as significant molecules related to cell–cell interaction and specific cell markers, and may play putative functional roles in the nervous system [11–19]. For example, in the asialo-series GSLs asialo-$G_{M1}$ is known as

Abbreviations used: the nomenclature used for gangliosides is based on the system of Svennerholm [1]; Cer, ceramide; CDH, Galβ1-4Glcβ1-1Cer; CTH, Galα1-4Galβ1-4Glcβ1-1Cer; asialo-$G_{M2}$, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; SPG, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; DMEM, Dulbecco's modified Eagle medium; PA, pyridylaminated; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; GalNAc, N-acetylgalactosamine; GalNAc-T, β1-4N-acetylgalactosaminyltransferase; GSLs, glycosphingolipids.

§ To whom correspondence should be sent.

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D17809.




**Scheme 1 Pathways of GSL synthesis**

The Scheme is summarized on the basis of previous studies [9,10,52].

a specific and common marker in mouse immune and nervous systems [11–13,15]. The α-series gangliosides, $G_{M1b}$ and $G_{D1\alpha}$ are synthesized through asialo-series GSLs and are characteristically found in mammalian brain [18,19]. Therefore, isolation of a cDNA encoding asialo-$G_{M2}$ synthase helps us to understand the biological significance of the asialo-series GSLs and α-series gangliosides at the molecular and genetic levels.

Our previous work showed that the non-adhesive variant of the rat hepatoma cell line AH7974F had a unique GSL composition [20–25]. The cells expressed asialo-series GSLs and α-series gangliosides such as asialo-$G_{M2}$, asialo-$G_{M1}$, $G_{M1b}$, and $G_{D1\alpha}$. On the other hand, the same cells did not express other gangliosides, including $G_{M3}$, $G_{M2}$ and $G_{M1a}$. Therefore, the cells were expected to have a GalNAc-T responsible for the synthesis of asialo-$G_{M2}$.

Recent studies have also shown that all glycosyltransferases have a common topology, which consists of a short N-terminal cytoplasmic domain, a membrane-spanning region and a large C-terminal region, oriented to the lumen of the Golgi apparatus, which includes the catalytic and substrate-binding sites [6]. The studies also indicated that there was no significant similarity in the deduced amino acid sequences between glycosyltransferases, even for those using the same acceptor substrates. On the other hand, some regions in the C-terminal domain are conserved within each glycosyltransferase family [26,27]. For example, sialyltransferases have conserved amino acid sequences, termed the 'sialylmotif', in the centre of their C-terminal region. The sialylmotif is predicted to be a catalytic or substrate-binding site. In addition, amino acid sequences in the C-terminal region of fucosyltransferases have also been shown to be highly conserved.

From the findings mentioned above we anticipated that a catalytic or substrate-binding site would also be located near the centre of the C-terminal region in GalNAc-T, and that its amino acid sequence would be conserved to some extent within GalNAc-Ts. We planned to isolate the gene encoding asialo-$G_{M2}$ synthase by using PCR. PCR was carried out using cDNA from AH7974F cells as template DNA.

In the present study we isolated the gene encoding GalNAc-T from AH7974F cells, examined the substrate specificity of the enzyme, and introduced the cDNA into a cultured cell line to examine the formation of asialo-$G_{M2}$ *in vivo*.

## MATERIALS AND METHODS

### Materials

T.l.c. plates were purchased from E. Merck, Darmstadt, Germany. CDH, $G_{M3}$ and $G_{D3}$were purified from buttermilk. Asialo-$G_{M1}$, $G_{M1a}$, $G_{M1b}$, $G_{D2}$, $G_{D1\alpha}$ and SPG (NeuAc, NeuGc) were purified from bovine brain. CTH was isolated from porcine intestine. $G_{M2}$ was from Tay–Sachs-disease brain, and asialo-$G_{M2}$ was obtained by hydrolysis of $G_{M2}$ with 0.05 M HCl. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgM were purchased from Organon Teknika N. V.–Cappel Products. Horseradish peroxidase (HRP)-conjugated cholera-toxin B-subunit was purchased from List Biological Laboratories. Fetuin, asialo-fetuin, and UDP-*N*-acetylgalactosamine (UDP-GalNAc) were obtained from Sigma. UDP-[1-$^{14}$C]GalNAc (2.0 GBq/mmol) was purchased from NEN Research Products. [α-$^{32}$P]dCTP (220 GBq/mmol) and

[$\gamma$-$^{32}$P]ATP (220 GBq/mmol) were from Amersham International. All other chemicals were of the highest purity available.

### Cell cultures

Adhesive AH7974 cells (island-forming cell type) and a non-adhesive variant of rat ascites hepatoma cells, AH7974F (free-cell type), a subclone derived from AH7974, were obtained from The Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University, Tohoku, Japan [28]. Both cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal-calf serum (FCS). CHO-Py-leu cells were a gift from Dr. M. Fukuda, Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, CA, U.S.A. [29]. The cells were grown in complete medium [Ham's F12/DMEM (1:1, v/v) medium containing 10% FCS with 0.1 mg/ml geneticin]. CHO-K1 cells were obtained from the RIKEN Cell Bank (Saitama, Japan). The cells were grown in Ham's F12 medium containing 10% FCS. All cells were cultured at 37 °C under an air/$CO_2$ (19:1) atmosphere.

### Construction and screening of the cDNA library

cDNA was synthesized from mRNA that was extracted from AH7974F cells, using cDNA Synthesis System Plus (Amersham International). cDNA library was constructed by cDNA Cloning System, $\lambda$gt10 (Amersham). The primers for PCR were designed using the nucleotide sequence of human GalNAc-T. The sequences of primers were indicated as follows: P-1 (5′-CTGGACCAACTCAACAGGCAA-3′), P-2 (5′-GTTACCGTGGTCATCGCTGAC-3′), P-3 (5′-CAGCAGCTGCCGATAAGTGGT-3′), and P-4 (5′-TGGCTCTCGTCCAGTGATCCT-3′) represented nucleotide positions at 637–657, 916–936, 1180–1200 and 1519–1539 of human GalNAc-T respectively [7]. The conditions for PCR were 40 cycles of denaturation (94 °C, 1 min), annealing (61 °C, 1 min) and extension (72 °C, 1.5 min) in 100 $\mu$l of the reaction mixture containing 50 mM KCl, 10 mM Tris/HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.2 $\mu$M of each primer, cDNA from AH7974F cells as a template and 2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) [30]. The 285 bp-long rat cDNA amplified using a combination of P-2/P-3 primer was labelled with [$\alpha$-$^{32}$P]dCTP (220 GBq/mmol) by Multiprime DNA Labeling System (Amersham), and used as a probe for screening of cDNA library [31]. The solution for plaque hybridization was 6 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.4), 1 × Denhardt's solution [0.02% Ficoll 400, 6 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.02% BSA], 50% formamide, 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA. The hybridization was carried out for 40 h at 42 °C, and the filters were washed twice with 6 × SSC/0.5% SDS for 15 min at room temperature and once with 2 × SSC/0.5% SDS for 15 min at 55 °C [31]. Positive clones were subcloned into the *Not*I site of the Bluescript KS vector.

### DNA sequencing

The dideoxy chain-termination method for sequencing of double-stranded templates from different clones by *Exo*III/mung-bean nuclease deletion was carried out using *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems) [32]. The nucleotide sequence of the isolated cDNA clone was confirmed by sequencing both strands.

### DNA transfection

The full-length cDNA from a positive clone was subcloned into the eukaryotic cell expression vector pCDM8 [33], and the subcloned plasmid was designated pCD-GNA56.

#### Transient expression for *in vitro* enzyme assay

The DNA transfection into CHO-Py-leu cells was performed by DEAE-dextran method [34] using the CellPhect Transfection Kit (Pharmacia, Sweden). Since CHO-Py-leu cells were stably expressing polyoma-virus large tumour (T-) antigen [29], pCD-GNA56 became replicated epigenetically. CHO-Py-leu cells were plated at (2–3) × $10^6$ cells/150 mm dish and cultured for 20–24 h at 37 °C. After removal of the medium and washing twice with SF-DMEM (serum-free DMEM containing 50 mM Tris/HCl buffer, pH 7.5), the cells were loaded with 10 $\mu$g of plasmid DNA/0.5 mg/ml DEAE-dextran mixture to a final volume of 5 ml for 3 h at 37 °C. Subsequently the cells were treated with 10% dimethyl sulphoxide in SF-DMEM for 1.5 min at room temperature as described previously [34]. After washing once with SF-DMEM, the cells were incubated in complete medium for 48 h at 37 °C. The cells were subjected to enzyme assay.

#### Establishment of stable transfectants

The DNA transfection of CHO-K1 cells was carried out by a calcium phosphate method [35] using a CellPhect Transfection Kit. Briefly, CHO-K1 cells were plated at 1.5 × $10^6$ cells in a 100 mm dish and incubated for 21 h at 37 °C. For each dish, 20 $\mu$g of pCD-GNA56 and 2 $\mu$g of pSV2neo (a eukaryotic expression vector containing a neomycin-resistant gene) were co-precipitated with calcium phosphate and incubated for 10 h at 37 °C. The cells were treated for 30 s at room temperature with 15% glycerol in isotonic Hepes buffer, pH 7.5. Stable transfectants were selected for 2 weeks in medium containing 0.6 mg/ml of G418 (geneticin; Gibco–BRL). G418-resistant cells were cloned by limiting dilution, and subsequently screened for asialo-$G_{M2}$ expression by immunocytochemical procedure. As a control experiment, pCDM8 and pSV2neo were transfected by the same procedures above. Finally we obtained stable transfectants, CHO-56b and CHO-N8, corresponding to CHO-K1 cells transfected with pCD-GNA56 and with pCDM8 respectively. Stable transfectants were subjected to *in vivo* assays such as lipid analysis and immunocytochemical analysis.

### Enzyme assay

Parental rat ascites hepatoma cells and transfected cells were harvested and suspended in 200–400 $\mu$l of 3 mM Tris/HCl, pH 7.4, containing 250 mM sucrose and 1 mM EDTA. The activity of GalNAc-T was measured with three assay methods (I–III) described below.

#### Assay method (I)

The assay for GSLs as acceptors described by Senn et al. [36] was followed with a slight modification. In a final volume of 50 $\mu$l, the reaction mixture contained 0.15 mM GSLs, 0.1 mM UDP-[1-$^{14}$C]GalNAc (2.0 GBq/mmol), 0.5% (w/v) Triton X-100, 10 mM $MnCl_2$, 5 mM CDP-choline, 64 mM sodium cacodylate/HCl buffer, pH 7.0, and cell suspension (50–100 $\mu$g as protein). The reaction mixture was incubated at 37 °C for 2 h and was terminated with 450 $\mu$l of distilled water. Products were desalted with a C18 reverse-phase column (M&S Aspec pak; M&S Instrument Trading Inc., Tokyo, Japan) and developed on a t.l.c.

plate with the solvent system chloroform/methanol/12 mM $MgCl_2$ (5:40:1, by vol.). Radiolabelled products were detected with a bioimaging analyser (BAS 2000; Fuji Film, Tokyo, Japan), and the radioactivity of spots corresponding to standard GSLs was preparatively quantified by using a liquid-scintillation counter.

Assay method (II)

The assay for pyridylaminated (PA-) oligosaccharide as an acceptor was carried out for 2 h at 37 °C as described by Dohi et al. [37]. The reaction mixture had the following final concentrations in a total volume of 50 $\mu$l: 80 $\mu$M PA-3′-sialyl-lactose, 10 mM UDP-GalNAc, 0.45% (w/v) Triton X-100, 10 mM $MnCl_2$, 100 mM sodium cacodylate/HCl buffer, pH 7.5, and cell suspension (50–100 $\mu$g as protein).

Assay method (III)

The assay for glycoproteins as acceptors was carried out for 2 h at 37 °C by the method of Takeya et al. [38] and Malagolini et al. [39]. The reaction mixture had the following final concentrations in a total volume of 50 $\mu$l: 0.5 mg glycoproteins, 0.1 mM UDP-[1-$^{14}$C]GalNAc (2.0 GBq/mmol), 0.5% (w/v) Triton X-100, 10 mM $MnCl_2$, 80 mM sodium cacodylate/HCl buffer, pH 7.5, and cell suspension (50–100 $\mu$g as protein).

### Lipid extraction

Parental rat ascites hepatoma cells and transfected cells were harvested and freeze-dried. Total lipids were sequentially extracted from dried cell pellets (3.0–4.0 mg dry weight) with 50 vol. of chloroform/methanol/water (5:5:1, by vol.) and chloroform/methanol (1:1, v/v). Total extracts were evaporated and subjected to a phenyl boronate–agarose column (Matrex gel PBA-60; Amicon Division, W. R. Grace and Co.) to remove neutral lipids and phospholipids. The extracts were applied to the column equilibrated with chloroform/methanol (4:1, v/v). The column was washed with 5 vol. of the same solvent, and then GSLs were eluted with 4 vol. of chloroform/methanol/water (5:5:1, by vol.). GSL composition in stable transfectants was analysed on t.l.c. plate using two solvent systems: (I) chloroform/methanol/12 mM $MgCl_2$ (5:4:1, by vol.) and (II) (60:35:8, by vol.). T.l.c.–immunostaining was carried out by the procedure of Higashi et al. [40]. Mouse anti-$G_{D1\alpha}$ monoclonal antibody, KA-17, anti-$G_{M1b}$ monoclonal antibody, NA-6 [19], affinity-purified rabbit anti-asialo-$G_{M2}$, and anti-asialo-$G_{M1}$ polyclonal antibodies were used as primary antibodies.

### Immunocytochemical methods

Fluoromicroscopic analysis was performed as described below. Stable transfectants, CHO-56b and CHO-N8 cells, were seeded at $2 \times 10^4$ cells/well in an eight-chamber slide (Lab-Tek; Nunc Inc.) and cultured for 48 h at 37 °C. The cells were washed three times with solution A (PBS containing 0.25% BSA and 0.5% normal goat serum) and fixed for 10 min at room temperature with 1.3% paraformaldehyde in solution B (PBS containing 2.5% BSA and 5% normal goat serum). After three washes with PBS, the cells were incubated for 1 h at room temperature in primary antibodies diluted with solution B. The cells were washed three times with solution A, and subsequently reacted for 30 min at room temperature in FITC- and TRITC-conjugated secondary antibody. Finally the cells were washed three times with solution A and twice with PBS, and then mounted with 50% glycerol in PBS.

Flow-cytometric analysis was carried out as follows. Briefly, stable transfectants were treated with trypsin and washed well with PBS. The cells were incubated with primary antibody, diluted with PBS on ice for 1 h, and subsequently in the FITC- and TRITC-conjugated secondary antibodies diluted with PBS on ice for 30 min. After several washings with PBS, the cells were suspended in 500 $\mu$l of PBS and analysed with a FACSort instrument (Becton–Dickinson Immunocytometry Systems). Mouse anti-$G_{M2}$ monoclonal antibody, YHD-06 [41], and affinity-purified rabbit anti-asialo-$G_{M2}$ polyclonal antibody were used as the primary antibodies.

### Northern-blot hybridization

Total cellular RNA was prepared from cultured cell lines and rat tissues by the guanidium thiocyanate method [31]. RNA (10 $\mu$g) was electrophoresed on a denatured formaldehyde–agarose gel (1.0%) and transferred on to a nylon membrane (Hybond N; Amersham). Rat GalNAc-T cDNA was labelled with [$\alpha$-$^{32}$P]dCTP by using random hexamers as primers. The cDNA hybridization solution consisted of 5 × SSPE, 1 × Denhardt's solution, 50% formamide, 0.5% SDS, 0.05 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA. The hybridization was carried out for 42 h at 42 °C, and the filters were washed once with 2 × SSC/0.1% SDS for 30 min at room temperature and three times with 0.2 × SSC/0.1% SDS for 30 min at 68 °C.

## RESULTS

### Isolation of a GalNAc-T cDNA clone from rat ascites hepatoma cell line AH7974F

We isolated a full-length GalNAc-T cDNA clone from rat ascites hepatoma cell line AH7974F by screening the $\lambda$gt10 library using a rat GalNAc-T cDNA probe. The probe (285 bp) was obtained by PCR of rat cDNA from the cell line using a combination of P-2 and P-3 primers taken from a published human sequence [7]. Comparison of the nucleotide sequence of the fragment with that of human GalNAc-T cDNA revealed 87% similarity. We assumed the fragment to be a part of an asialo-$G_{M2}$ synthase, and subsequently screened the cDNA library from AH7974F mRNA. In all, 22 positive clones were obtained by screening of $5.5 \times 10^5$ independent plaques. Finally, four clones were purified and digested with *Not*I to yield inserts. Figure 1 shows the structure of the pGNA56 cDNA that has the longest insert (2166 bp).

### Nucleotide and deduced amino acid sequence of the cDNA clone

The isolated cDNA was subcloned into the *Not*I site of the Bluescript KS vector, and the deletion clones were sequenced by

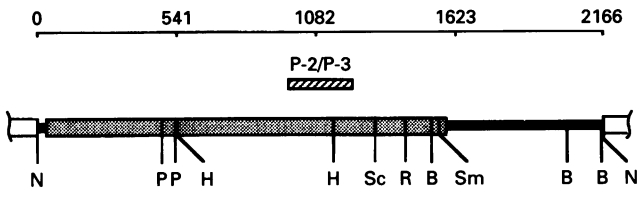



**Figure 1 Schematic structure and restriction map of pGNA56 cDNA**

The open reading frame is indicated by the stippled box and the non-coding regions by the black box. A PCR fragment (P-2/P-3) was used for screening of $\lambda$gt10 library. Restriction sites B, H, N, P, R and Sc represent *Bam*HI, *Hin*dIII, *Not*I, *Pst*I, *Eco*RI, and *Sac*I respectively.

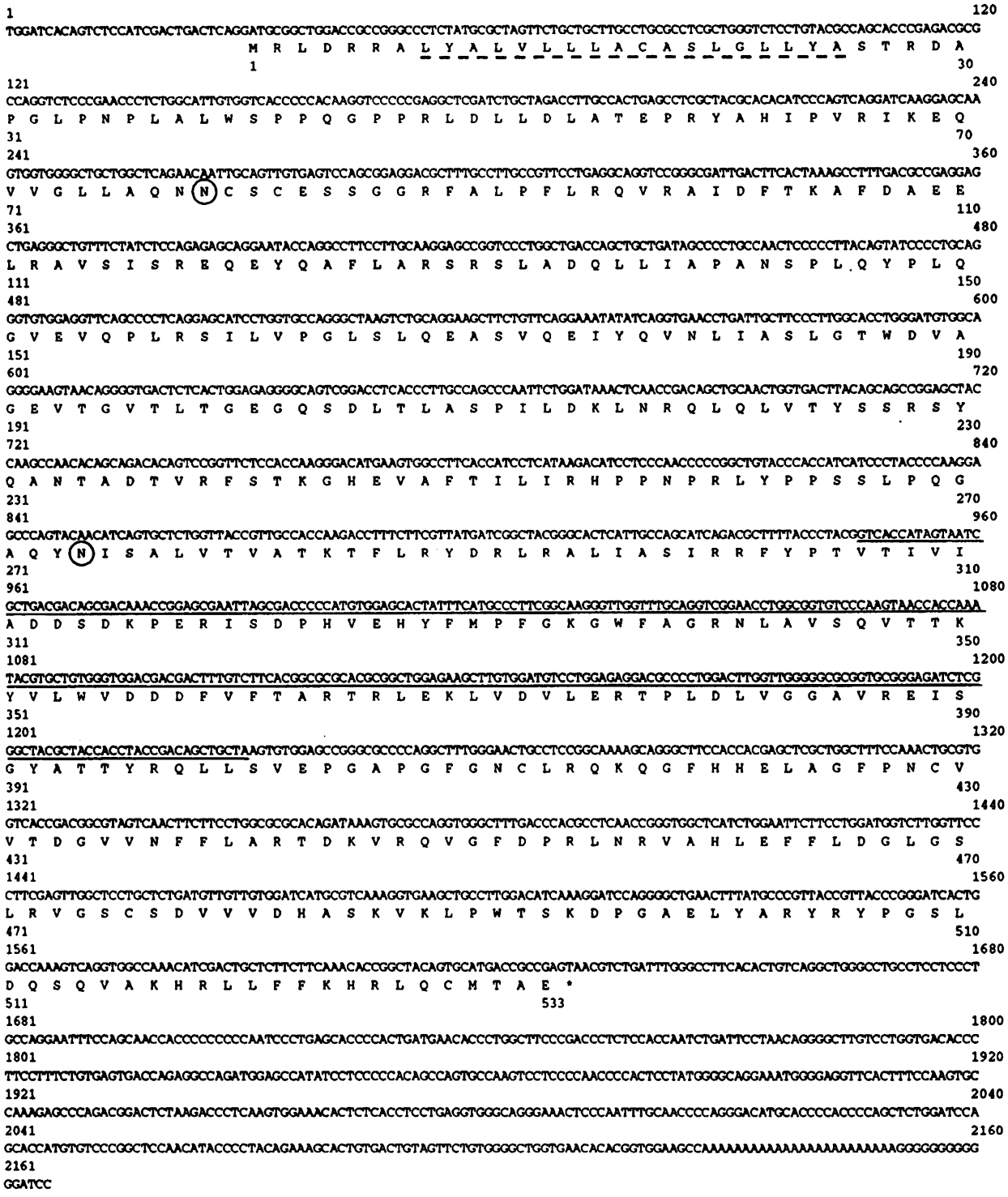



**Figure 2 Nucleotide and deduced amino acid sequence of pGNA56 cDNA**

The predicted transmembrane region is indicated by a broken line. The possible N-glycosylation sites are circled. The fragment obtained by PCR, termed the 'P-2/P-3 fragment', is underlined.

the dideoxy-chain-termination method. The pGNA56 cDNA has 2166 bp of an insert and consists of 30 bp of a short 5′-untranslated region, 1599 bp of an open reading frame and 637 bp of 3′-untranslated region (Figure 2). The open reading frame encodes a protein of 533 amino acids with a predicted molecular mass of 59 kDa. Two possible N-glycosylation sites (Asn-Xaa-Ser/Thr) are found at positions 79 and 274. These sites are well conserved between rat and human GalNAc-T clones [7]. When the amino acid sequences of rat and human GalNAc-Ts were compared, about 400 amino acid residues were well conserved in the N-terminal region (88 % similarity). On the other hand, there was no significant similarity in the 120-amino-

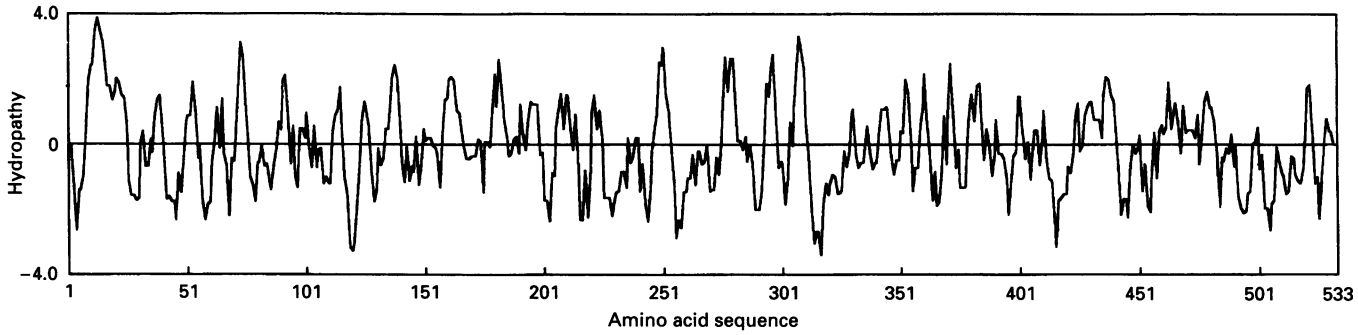



**Figure 3 Hydropathy profile of the protein encoded by pGNA56 cDNA**

The profile was determined by the program of Kyte and Doolittle [53]. Positive values represent hydrophobic areas.

acid C-terminal region. However, when we re-examined the nucleotide sequence of human GalNAc-T cDNA, we found the omission of two nucleotides (revised nucleotide and deduced amino acid sequences were reported by Nagata et al. [54]). Finally, the deduced amino acid sequence of rat GalNAc-T exhibited 88% similarity with that of human GalNAc-T in the whole region. A hydropathy profile indicates that the protein predicted from the nucleotide sequence of pGNA56 cDNA is a typical type II membrane protein, which consists of a short cytoplasmic domain, a transmembrane region and a long C-terminal region (Figure 3). 'Homology' searches in protein databases (PIR Release 34.0 and SWISS-PLOT Release 23.0) demonstrated that there were no other proteins with significant sequence similarity.

## Determination of substrate specificity of the enzyme encoded by pGNA56 cDNA

The cDNA clone was subcloned into a eukaryotic expression vector pCDM8 [33]. We transfected pCD-GNA56 into CHO-Py-leu cells, which have no GalNAc-T activity (results not shown). Subsequently, we determined substrate specificity of the transiently expressed GalNAc-T. As shown in Table 1, the total homogenate from cells transfected with pCD-GNA56 had the ability to transfer GalNAc to $G_{M3}$ and $G_{D3}$. GalNAc was also incorporated into CDH, $G_{M1b}$, SPG (NeuAc and NeuGc), and

**Table 1 Substrate specificity of GalNAc-T encoded by pGNA56 cDNA**

The GalNAc-T assay was performed as described in the Materials and methods section. Each value is taken from duplicate assays and is shown as a percentage of the rate obtained with $G_{M3}$ as a substrate. Comparable results were achieved in another independent experiment. N.D., not detected; N.T., not tested.

| Substrate | Transfected cells (pCD-GNA56) | pCDM8 | Original cells (AH7974F) | AH7974 |
|---|---|---|---|---|
| CDH | 1.2 | N.D | 1.6 | N.D |
| $G_{M3}$ | 100 | N.D | 100 | N.D |
| $G_{D3}$ | 77.3 | N.D | 82.4 | N.D |
| $G_{M1b}$ | 3.3 | N.D | 7.5 | N.D |
| SPG (NeuAc) | 0.8 | N.D | 7.4 | N.D |
| SPG (NeuGc) | 11.6 | N.D | 20.9 | N.D |
| $G_{D1a}$ | 21.0 | N.D | 14.8 | N.D |
| CTH | N.D | N.D | N.D | N.D |
| Asialo-$G_{M1}$ | N.D | N.D | N.D | N.D |
| $G_{M1a}$ | N.D | N.D | N.D | N.D |
| PA-3′-sialyl-lactose | N.D | N.D | N.D | N.D |
| Fetuin | N.D | N.D | N.T. | N.T. |
| Asialo-fetuin | N.D | N.D | N.T. | N.T. |

**Table 2 Correlation between GalNAc-T activity and the expression of $G_{M2}$ and asialo-$G_{M2}$ in transfected cells**

The GalNAc-T assay was performed as described in the Materials and methods section. Values indicate $G_{M2}$ formation from $G_{M3}$ as an acceptor substrate. Quantitative analysis of GSLs was carried out using a densitometer, CS-9000 (Shimadzu, Kyoto, Japan). N.D., not detected.

| Clone | Content of GSLs (nmol/mg dry weight) $G_{M2}$ | Content of GSLs (nmol/mg dry weight) Asialo-$G_{M2}$ | GalNAc-T activity (nmol/h per mg of protein) |
|---|---|---|---|
| CHO-N8 | N.D. | N.D. | N.D. |
| CHO-56a | 0.31 | 0.63 | 0.45 |
| CHO-56b | 1.21 | 1.53 | 2.58 |

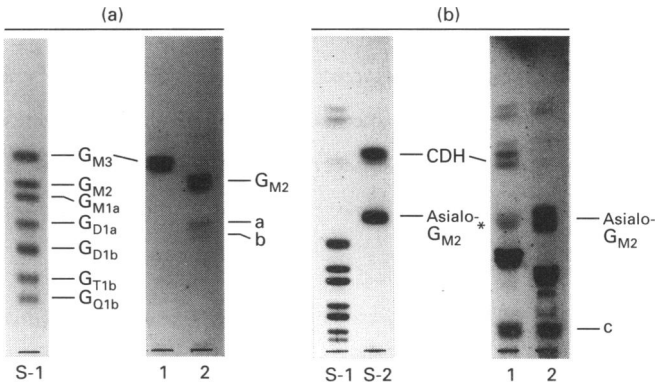



**Figure 4 Composition of GSLs synthesized in stable transfectants**

Lipid analysis was performed as described in the Materials and methods section. (**a**) Resorcinol staining of the t.l.c. plate developed with solvent system (I). (**b**) Orcinol staining, with solvent system (II). Lane S-1, standard gangliosides ($G_{M3}$, $G_{M2}$, $G_{M1a}$, $G_{D1a}$, $G_{D1b}$, $G_{T1b}$, and $G_{Q1b}$); lane S-2, standard GSLs (CDH and asialo-$G_{M2}$); lane 1, lipid fraction from the stable transfectant CHO-N8; lane 2, lipid fraction from CHO-56b. (**a**) a and b, $G_{D1a}$ and GalNAc-$G_{D1a}$ respectively. (**b**) c, free glucose; *, phospholipid.

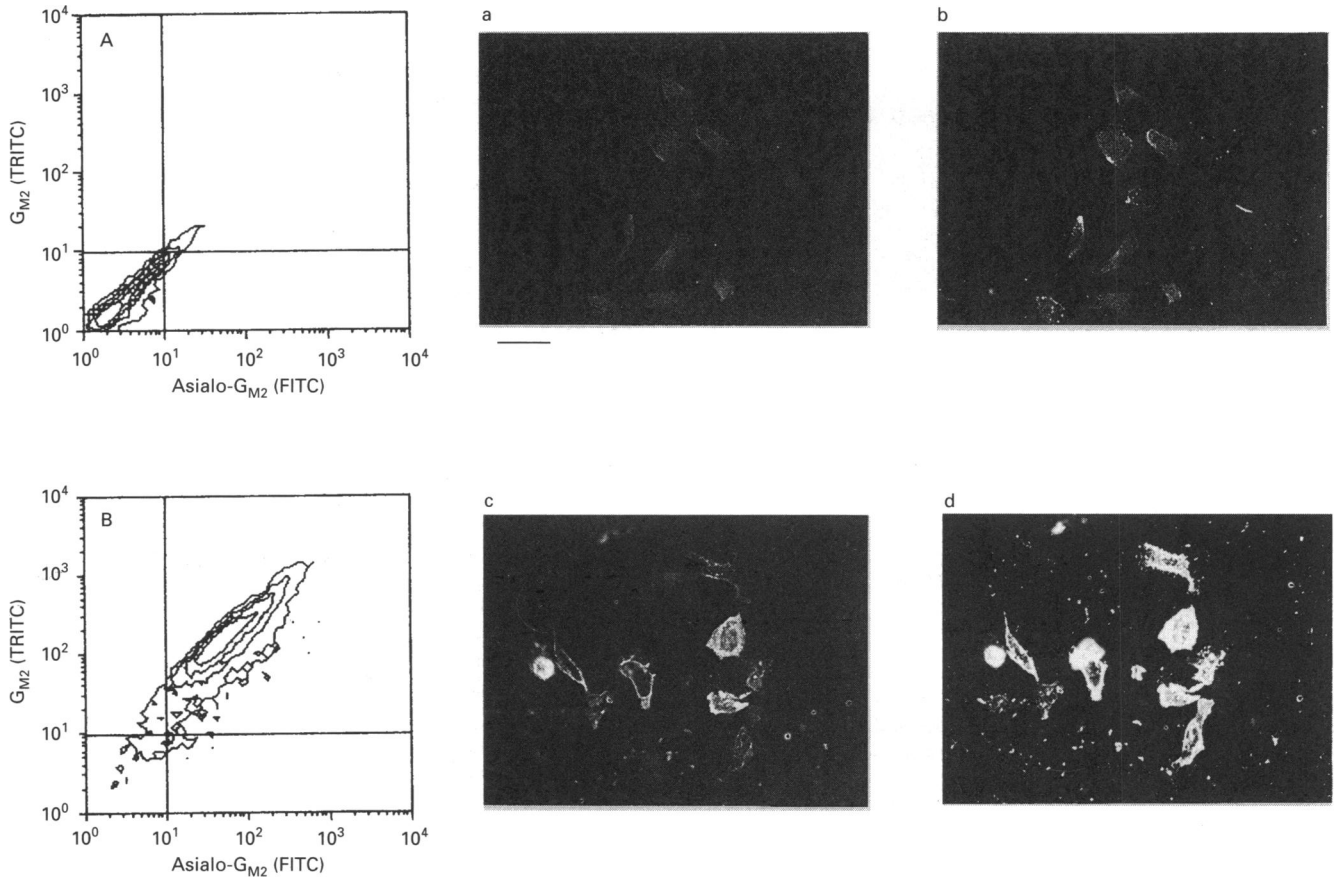



**Figure 5 Expression of GSLs on the cell surface of stable transfectants**

Immunocytochemical procedures were performed as described in the Materials and methods section. Flow-cytometric profiles A and B represent stable transfectants, CHO-N8 and CHO-56b respectively. Fluorescence photographs a and b represent CHO-N8 cells stained with anti-asialo-$G_{M2}$ antibody and YHD-06 respectively, and c and d, CHO-56b cells with anti-asialo-$G_{M2}$ antibody and YHD-06 respectively. The bar represents 50 $\mu$m.

$G_{D1a}$. On the other hand, asialo-$G_{M1}$, $G_{M1a}$, and CTH did not serve as substrates at all. Since the enzyme did not act on oligosaccharides and glycoproteins as substrates, we concluded that the enzyme specifically transferred GalNAc to GSLs as substrates. A similar result was obtained in the case of human recombinant fusion GalNAc-T protein (data not shown). The substrate specificity of cell lysate from the original cell line, AH7974F, was similar to that of the lysate from the pGNA56-cDNA-transfected cells. Since the AH7974F cells intensely expressed asialo-$G_{M2}$, but not $G_{M2}$ [20–25], the enzyme was thought to participate in the synthesis of asialo-$G_{M2}$. The relative activity of asialo-$G_{M2}$ formation, however, was 1–2% of that of $G_{M2}$ synthesis *in vitro*. Thus we examined whether asialo-$G_{M2}$ could be synthesized by the enzyme *in vivo* as described below.

## Expression of GSLs in stable transfectants

We obtained two clones, CHO-56a and 56b, from several asialo-$G_{M2}$-positive clones by immunocytochemical screening. The two clones expressed different levels of asialo-$G_{M2}$. As a negative control, CHO-N8 cells were obtained by co-transfection of pCDM8 and pSV2neo into CHO-K1 cells. The control cells did not react with anti-asialo-$G_{M2}$ antibody. The cellular expression of $G_{M2}$ and asialo-$G_{M2}$ was clearly contributed by the single GalNAc-T (Table 2). Furthermore, the level of expresssion of the GSLs was positively correlated with the GalNAc-T activity. Figure 4 shows the GSL composition of CHO-56b and CHO-N8 cells. The GSL composition of recipient CHO-K1 cells was very simple, consisting of only CMH, CDH, and $G_{M3}$ (results not shown). CHO-N8 cells showed a similar GSL pattern to that shown by CHO-K1 cells (Figure 4, lane 1). In contrast, $G_{M2}$ and asialo-$G_{M2}$ were newly synthesized in CHO-56b cells. In addition, the cells had a detectable amount of gangliosides with longer carbohydrate chains corresponding to $G_{D1a}$ and GalNAc-$G_{D1a}$ on t.l.c. (Figure 4, lane 2, a and b). Interestingly, $G_{M3}$and CDH were not apparently detected, indicating that these two GSLs were taken up by the GalNAc-T as substrates (Figure 4, lane 2). The GSLs expressed in the stable transfectants were identified by t.l.c.–immunostaining with the exception of $G_{D1a}$ (results not shown). Asialo-$G_{M1}$, $G_{M1a}$ and $G_{M1b}$ as minor components were also detected in CHO-56b cells by t.l.c.–immunostaining with each specific antibody (results not shown).

In order to confirm simultaneous expression of asialo-$G_{M2}$ and $G_{M2}$ on the cell surface, the stable transfectants were analysed by flow cytometry using the appropriate primary antibodies. Figure 5 showed that the positive clone, CHO-56b cells, simultaneously expressed the GSLs (Figure 5, panels A and B). We further examined the cellular distribution of the newly synthesized GSLs by fluoromicroscopic analysis (Figure 5, panels c and d). CHO-56b cells were intensely stained on the cell surface with both anti-

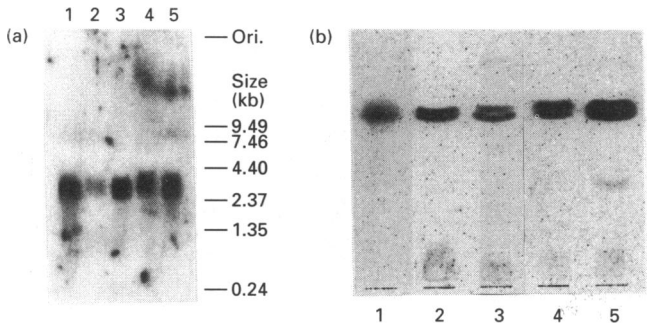



**Figure 6 Northern-blot analysis of GalNAc-T expression and detection of asialo-$G_{M1}$ in rat tissues**

Northern-blot analysis and t.l.c.-immunostaining were performed as described in the Materials and methods sction. (**a**) Hybridization with $^{32}$P-labelled pGNA56 cDNA as a probe. Lane 1, rat ascites hepatoma cell line AH7974F; lanes 2–5, rat liver, brain, testis and spleen respectively. Ori., origin. (**b**) T.l.c.–immunostaining with affinity-purified anti-asialo-$G_{M1}$ polyclonal antibody. Lane 1, standard asialo-$G_{M1}$ (20 pmol); lanes 2–5, rat liver, brain, testis and spleen respectively. The amount of glycolipid applied per lane corresponded to 10 mg dry weight of tissue.

asialo-$G_{M2}$ antibody and anti-$G_{M2}$ monoclonal antibody, YHD-06, also indicating that both GSLs are expressed on the plasma membrane.

### Northern-blot analysis

The expression of GalNAc-T mRNA in rat cultured cell lines and tissues were examined by Northern-blot analysis (Figure 6). A single GalNAc-T mRNA of 2.9 kb was strongly expressed in parental cells, AH7974F (Figure 6a, lane 1), but not detected in its original clone, AH7974, deficient in GalNAc-T activity (results not shown; see also Table 1) [22,23]. The GalNAc-T transcript was also detected in adult rat tissues, although the intensity of its expression varied among tissues examined (Figure 6a, lanes 2–5). However, asialo-$G_{M2}$ could not be detected in adult rat tissues, when examined by t.l.c.-immunostaining with anti-asialo-$G_{M2}$ polyclonal antibody. Instead, the presence of asialo-$G_{M1}$ was demonstrated in rat tissues by t.l.c.–immunostaining with anti-asialo-$G_{M1}$ polyclonal antibody (Figure 6b). Both a- and $\alpha$-series gangliosides, such as $G_{M2}$ and $G_{D1\alpha}$ respectively, were also shown in all tissues examined (results not shown). These observations strongly support our conclusion that $G_{M2}$ and asialo-$G_{M2}$ are synthesized by the single GalNAc-T.

## DISCUSSION

Since the transfer of GalNAc to CDH is an initial step in the synthesis of asialo-series GSLs and the subsequent synthesis of $\alpha$-series ganglioside, it is very important to isolate the gene encoding this enzyme and to analyse the mechanism regulating its expression [9]. Pohlentz et al. [8] suggested that asialo-$G_{M2}$, $G_{M2}$, and $G_{D2}$ were synthesized by a single GalNAc-T from rat liver by kinetic analysis. On the other hand, Nagata et al. [7] isolated GalNAc-T cDNA from a human melanoma cell line. When they analysed GSLs from stable clones into which were introduced the human GalNAc-T cDNA, they could not detect asialo-$G_{M2}$, despite the strong expression of $G_{M2}$ and $G_{D2}$ in these cells. This observation suggested the existence of another GalNAc-T responsible for asialo-$G_{M2}$ synthesis. Thus we undertook to isolate the gene encoding asialo-$G_{M2}$ synthase from the rat ascites hepatoma cell line AH7974F, because the cells expressed asialo-$G_{M2}$ strongly, but did not express $G_{M2}$ at all [20–25].

GalNAc-T encoded by pGNA56 cDNA exhibited high $G_{M2}$ and GD2 synthase activity, whereas the relative asialo-$G_{M2}$ synthase activity was only 1–2% of $G_{M2}$ synthase (Table 1). Substrate-specificity experiments also indicated that the rat GalNAc-T predominantly recognized the non-reducing terminal NeuAc$\alpha$2-3Gal$\beta$1- structure on GSLs irrespective of core-sugar-chain structures and formed a GalNAc$\beta$1-4 residue. However, it did not act on soluble substrates such as glycoproteins and oligosaccharides. Previous work has already shown the presence of several GalNAc-Ts having a substrate specificity different from that of the rat GalNAc-T. Examples include enzymes that transfer GalNAc to both GSLs and oligosaccharides [42], to both GSLs and glycoproteins, such as Cad and Sda antigens [43–45], and to pituitary glycoprotein hormones such as lutropin, follitropin and thyrotropin [46–48]. It is an intriguing issue whether these GalNAc-Ts have a conserved sequence, as is seen in sialyltransferases. Further isolation and characterization of other cDNA clones will resolve this problem.

*In vivo* assays showed the simultaneous expression of asialo-$G_{M2}$ and $G_{M2}$ with high levels on the plasma membrane of the stable transfectant CHO-56b. This means that the cellular expression of asialo-$G_{M2}$ was carried out by the single GalNAc-T that had 1–2% relative activity of asialo-$G_{M2}$ synthase to that for $G_{M2}$ synthase. Besides asialo-$G_{M2}$ and $G_{M2}$, GSLs with longer carbohydrate chains, such as asialo-$G_{M1}$, $G_{M1a}$, $G_{M1b}$ and GalNAc-$G_{D1a}$, were neo-synthesized. These findings indicate that new metabolic pathways of GSLs, asialo-series GSLs and $\alpha$-series gangliosides are opened by introduction of the single GalNAc-T. Since recipient cells, CHO-K1, had no $G_{D3}$, b-series gangliosides including $G_{D2}$, were not detected.

Rat GalNAc-T mRNA was expressed in normal liver (Figure 6a). Indeed, Pohlentz et al. [8,9] reported the presence of GalNAc-T activity in Golgi membrane fraction from rat liver. However, we could not find asialo-$G_{M2}$ in the tissue as previously described [49–51]. On the other hand, asialo-$G_{M1}$ and $G_{D1\alpha}$ belonging to asialo- and $\alpha$-series were shown to exist in rat liver for the first time by sensitive t.l.c.–immunostaining with specific antibodies. This finding indicates that asialo-$G_{M1}$ and $\alpha$-series gangliosides with longer carbohydrate chains are sequentially formed from asialo-$G_{M2}$ in the tissue. The apparent absence of asialo-$G_{M2}$ may result from its immediate utilization as a metabolic precursor for asialo-$G_{M1}$ synthesis. From Northern-blot and GSL analyses, the expression of GalNAc-T mRNA was clearly coincident with the presence of $G_{M2}$ and asialo-series GSLs in rat tissues examined (Figure 6). This strongly suggests that asialo-$G_{M2}$ and $G_{M2}$ are synthesized by a single GalNAc-T.

The above observations made us consider further the inability of the human GalNAc-T encoded by pM2T1-1 to synthesize asialo-$G_{M2}$ *in vivo*. We found that human recombinant GalNAc-T also exhibited about 1% activity of asialo-$G_{M2}$ synthase compared with $G_{M2}$ synthase *in vitro*. Nagata et al. [7] examined the activity of GalNAc-T in GSL synthesis by a cellular expression method using melanoma cells. They did not find asialo-$G_{M2}$ expression in transfected cells. However, according to our results, it is expected that asialo- and $\alpha$-series GSLs may be detected in the melanoma cells by sensitive t.l.c.–immunostaining with specific antibodies.

In conclusion, we found that the expression of asialo-$G_{M2}$, $G_{M2}$, and $G_{D2}$ was carried out by the single GalNAc-T from AH7974F cells on a molecular basis. This finding may be helpful for understanding the biological significance of asialo-series GSLs found in rat malignant and normal cells and tissues.

We are grateful to Dr. Yoshitaka Nagai, Tokyo Metropolitan Institute of Medicine, and Dr. Thomas N. Seyfried, Department of Biology, Boston College, Chestnut Hill, MA, U.S.A., for helpful comments, and Dr. Kenji Yamamoto, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, for PA-oligosaccharide analysis. We thank Dr. Minoru Fukuda, Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, CA, U.S.A., for kindly providing cells and for helpful comments. This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (no. 05274106) from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

1 Svennerholm, L. (1963) J. Neurochem. **10**, 613–623
2 Feizi, T. (1985) Nature (London) **314**, 53–57
3 Hannun, Y. A. and Bell, R. M. (1989) Science **243**, 500–507
4 Hakomori, S. (1990) J. Biol. Chem. **265**, 18713–18716
5 Varki, A. (1993) Glycobiology **3**, 97–130
6 Paulson, J. C. and Colley, K. J. (1989) J. Biol. Chem. **264**, 17615–17618
7 Nagata, Y., Yamashiro, S., Yodoi, J., Lloyd, K. O., Shiku, H. and Furukawa, K. (1992) J. Biol. Chem. **267**, 12082–12089
8 Pohlentz, G., Klein, D., Schwarzmann, G., Schmitz, D. and Sandhoff, K. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7044–7048
9. Pohlentz, G., Klein, D., Schmitz, D., Schwarzmann, G., Peter–Katalinic, J. and Sandhoff, K. (1988) Biol. Chem. Hoppe-Seyler **369**, 55–63
10 Taki, T., Hirabayashi, Y., Ishikawa, H., Ando, S., Kon, K., Tanaka, Y. and Matsumoto, M. (1986) J. Biol. Chem. **261**, 3075–3078
11 Kasai, M., Iwamori, M., Nagai, Y., Okumura, K. and Tada, T. (1980) Eur. J. Immunol. **10**, 175–180
12 Young,W. W., Jr., Hakomori, S., Durdik, J. M. and Henney, C. S. (1980) J. Immunol. **124**, 199–201
13 Dennert, G., Yogeeswaran, G. and Yamagata, S. (1981) J. Exp. Med. **153**, 545–556
14 Okada, Y., Matsuura, H. and Hakomori, S. (1985) Cancer Res. **45**, 2793–2801
15 Kusunoki, S., Tsuji, S. and Nagai, Y. (1985) Brain Res. **334**, 117–124
16 Kojima, N. and Hakomori, S. (1989) J. Biol. Chem. **264**, 20159–20162
17 Kojima, N. and Hakomori, S. (1991) J. Biol. Chem. **266**, 17552–17558
18 Hirabayashi, Y., Nakao, T., Matsumoto, M., Obata, K. and Ando, S. (1988) J. Chromatogr. **445**, 377–384
19 Hirabayashi, Y., Hyogo, A., Nakao, T., Tsuchiya, K., Suzuki, Y., Matsumoto, M., Kon, K. and Ando, S. (1990) J. Biol. Chem. **265**, 8144–8151
20 Matsumoto, M., Taki, T. and Kojima, K. (1976) Jpn. J. Exp. Med. **46**, 135–138
21 Hirabayashi, Y., Taki, T., Matsumoto, M. and Kojima, K. (1978) Biochim. Biophys. Acta **529**, 96–105
22 Taki, T., Hirabayashi, Y., Suzuki, Y., Matsumoto, M. and Kojima, K. (1978) J. Biochem. (Tokyo) **83**, 1517–1520
23 Taki, T., Hirabayashi, Y., Matsumoto, M. and Kojima, K. (1979) Biochim. Biophys. Acta **572**, 105–112
24 Taki, T., Hirabayashi, Y., Ishiwata, Y., Matsumoto, M. and Kojima, K. (1979) Biochim. Biophys. Acta **572**, 113–120
25 Hirabayashi, Y., Taki, T. and Matsumoto, M. (1979) FEBS Lett. **100**, 253–257
26 Wenn, D. X., Livingston, B. D., Medzihradszky, K. F., Kelm, S., Burlingame, A. L. and Paulson, J. C. (1992) J. Biol. Chem. **267**, 21011–21019
27 Weston, B. W., Nair, R. P., Larsen, R. D. and Lowe, J. B. (1992) J. Biol. Chem. **267**, 4152–4160
28 Koizumi, K., Ito, Y., Kojima, K. and Fujii, T. (1976) J. Biochem. (Tokyo) **79**, 739–748
29 Bierhuizen, M. F. A. and Fukuda, M. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9326–9330
30 Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985) Science **230**, 1350–1354
31 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
33 Seed, B. (1987) Nature (London) **329**, 840–842
34 Sussman, D. J. and Milman, G. (1984) Mol. Cell. Biol. **4**, 1641–1643
35 Graham, F. L. and Van der Eb, A. J. (1973) Virology **52**, 456–467
36 Senn, H.-J., Cooper, C., Warnke, P. C., Wagner, M. and Decker, K. (1981) Eur. J. Biochem. **120**, 59–67
37 Dohi, T., Nishikawa, A., Ishizuka, I., Totani, M., Yamaguchi, K., Nakagawa, K., Saitoh, O., Ohshiba, S. and Oshima, M. (1992) Biochem. J. **288**, 161–165
38 Takeya, A., Hosomi, O. and Kogure, T. (1987) J. Biochem. (Tokyo) **101**, 251–259
39 Malagolini, N., Dall'Olio, F., Di Stefano, G., Minni, F., Marrano, D. and Serafini-Cessi, F. (1989) Cancer Res. **49**, 6466–6470
40 Higashi, H., Fukui, Y., Ueda, S., Kato, S., Hirabayashi, Y., Matsumoto, M. and Naiki, M. (1984) J. Biochem. (Tokyo) **95**, 1517–1520
41 Sanai, Y., Yamasaki, M. and Nagai, Y. (1988) Biochim. Biophys. Acta **958**, 368–374
42 Yanagisawa, K., Taniguchi, N and Makita, A. (1987) Biochim. Biophys. Acta **919**, 213–220
43 Blanchard, D., Piller, F., Gillard, B., Marcus, D. and Cartron, J.-P. (1985) J. Biol. Chem. **260**, 7813–7816
44 Piller, F., Blanchard, D., Huet, M. and Cartron, J.-P. (1986) Carbohydr. Res. **149**, 171–184
45 Dohi, T., Hanai, N., Yamaguchi, K. and Oshima, M. (1991) J. Biol. Chem. **266**, 24038–24043
46 Smith, P. L. and Baenziger, J. U. (1988) Science **242**, 930–933
47 Smith, P. L. and Baenziger, J. U. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7275–7279
48 Smith, P. L. and Baenziger, J. U. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 329–333
49 Ueno, K., Kushi, Y., Rokukawa, C. and Handa, S. (1982) Biochem. Biophys. Res. Commun. **105**, 681–687
50 Rokukawa, C., Kushi, Y., Ueno, K. and Handa, S. (1982) J. Biochem. (Tokyo) **92**, 1481– 1488
51 Iwamori, M., Shimomura, J., Tsuyuhara, S. and Nagai, Y. (1984) J. Biochem. (Tokyo) **95**, 761–770
52 van Echten, G. and Sandhoff, K. (1993) J. Biol. Chem. **268**, 5341–5344
53 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132
54 Nagata, Y., Yamashiro, S., Yodoi, J., Lloyd, K. O., Shiku, H. and Furukawa, K (1992) J. Biol. Chem. **269**, 7045

Received 4 January 1994/16 May 1994; accepted 7 June 1994