The effects of the site-directed removal of N-glycosylation sites from β-1,4-*N*-acetylgalactosaminyltransferase on its function

Masashi HARAGUCHI,* Shuji YAMASHIRO,* Keiko FURUKAWA,* Kogo TAKAMIYA,* Hiroshi SHIKU† and Koichi FURUKAWA*‡

*Department of Oncology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan, and †Second Department of Internal Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu 514, Japan

The amino acid sequence deduced from the cloned human cDNA of β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T; EC 2.4.1.92) gene predicted three potential sites for N-linked glycosylation. Although many glycosyltransferases isolated contain from 2 to 6 N-glycosylation sites, their significance has not been adequately demonstrated. To clarify the roles of N-glycosylation in GalNAc-T function, we generated a series of mutant cDNAs, in which some or all of the glycosylation recognition sites were eliminated by polymerase chain reaction (PCR)-mediated site-directed mutagenesis. Using transcription/translation *in vitro*, we confirmed that all potential N-glycosylation sites could be used. Although cell lines transfected with mutant cDNAs showed equivalent levels of GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc-Cer (G_{M2}) to that of the wild-type, the extracts from mutant cDNA transfectants demonstrated lower enzyme activity than in the wild-type. The decrease in enzyme activity was more evident as the number of deglycosylated sites increased, with about 90% decrease in a totally deglycosylated mutant. The enzyme kinetics analysis revealed no significant change of K_m among wild-type and mutant cDNA products. The intracellular localization of GalNAc-T expressed in transfectants with wild-type or mutant cDNAs also showed a similar perinuclear pattern (Golgi pattern). These results suggest that N-linked carbohydrates on GalNAc-T are required for regulating the stability of the enzyme structure.

INTRODUCTION

Glycosyltransferases involved in the biosynthesis of carbohydrate structures in glycoproteins and glycolipids are resident membrane proteins of the endoplasmic reticulum and Golgi apparatus [2]. Although more than thirty glycosyltransferase genes have been isolated, the molecular basis for the subcellular organization of the glycosylation machinery and for the regulation of the expression of specific carbohydrate sequences has not been adequately demonstrated. Generally they have a characteristic topology in the Golgi apparatus, consisting of a short N-terminal cytoplasmic tail, a signal-anchor domain that spans the Golgi membrane, an extended stem region, and a large C-terminal catalytic domain oriented within the lumen of the Golgi cisternae [2]. This type II glycoprotein organization [3] is common among the glycosyltransferases cloned so far [2,4].

Except for the β-1,2-*N*-acetylglucosaminyltransferase (GnT I; EC 2.4.1.101) gene [5–7], the predicted amino acid sequences of all known glycosyltransferases indicate the presence of 2–6 potential N-glycosylation sites, most of which are in the stem region. However, the roles of these N-linked carbohydrate components in glycosyltransferases have not yet been analysed except for the study of α-2,6-sialyltransferase (ST; EC 2.4.99.1) with purified enzyme [8]. Even the actual glycosylation in glycosyltransferases has not been convincingly demonstrated.

The amino acid sequence of β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T; EC 2.4.1.92), the cDNA for which we isolated [9], predicted three potential sites for N-glycosylation, Asn-X-Thr/Ser at Asn^{79}, Asn^{179} and Asn^{274}. In contrast, comparison of the enzyme activity with the mRNA levels of the gene in various cell lines suggests that regulatory mechanisms at the translational or post-translational level such as phosphorylation and glycosylation should be of importance as well as those at transcriptional level [10].

Generally, the roles of N-glycosylation have been studied by means of the deglycosylation of purified compounds with various glycosidases [8,11,12], incubating cultured cells with various processing inhibitors such as tunicamycin [13,14], or site-directed mutagenesis of the amino acids essential for N-glycosylation [15–19]. These studies have demonstrated that carbohydrate deletion can have a variety of effects on the properties of surface molecules, secreted proteins and intracellular molecules.

In this study we generated a series of mutant cDNAs in which 1, 2 or all 3 glycosylation recognition sites were eliminated, to clarify the role of N-linked carbohydrates in the expression of GalNAc-T activity. Using expression systems *in vitro* and *in vivo*, we confirmed that all three N-glycosylation sites could be used and showed that glycosylation at these locations is important for the full expression of enzyme function.

MATERIALS AND METHODS

Site-directed mutagenesis of the human GalNAc-T cDNA and sequence analysis

All GalNAc-T cDNA mutants were constructed starting from CDM8/pM2T1-1. The amino acid substitutions in GalNAc-T cDNA were generated by using PCR as described [20], and the oligonucleotides used for PCR are listed below; substituted nucleotide positions are shown in bold type: A, 5′-CACAGC-CCGGACCGAAATTT-3′; B, 5′-CTTGGAACCAGTGCAGT-TGT-3′; C, 5′-ACAACTGCACTGGTTCCAAG-3′; D, 5′-

Abbreviations used: CHO, Chinese hamster ovary; FCM, flow cytometry; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Gal-T, β-1,4-galactosyltransferase; GalNAc-T, β-1,4-*N*-acetylgalactosaminyltransferase; GnT I, β-1,2-*N*-acetylglucosaminyltransferase; GST, glutathione S-transferase; IF, immunofluorescence; mAb, monoclonal antibody; PCR, polymerase chain reaction; RT, reverse transcription. Ganglioside nomenclature is based on that of Svennerholm [1]: G_{M2}, GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc-Cer; G_{M3}, NeuAcα2 → 3Galβ1 → 4Glc-Cer; G_{D3}, NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4Glc-Cer; G_{D2}, GalNAcβ1 → 4(NeuAcα2 → 8NeuAcα2 → 3)Galβ1 → 4Glc-Cer; NeuAc, *N*-acetylneuraminic acid.

‡ To whom correspondence should be addressed.

GTTCACCTGGTATACCTCCT-3′; E, 5′-ACAGGGTGTGG-AAGTTCAG-3′; F, 5′-TACCAGGTGCAGCTGACTGC-3′; G, 5′-GCAGTCAGCTGCACCTGGTA-3′; H, 5′-AGCCCG-TAGCCGATCATAA-3′; I, 5′-AGAGCGCTGATCTGGTAC-TG-3′.

These primers were constructed on an Applied Biosystems Model 394 DNA/RNA Synthesizer. The mutant cDNAs (MT1, MT2, MT3, MT2,3 and MT1,2,3) were constructed as shown in Figure 1c, which is described in detail below.

MT1 construction

To construct MT1 (replacing the codon for the asparagine residue at 79 by that for glutamine), two fragments containing the target gene sequence were amplified by separate PCR. Each reaction used one flanking primer that hybridized at one end of the target sequence (primer A or D in Figure 1c) and one internal primer that hybridized at the site of the mutation and contained the mismatched bases (primers B and C in Figure 1c). First-step PCR proceeded with primer A, B, C or D independently. Two fragments sharing a common site containing the mutational point were generated. The second PCR was performed with a mixture of these products as templates and the flanking primers (primer A and primer D in Figure 1c), resulting in the generation of a fusion product. This product was cut with *Sma*I and *Sna*I, and then inserted into an *Sma*I–*Sna*I site of CDM8/pM2T1-1, yielding MT1.

MT2 construction

To construct MT2 (replacing the codon for asparagine residue 179 in GalNAc-T with that for glutamine), the first-step PCR reaction used primers E, F, G and H. The second-step PCR used the products of the first-step PCR as a template, primers E and H. The fusion product was cut with *Sna*I and *Eco*47III, then inserted into a *Sna*I–*Eco*47III site of plasmid CDM8/pM2T1-1, yielding MT2.

MT3 construction

To construct MT3 (replacing the codon for asparagine residue 274 in GalNAc-T with that for glutamine), the MT3 fragment generated by PCR with primers E and I was cut with *Sna*I and *Eco*47III, then inserted into an *Sna*I–*Eco*47III site of plasmid CDM8/pM2T1-1, yielding MT3.

MT2,3 construction

To construct MT2,3 (replacing the codons for the asparagine residues 179 and 274 in GalNAc-T with those for glutamine), the first-step PCR used primers E, F, G and I. The second-step PCR used the product of the first-step PCR as a template and primers E and I. The fusion product was cut and inserted as in MT2, yielding MT2,3.

MT1,2,3 construction

To construct MT1,2,3 (replacing codons for the asparagine residues 79, 179 and 274 in GalNAc-T with those for glutamine), the *Sma*I–*Sna*I fragment of MT1 was inserted into the *Sma*I–*Sna*I site of MT2,3.

Sequencing

The full sequence of each PCR-generated fragment containing a mutation was checked. The presence of the mutations and the sequence across the vector and insert junction were confirmed by *Taq* DyeDeoxy Terminator Cycle Sequencing (Applied Biosystems).

Transcription/translation *in vitro*

The plasmid Bluescript SK(−) containing GalNAc-T wild-type or mutant cDNA was linearized downstream from the cloned cDNA insert by digestion with *Hin*dIII. Capped RNA transcripts were then generated *in vitro* from these linearized templates using a T3 polymerase promoter-based transcription kit (Stratagene). Transcripts were initiated from the T3 promoter proximal to the cDNA cloning site in Bluescript. RNA transcripts produced *in vitro* were used to programme a rabbit reticulocyte lysate translation system (Promega) in the presence of [^{35}S]methionine (Amersham) according to the manufacturer's instructions. Membrane-associated radiolabelled translation products were generated *in vitro* in the presence or absence of canine pancreatic microsomal membranes (Promega). The various radiolabelled translation products were then denatured in SDS sample buffer, and were fractionated through 12% SDS/polyacrylamide gels, which were then detected by autoradiography.

Cells and cell culture

The mouse melanoma line KF3027 containing the polyoma T antigen was prepared as described [9], and maintained in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum (FCS) and G418 (750 μg/ml). The Chinese hamster ovary cell line (CHO-K1) was obtained from the RIKEN Cell Bank (Tsukuba, Japan). MeWo, used as a recipient cell in the stable expression system, was a human melanoma line provided by Dr. L. J. Old (Sloan-Kettering Cancer Center, New York).

Transfection

KF3027 was plated 48 h before transfection in flasks (250 ml) (Greiner GmbH, Frickenhausen, Germany) at a density of 1.5×10^6 cells per flask (for enzyme assay), or 24 h before transfection in 6 cm dishes (Iwaki Glass, Tokyo) at a density of 5×10^5 cells per dish (for flow cytometry). CHO cells were plated 24 h before transfection in 10 cm dishes (Iwaki Glass) at a density of 10^6 cells per dish. Cells were transfected with GalNAc-T wild-type or mutant plasmid DNAs by using DEAE-dextran coupled with a dimethyl sulphoxide shock (Nacalai, Kyoto, Japan) as described [21]. Forty-eight hours after transfection, the cells were trypsinized and collected by centrifugation, then used for enzyme assay or flow cytometry. For the preparation of stable transfectants, MeWo cells were plated 24 h before transfection in 10 cm dishes (Iwaki Glass) at a density of 10^6 cells per dish. Cells were transfected with GalNAc-T wild-type or mutant plasmid DNAs by the calcium phosphate precipitation method [22].

Immunofluorescence

Cells were incubated with monoclonal antibodies (mAbs) for 45 min on ice and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (heavy and light chains) (Cappel). After washing, the cells were mounted in PBS on glass slides and examined under a fluorescence microscope (Nikon, Tokyo).

Flow cytometry

Glycolipid expression was analysed with mouse mAb 10-11 (anti-G_{M2}) [23], which was a gift from Dr. P. O. Livingston of the Sloan-Kettering Cancer Center. Cells were incubated with the

mAb for 45 min on ice and stained with FITC-conjugated goat anti-mouse IgM (heavy and light chains) (Cappel), then on a FACScan as previously described [9].

Enzyme assay of transient transfectants with GalNAc-T wild-type and mutant cDNAs

The membrane fractions of the transient transfectants were prepared as described by Thampoe et al. [24]. The enzyme assay was performed as described previously [10,25]. The level of radioactivity was measured in 20% of the product and the remainder was analysed by TLC and fluorography.

Reverse transcriptase PCR

Reverse transcriptase

Single-strand cDNAs of the transient transfectants were synthesized with oligo$(dT)_{14}$ primer as described [26]. Total RNAs (3 μg) were dissolved in 50 μl of 50 mM Tris/HCl (pH 8.3) containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dATP, dGTP, dCTP, dTTP, 0.5 μg oligo$(dT)_{14}$, and 200 units of Moloney murine leukaemia virus reverse transcriptase (RT) (Gibco-BRL, Grand Island, NY), then incubated for 90 min at 37 °C.

PCR

PCR was performed basically according to previous methods [10,26]. The PCR primers for the GalNAc-T gene were an M2T1-1 sense primer (GalNAc-T cDNA clone M2T1-1, nucleotides 142–161), 5′-AGGCCCGGGCTGCCAGATCT-3′, and an M2T-AS2 antisense primer (nucleotides 677–696), 5′-GGTCT-GGAAGCTTCGGCTGG-3′. Reverse transcription products (5 μl) were diluted in a 50 μl reaction mixture consisting of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (0.2 mM each), 0.002% gelatin, and PCR proceeded after the addition of 0.325 μg M2T1-1 sense primer, 0.325 μg M2T-AS2 antisense primer and 0.25 units of *Taq* DNA polymerase (Wako Junyaku, Ohsaka, Japan). Samples were amplified by 15 and 20 cycles, each of which consisted of denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C and polymerization for 2 min at 72 °C.

Southern blotting

RT–PCR products (10 μl) were resolved by electrophoresis on a 10% (w/v) polyacrylamide gel, hydrolysed with 0.4 M NaOH containing 0.6 M NaCl, then transferred to nylon membranes. These were prehybridized with [^{32}P]dCTP-labelled full-length cDNA probe as described [9], then analysed with a BAS2000 Imaging Analyzer.

Immunohistochemistry

Cells were cultured in an eight-well tissue culture chamber (Lab Tek) for 48 h. After washing, the slide with cells was incubated with mAbs for 3 h at room temperature. Immunostaining was performed with mouse monoclonal antibodies, P3 (anti-human GalNAc-T) or mAb8628 [anti-human β-1,4-galactosyltransferase (Gal-T); EC 2.4.1.38], used at dilutions of 1:200 and 1:2000, respectively. MAbP3 was generated by immunization of mice with glutathione S-transferase (GST)-fusion protein with some portion of human GalNAc-T (M. Haraguchi, Y. Nagata, H. Shiku and K. Furukawa, unpublished work). MAb8628 was provided by Dr. Narimatsu (Soka University, Tokyo, Japan). Staining was performed with 0.2 mg/ml diaminobenzidine tetrahydrochloride (Nacalai, Kyoto, Japan) and 0.005% H_2O_2.

RESULTS

Construction of mutant GalNAc-T

We predicted that in GalNAc-T the asparagine residues in amino acid positions 79, 179 and 274 were sites of recognition sequences (Asn-X-Thr/Ser) for the potential attachment of N-linked oligosaccharide chains (Figure 1a). We generated five kinds of mutated GalNAc-T cDNAs by site-directed mutagenesis with PCR, in which the asparagine residues of one, two or all three of the potential N-glycosylation sites were replaced by glutamine (Figures 1b and 1c). The sequence Gln-X-Thr/Ser is not a substrate for N-glycosylation. Mutants 1–3 (MT1–3) were missing one of the N-glycosylation sites. Mutant 2,3 (MT2,3) lacked two of the three, and mutant 1,2,3 (MT1,2,3) lacked all three sites. These cDNAs were cloned into the expression vector pCDM8, as the original wild- type clone pM2T1-1.

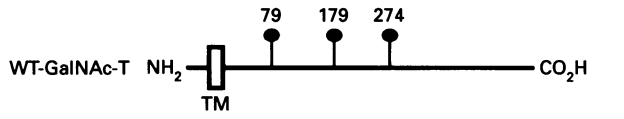
In vitro transcription of wild-type and mutant cDNAs

To confirm the presence of N-glycosylation at the predicted sites, wild-type and mutant GalNAc-T complementary RNAs (cRNAs) were prepared and analysed *in vitro* by a series of transcription and translation experiments. The cRNAs produced from wild-type and all mutant cDNA templates migrated as a 2.55 kb band in agarose gel (Figure 2a).

In vitro translation of wild-type cRNA

The [^{35}S]methionine-labelled primary translation products of wild-type GalNAc-T generated by translation *in vitro* migrated with a molecular mass of 59.5 kDa (Figure 2b). When this radiolabelled protein was generated by translation *in vitro* in the

(a)



(b)

Construct	Amino acid change	DNA codon mutation
WT-GalNAc-T	None	None
MT1-Gln79	Asn to Gln	AAC to CAG
MT2-Gln179	Asn to Gln	AAC to CAG
MT3-Gln274	Asn to Gln	AAC to CAG
MT2, 3-Gln179, 274	Asn to Gln	AAC to CAG
MT1, 2, 3-Gln79, 179, 274	Asn to Gln	AAC to CAG

(c)

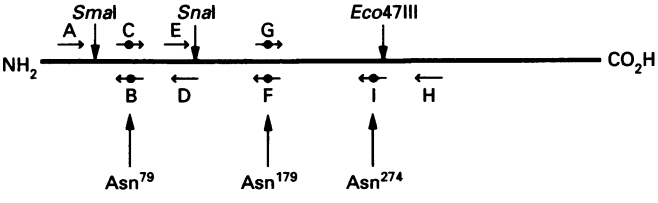


Figure 1 N-glycosylation sites in GalNAc-T and the strategy for mutant construction

(**a**) Site of potential N-glycosylation in GalNAc-T. All three sites are in the intraluminal domain of the Golgi apparatus. Numbers indicate the positions of the asparagine residues in the N-glycosylation sites in the wild-type GalNAc-T, as previously reported [8]. (**b**) Mutant GalNAc-T cDNA plasmids constructed in this work. (**c**) Schematic diagram of site-directed mutagenesis by overlap extension. The enzyme sites used for insertion are indicated by arrows. The sites of mutagenesis are indicated by small filled circles. Arrows represent the various PCR primers used (A–I).

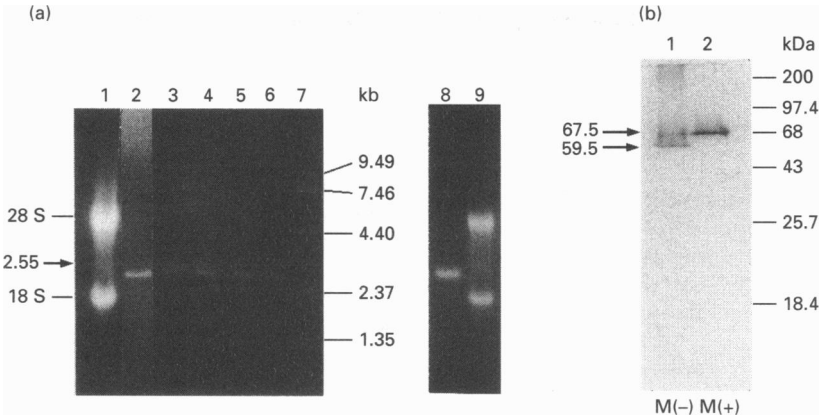


Figure 2 Transcription and translation of cDNAs *in vitro*

(**a**) Transcription of wild-type and mutant cDNAs *in vitro*. Wild-type and mutant cRNA were synthesized as described in Materials and methods. They were separated in 1.25% (w/v) agarose/formaldehyde gel and stained with ethidium bromide. Lane 1, control (total RNA); lane 2, wild-type; lane 3, MT1 ($Asn^{79} \rightarrow Gln$); lane 4, MT2 ($Asn^{179} \rightarrow Gln$); lane 5, MT3 ($Asn^{274} \rightarrow Gln$); lane 6, MT2,3 ($Asn^{179}$, $Asn^{274} \rightarrow Gln$); lane 7, marker; lane 8, MT1,2,3 (Asn^{79}, Asn^{179} and $Asn^{274} \rightarrow Gln$); lane 9, control. (**b**) Translation of wild-type cRNA *in vitro*. The translation products labelled with [^{35}S]methionine were fractionated by SDS/PAGE and revealed by autoradiography as shown. Translation proceeded in the absence (lane 1) or presence (lane 2) of canine pancreatic microsomes (M).

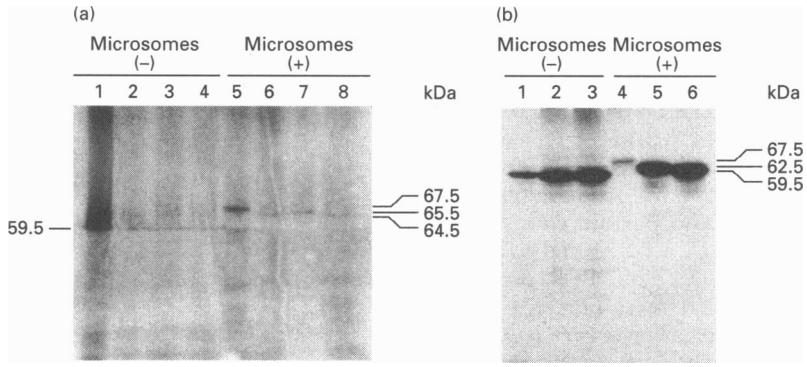


Figure 3 Translation of wild-type and mutant cRNAs *in vitro*

Autoradiograph of [^{35}S]methionine-labelled *in vitro* translation products fractionated by SDS/PAGE. (**a**) Translations were done in the absence (lanes 1–4) or presence (lanes 5–8) of canine pancreatic microsomes. Lanes 1 (59.5 kDa) and 5 (67.5 kDa), wild-type; lanes 2 (59.5 kDa) and 6 (64.5 kDa), MT1; lanes 3 (59.5 kDa) and 7 (65.5 kDa), MT2; lanes 4 (59.5 kDa) and 8 (64.5 kDa), MT3. (**b**) Translations in the absence (lanes 1–3) or presence (lanes 4–6) of canine pancreatic microsomes. Lanes 1 (59.5 kDa) and 4 (67.5 kDa), wild-type; lanes 2 (59.5 kDa) and 5 (62.5 kDa), MT2,3; lanes 3 (59.5 kDa) and 6 (59.5 kDa), MT1,2,3. The sizes of the bands were estimated from the standard curve based on the prestained molecular mass standards, high range (Life Technologies, Inc., Gaithersburg, MD, U.S.A.).

presence of canine pancreatic microsomes, it migrated more slowly, with a molecular mass of 67.5 kDa (Figure 2b). This 8 kDa increase in molecular mass that occurred when the translation proceeded in the presence of microsomes, suggested that core glycosylation structures were added by microsomal oligosaccharyltransferases to some or all of the potential asparagine-linked glycosylation sites during co-translational translocation across the microsomal membrane.

Translation of mutant cRNAs *in vitro*

We analysed the translation products of all mutants with or without microsomes (Figure 3). When generated by translation *in vitro* without microsomes, all products migrated with a molecular mass of 59.5 kDa, as did the wild-type. In contrast, when generated by translation *in vitro* with microsomes, the MT1, MT2, MT3 and MT2,3 products migrated with molecular masses of 64.5, 65.5, 64.5 and 62.5 kDa respectively. However, the MT1,2,3 product migrated in the same manner as that produced without microsomes at 59.5 kDa. The translation products *in vitro* in the presence of microsomes using any mutant cRNA had smaller molecular masses than that of the wild-type: MT1, MT3, MT2, MT2,3 and MT1,2,3 products were 3, 3, 2, 5 and 8 kDa smaller than the wild-type product, respectively (Figures 3a and 3b). The consecutive loss of one, two and three N-glycosylation sites resulted in a stepwise decrease in the apparent molecular mass. These results suggested that the migration differences between wild-type and mutant products were indeed due to differences in the number of N-glycosylations. The amounts of the translation products were markedly different between mutants and wild-type (Figures 3a and 3b), although the same amounts of cRNA were used. This might be because the quality of cRNA preparations and the quality of the reagents for translation *in vitro* were not uniform. We concluded that all three

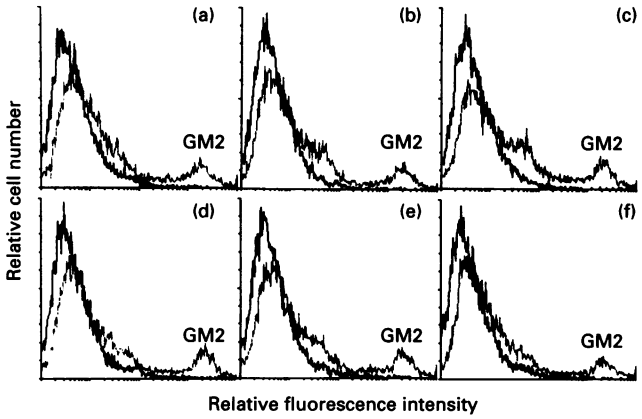


Figure 4 Flow cytometry of G_{M2} expression on KF3027 cells after transfection with GalNAc-T cDNA, wild-type or mutants

Cells were incubated with anti-G_{M2} antibody 10-11, then stained with FITC-conjugated goat anti-mouse IgM (thin lines). Bold lines indicate the profile of the controls in which the cells were stained with FITC-conjugated goat anti-mouse IgM alone. The small peak at the right of each profile is G_{M2}. (**a**) Wild-type; (**b**) MT1; (**c**) MT2; (**d**) MT3; (**e**) MT2,3; (**f**) MT1,2,3.

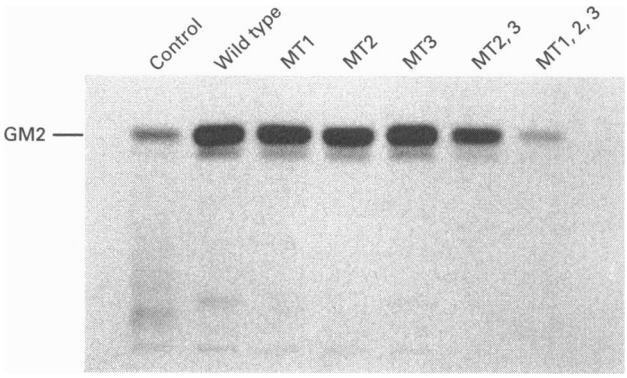


Figure 5 Products of the GalNAc-T enzyme assay on the cell extracts transfected with wild-type or mutant cDNAs

The TLC profile of the radioactive enzyme assay products with membranes (50 μg) from KF3027 transfected with wild-type or mutant GalNAc-T cDNAs is shown. Precursor ganglioside, G_{M3} (20 μg), was used as an acceptor in the enzyme reaction as described in Materials and methods. The products were applied to TLC and developed in chloroform/methanol/0.22% $CaCl_2$ (55/45/10 by vol.), then exposed to X-ray film.

potential N-glycosylation sites in GalNAc-T can be glycosylated, and that no other N-glycosylation sites were detectable in the wild-type or mutants.

Ganglioside expression on transfectants of wild-type and mutant cDNAs

To compare enzyme activities between the products of wild-type and glycosylation mutant cDNAs, we transfected wild-type and mutant cDNAs into KF3027, a mouse melanoma cell line, and examined them for surface expression of G_{M2} by flow cytometry (FCM) using the anti-G_{M2} mAb 10-11. As shown in Figure 4, FCM analysis revealed that the cell-surface G_{M2} was expressed equally well and a significant difference in G_{M2} expression was not recognized in cells transfected with wild-type or mutant

Table 1 Comparison of the enzyme activity in transient transfectants of wild-type and mutant cDNAs

Upper and lower panels are results of independent experiments.

	A	B		
Construct	IF-positive* (%)	Enzyme activity (pmol/mg protein per h)	B/A†	Percentage activity‡
WT	13.0	14436	1110	100.0
MT1	22.8	9669	424	38.2
MT2	19.2	10068	524	47.2
MT3	19.2	11808	615	55.4
MT2,3	15.6	5764	369	33.2
MT1,2,3	10.0	1189	119	10.7
WT	9.6	19244	2005	100.0
MT1	9.8	5896	602	30.0
MT2	7.8	5929	760	37.9
MT3	8.1	11427	1410	70.3
MT2,3	12.6	6578	522	26.0
MT1,2,3	8.1	1418	175	8.7

* IF-positive cells were calculated by counting the number of immunofluorescing cells per 500 cells.

† B/A is the ratio of enzyme activity to IF-positive cells, to compare the enzyme activity per positive cell between wild-type and mutants.

‡ Percentage activity is the ratio of enzyme activity of wild-type or mutants to that of the wild-type.

cDNAs. In addition to KF3027, we compared the enzyme activity in CHO cells by FCM after transfecting various cDNAs: no significant differences were detected (results not shown).

Enzyme activity of the tranfectants

The levels of expressed GalNAc-T activity were compared in membrane fractions from KF3027 transiently transfected with wild-type or mutant cDNAs. Enzyme products under standard conditions were analysed by TLC; the results are shown in Figure 5. The enzyme activities of MT2,3 and MT1,2,3 products were markedly lower than that of the wild-type product (Figure 5). Table 1 summarizes these results. Transfectants of MT1, MT2 and MT3 expressed 30–70% less activity than that of wild-type cDNA. Transfectants of MT2,3 decreased by about 70% and transfectants of MT1,2,3 decreased markedly, by about 90%. These results suggest that N-linked oligosaccharide chains have an important role in the enzyme activity of GalNAc-T.

Kinetic studies of GalNAc-Ts in the transfectants of wild-type and mutant cDNAs

To examine whether the difference in the enzyme activity among various transfectants is due to an alteration in their affinity for the substrate, a kinetic analysis was performed. As shown in Figure 6a, different saturation curves were obtained for GalNAc transfer among the wild-type and mutant transfectants. These results show a difference for the apparent $V_{max.}$ of GalNAc-Ts in the transfectants of wild-type and mutant cDNAs, with the MT1,2,3 mutant having the lowest value. On the other hand, they had similar K_m values for GalNAcβ1 → 4(NeuAcα2 → 3)Galβ1 → 4Glc-Cer (G_{M3}) (Figure 6b). Double-reciprocal plots revealed that the K_m values of wild-type, MT1, MT2, MT3, MT2,3 and MT1,2,3 were 73.3, 70.6, 71.6, 63.1, 109.7 and 88.7 μM respectively. We therefore concluded that there were no

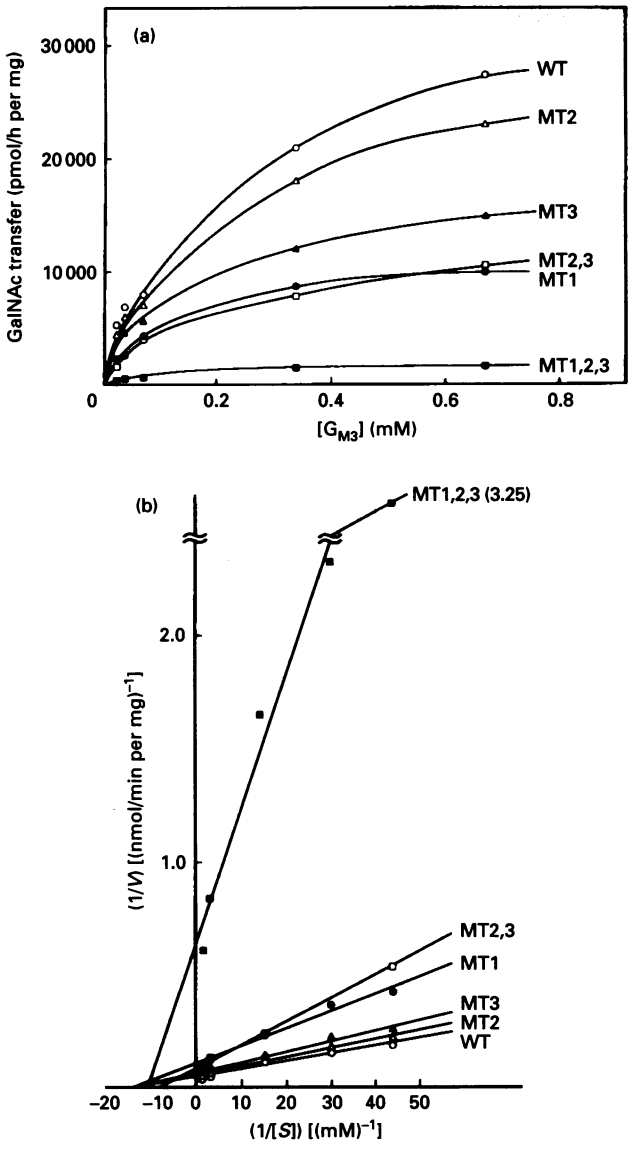


Figure 6 Kinetic analysis of wild-type and mutant cDNAs

(**a**) Increasing concentrations of G_{M3} were incubated with UDP-[^{14}C]GalNAc, membrane fractions (150 μg) from the wild-type and mutant cDNAs for 3 h at 37 °C. GalNAc transfer at the various concentrations of G_{M3} is shown. (**b**) Enzyme activity measured at various concentrations of G_{M3} expressed as double-reciprocal plots for each membrane fraction of the wild-type and mutant cDNAs. Symbols: ○, wild-type; ●, MT1; △, MT2; ▲, MT3; □, MT2,3; ■, MT1,2,3.

significant differences in the affinity for the acceptor among GalNAc-Ts of the wild-type and N-glycosylation mutants.

RT–PCR/Southern blotting

The expression levels of transfected GalNAc-T cDNAs in the various cells were compared by semiquantitative RT–PCR; 15 and 20 cycles of amplification were performed. As shown in Figure 7, no significant differences were detected between the amounts of RT–PCR products from GalNAc-T wild-type and mutant mRNAs. This suggests that the difference in the enzyme activities between the transfectants of GalNAc-T wild-type and mutant cDNAs is not due to a difference in the expression levels of the mRNAs.

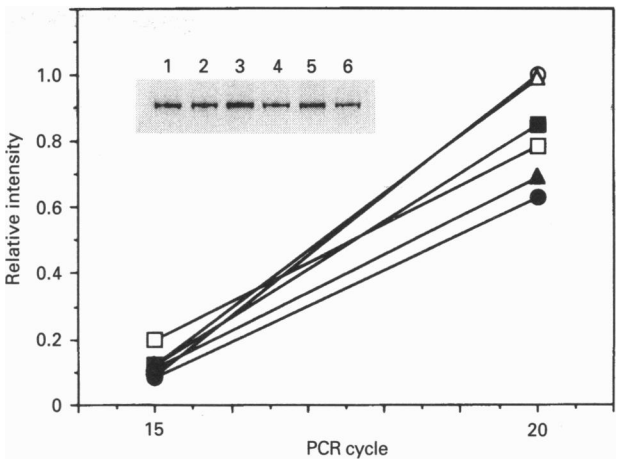


Figure 7 Semiquantitative analysis of GalNAc-T wild-type and mutant mRNAs by RT–PCR

The PCR products of 15 and 20 cycles were analysed by Southern blotting and quantified by an image analyser as described in Materials and methods. The cDNA insert of M2T1-1 clone was used as a probe after ^{32}P-labelling with Multiprime DNA labelling system (Amersham). Symbols: ○, wild-type; ●, MT1; △, MT2; ▲, MT3; □, MT2,3; ■, MT1,2,3. Inset is a Southern blot of 15-cycle PCR products. Lane 1, wild-type; lane 2, MT1; lane 3, MT2; lane 4, MT3; lane 5, MT2,3; lane 6, MT1,2,3.

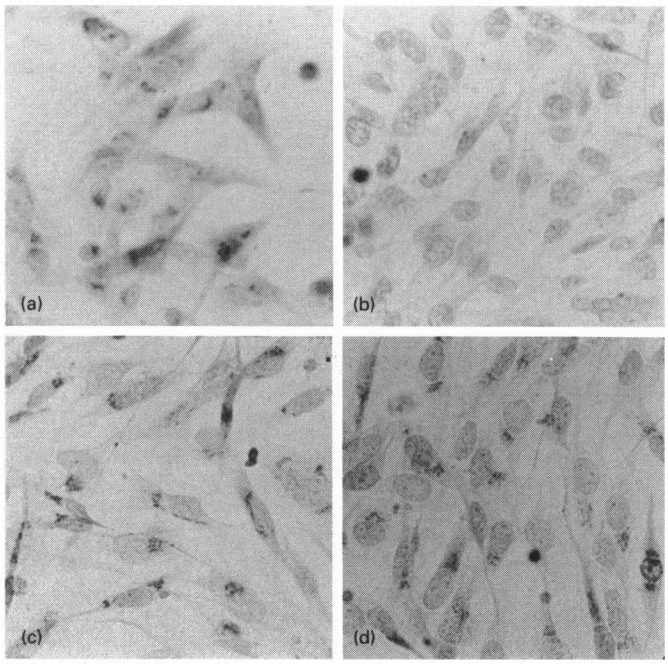


Figure 8 Immunohistochemical localization of GalNAc-T in MeWo and a stable transfectant line

MeWo was stained with mAb8628 (anti-human Gal-T) (**a**) and mAbP3 (anti-human GalNAc-T) (**b**) as described in Materials and methods. MeWo transfected with GalNAc-T wild-type cDNA (**c**) and mutant cDNA (MT3) (**d**) were stained with mAbP3.

Localization of GalNAc-T in stable transfectants

The intracellular distribution of GalNAc-T in MeWo and stable transfectants was analysed by immunohistochemical techniques (Figure 8). Staining of transfectant cells of wild-type cDNA by

anti-human GalNAc-T mAbP3 showed a juxtanuclear staining pattern (Figure 8c). It was characteristic of the Golgi complex as shown in Figure 8a, in which MeWo (a parent cell) was stained with mAb8628 (anti-human Gal-T mAb). Similar results were observed in a transfectant of a mutant cDNA (MT3) (Figure 8d) and in other transfectants (results not shown). No immunostaining was observed in MeWo (Figure 8b), as expected. The transient transfectants of the wild-type and mutant cDNAs of GalNAc-T also showed similar staining patterns (results not shown). These results indicated that the intracellular localization of GalNAc-T protein in the transfectants of the wild-type or mutant cDNAs of GalNAc-T were very similar, irrespective of the different levels of enzyme activity.

DISCUSSION

Except for GnT I [5–7], almost all glycosyltransferases studied so far contain 2–6 N-glycosylation sites in the stem region, on the basis of the predicted amino acid sequences. This fact suggests that those N-linked carbohydrates are required for the expression of glycosyltransferase function, and also that they may not be directly involved in the catalytic reaction because the stem region is separate from the catalytic domain [2]. No data on the significance of N-glycosylation in glycosyltransferases are available except for Galβ1,4GlcNAc-α-2,6-sialyltransferase [8]. Even the presence of carbohydrate chains in glycosyltransferases has not been demonstrated, except for the Lewis blood group α(1,3/1,4)-fucosyltransferase, for which N-glycosylation was verified by a transcription/translation assay *in vitro* [27].

Using similar *in vitro* experiments, we examined whether all three potential N-glycosylation sites on GalNAc-T could be used. Although the actual occurrence of glycosylation remains to be demonstrated, our data strongly suggest that these potential sites were N-glycosylated. Our experiments showed that a 59.5 kDa protein was generated from both wild-type and mutant cRNA by translation *in vitro* in the absence of canine pancreas microsomes, whereas in the presence of the microsomes a new band with a larger molecular mass (67.5 kDa) was obtained from the wild-type cRNA. When microsomes from mutant cells were added to the reaction mixture, several different bands were identified, indicating that genetic deglycosylation resulted in a loss of molecular mass of 2.0–3.0 kDa per glycosylation site. This value seemed to correspond with single core glycosylation.

When KF3027 cells were transfected with the mutant cDNAs, the expression of G_{M2} was equally high in all five transfectants. There were no significant differences in the intensity of the fluorescence in flow cytometry between wild-type and mutant transfectants. This was also true of transfected CHO cells. In contrast, the enzyme activities in the membrane fractions of mutant transfectants were apparently lower than that of the wild-type in repeated experiments. Because the transfection efficiency was very similar and the expression levels of the transfected cDNAs were almost equivalent, the difference in enzyme activity may be due to post-translational modifications or other cellular factors. Transient transfectants with wild-type or mutant cDNAs may have shown equal levels of G_{M2} expression because the catalytic reaction in each transfectant reached a plateau owing to the limitation of components other than the enzyme itself.

Elimination of any one of three N-glycosylation sites resulted in a significant, but not marked, decrease in the enzyme activity. Deletion of two sites resulted in a $\sim$ 70% decrease, and complete deglycosylation reduced the enzyme activity to $\sim$ 10% of that of the wild-type. These results indicate that N-glycosylation at any of the three potential sites significantly contributed to the full expression of GalNac-T activity, and a combination of all three N-glycosylations can give rise to optimal enzymic function.

Several studies have addressed the role of the Asn-linked carbohydrates on various functional proteins. Chemical or enzymic methods have been used to remove all or part of the carbohydrate chain. However, in those studies incomplete deglycosylation or denaturation of the proteins may have occurred, and the elimination of individual N-glycosylations between multiple sites is quite difficult. Tunicamycin, which blocks the synthesis of the lipid–carbohydrate carrier complex, cannot remove the glycosylation at particular sites, and this drug may have general effects on cell culture. The use of PCR-mediated site-directed mutagenesis [20] enabled us to evaluate the contribution of individual N-linked oligosaccharide chains more easily than the original method, which used uracil-containing DNA templates and M13 cloning vectors [28].

Several biological functions have been assigned to carbohydrates attached to proteins, such as cell-surface receptors for antigens and secreted hormones. The carbohydrates of glycoproteins are required for the biosynthesis of the proteins including correct folding during translation, correct sorting and intracellular processing [3,19,29–32], and for correct secretion from cells [17,18]. They are also needed to increase the stability of proteins resulting in resistance to degradation or in enhancement of survival in the circulation [2,11,15,17,18,33]. Their roles in the binding of ligands and subsequent intracellular signal transduction are also critical [13,15,16,34]. As for enzyme molecules, deglycosylation of some enzymes results in a decrease in the efficiency with which they act on substrate [8,18], although detailed mechanisms remain to be clarified.

The molecular mechanisms by which the elimination of single or multiple N-glycosylation sites in GalNAc-T resulted in an additive decrease of enzyme activity remain unclear. The kinetic analysis revealed that the K_m did not significantly change after mutation of the N-glycosylation sites, although the apparent $V_{max.}$ values varied between wild-type and mutant transfectants. These results suggested that N-linked carbohydrates are not directly involved in the interaction of the enzyme with substrates; rather they may help regulate the stability of the enzyme structure and are required for high enzyme activity. In studies on α-2,6-sialyltransferase using purified enzyme and exoglycosidases, Fast et al. [8] also reported that the presence of the trimannose core with GlcNAc attached is important for the expression of catalytic activity. An alternative possibility is that N-glycosylation serves a role in the correct synthesis and sorting of the enzyme to the Golgi membrane. In the immunostaining of GalNac-T in the stable transfectants as well as in transiently transfected cells, a similar staining of the Golgi pattern was observed in any transfectants, suggesting that there was no difference in enzyme sorting and localization between the transformants with wild-type and mutant cDNAs. Biochemical analysis of the subcellular localization of wild-type and mutant GalNac-T enzymes was difficult because few clones for multiple-site mutants could be obtained in spite of repeated attempts (results not shown). A similar problem was reported by Velan et al. [19] for the generation of transfectants with N-glycosylation mutants of human acetylcholinesterase (EC 3.1.1.7) cDNA. In summary, this study has demonstrated that all three N-glycosylation sites can be used in GalNac-T, and all three glycosylations are required for the stable conformation of GalNac-T and for the full expression of the catalytic activity of the enzyme. However, further studies are needed to address the precise role of each N-linked carbohydrate chain and the mechanism by which it affects enzyme activity.

We thank Dr. T. Maita for helpful discussion, and Dr. H. Narimatsu for mAb8628. We also thank T. Shimomura for excellent technical assistance and Y. Nakaji for preparation of the manuscript. This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, by a grant from the Ministry of Health and Welfare of Japan, and by a Grant-in-aid from the Nissan Foundation.

REFERENCES

1 Svennerholm, L. (1963) J. Neurochem. **10**, 613–623
2 Paulson, J. C. and Colley, K. J. (1989) J. Biol. Chem. **264**, 17615–17618
3 Wickner, W. T. and Lodish, H. F. (1985) Science **230**, 400–407
4 Joziasse, D. H. (1992) Glycobiology **2**, 271–277
5 Kumar, R., Yang, J., Larsen, R. D. and Stanley, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9948–9952
6 Sarkar, M., Hull, E. and Nishikawa, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 234–238
7 Pownall, S., Kozak, C. A. and Schachter, H. (1992) Genomics **2**, 669–704
8 Fast, D. G., Jamieson, J. C. and McCaffrey, G. (1993) Biochim. Biophys. Acta **1202**, 325–330
9 Nagata, Y., Yamashiro, S., Yodoi, J., Lloyd, K. O., Shiku, H. and Furukawa, K. (1992) J. Biol. Chem. **267**, 12082–12089
10 Yamashiro, S., Ruan, S., Furukawa, K., Tai, T., Lloyd, K. O., Shiku, H. and Furukawa, K. (1993) Cancer Res. **53**, 5395–5400
11 Higuchi, M., Oh-eda, M., Kuboniwa, H., Tomonoh, K., Shimonaka, Y. and Ochi, N. (1992) J. Biol. Chem. **267**, 7703–7709
12 Nag, B., Passmore, D., Kendrick, T., Bhayani, H. and Sharma, S. D. (1992) J. Biol. Chem. **267**, 22624–22629
13 Neefjes, J. J., DeBruijn, M. L. H., Boog, C. J. P., Nieland, J. D., Boes, J., Melief, C. J. M. and Ploegh, H. L. (1990) J. Exp. Med. **171**, 583–588
14 Koyama, T. and Hughes, R. C. (1992) J. Biol. Chem. **267**, 25939–25944
15 Matzuk, M. M. and Boime, I. (1988) J. Cell Biol. **106**, 1049-1059
16 Wright, A., Tao, M., Kabat, E. A. and Morrison, S. L. (1991) EMBO J. **10**, 2717–2723
17 Yamaguchi, K., Akai, K., Kawanishi, G., Ueda, M., Masuda, S. and Sasaki, R. (1991) J. Biol. Chem. **266**, 20434–20439
18 O, K., Hill, J. S., Wang, X., Mcleod, R. and Pritchard, P. H. (1993) Biochem. J. **294**, 879-884
19 Velan, B., Kronman, C., Ordentlich, A., Flashner, Y., Leitner, M. and Cohen, S. (1993) Biochem. J. **296**, 649–656
20 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Gene **77**, 51–59
21 Davis, L. G., Dibner, M. D. and Battey, J. (1986) in Basic Methods in Molecular Biology, pp. 290–292, Elsevier Science Publishing, New York
22 Davis, L. G., Dibner, M. D. and Battey, J. (1986) in Basic Methods in Molecular Biology, pp. 286–289, Elsevier Science Publishing, New York
23 Natoli, E. J., Livingston, P. O., Pukel, C. S., Lloyd, K. O., Wiegandt, H., Szalay, J., Oettgen, H. F. and Old, L. J. (1986) Cancer Res. **46**, 4116–4120
24 Thampoe, I. J., Furukawa, K., Vellve, E. and Lloyd, K. O. (1989) Cancer Res. **49**, 6258–6264
25 Ruan, S. and Lloyd, K. O. (1992) Cancer Res. **52**, 5725–5731
26 Furukawa, K., Furukawa, K. and Shiku, H. (1991) FEBS Lett. **295**, 141–145
27 Kurowska-Latallo, J. F., Larsen, R. D., Nair, R. P. and Lowe, J. B. (1990) Genes Dev. **4**, 1288–1303
28 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. **82**, 488–492
29 Tifft, C. J., Proia, R. L. and Camerini-Otero, R. D. (1992) J. Biol. Chem. **267**, 3268–3273
30 Gieselmann, V., Schmidt, B. and von Figura, K. (1992) J. Biol. Chem. **267**, 13262–13266
31 Qu, S. J., Fan, H.-Z., Blanco-Vaca, F. and Pownall, H. (1993) Biochemistry **32**, 8732–8736
32 Schinkel, A. H., Kemp, S., Dollé, M., Rudenko, G. and Wagenaar, E. (1993) J. Biol. Chem. **268**, 7474–7481
33 Kretz, K. A., Carson, G. S., Morimoto, S., Kishimoto, Y., Fluharty, A. L. and O'Brien, J. S. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2541–2544
34 Recny, M. A. et al. (1992) J. Biol. Chem. **267**, 22428–22434

Received 13 April 1995/23 June 1995; accepted 20 July 1995