Carbohydrate Research, 236 (1992) 281–299
Elsevier Science Publishers B.V., Amsterdam

Control of glycoprotein synthesis. Characterization of (1 → 4)-*N*-acetyl-β-D-glucosaminyltransferases acting on the α-D-(1 → 3)- and α-D-(1 → 6)-linked arms of *N*-linked oligosaccharides *,**

Inka Brockhausen [a,b], Gabriele Möller [a], Ji-Mao Yang [a], Shaheer H. Khan [c,1], Khushi L. Matta [c], Hans Paulsen [d], Arthur A. Grey [e], Rajan N. Shah [e] and Harry Schachter [a,b]

[a] *The Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8 (Canada)*
[b] *Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8 (Canada)*
[c] *Roswell Park Memorial Institute, Buffalo, New York (USA)*
[d] *Organisch-Chemisches Institut der Universität Hamburg, 2000 Hamburg 13 (Germany)*
[e] *Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8 (Canada)*

(Received June 26th, 1991; accepted in revised form April 28th, 1992)

ABSTRACT

Hen oviduct membranes contain at least three *N*-acetyl-β-D-glucosaminyltransferases (GlcNAc-T) that attach a βGlcNAc residue in (1-4)-linkage to a D-Man*p* residue of the *N*-linked oligosaccharide core, i.e., (1 → 4)-β-D-GlcNAc-T III which adds a "bisecting" GlcNAc group to form the β-D-GlcpNAc-(1 → 4)-β-D-Man*p*-(1 → 4)-D-GlcNAc moiety; (1 → 2)-β-D-GlcNAc-T IV which adds a GlcNAc group to the (1 → 3)-α-D-Man arm to form the β-D-Glc*p*NAc-(1 → 4)-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 → 4)-D-Glc*p*NAc component; and (1 → 4)-β-D-GlcNAc-T VI which adds a GlcNAc group to the α-D-Man*p* residue of β-D-Glc*p*NAc-(1 → 6)-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p*-R to form β-D-Glc*p*NAc-(1 → 6)-[β-D-Glc*p*NAc-(1 → 4)]-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p*-R. We now report a novel (1 → 4)-β-D-GlcNAc-T activity (GlcNAc-T VI′) in hen oviduct membranes that transfers GlcNAc to β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)-β-D-Man*p*-R to form β-D-Glc*p*NAc-(1 → 4)-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p*-(1 → 6)-β-D-Man*p*-R. The structure of the enzyme product was confirmed by ^{1}H NMR spectroscopy, FAB-mass spectrometry and methylation analysis. Previous work with GlcNAc-T IV was carried out with biantennary substrates; we now show that hen oviduct membrane GlcNAc-T IV can also transfer GlcNAc to monoantennary β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 3)-β-

Correspondence to: Dr. I. Brockhausen, The Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

* Dedicated to Professor Jean Montreuil.

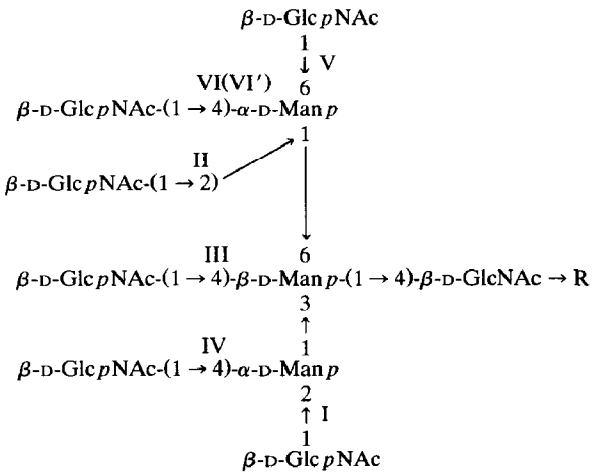
** This research was supported by grants from the Medical Research Council of Canada (to H.S. and I.B.), and grants MT-3732 and MA-6499 (to J.P. Carver, University of Toronto, Toronto, Canada), from the National Institute of Health (grant No. CA 35329 to K.L.M.), from the Deutsche Forschungsgemeinschaft (to H.P.), and by a NATO grant for international collaboration in research (to H.P. and I.B.).

[1] Present address: Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada.

D-Man*p*-R to form β-D-Glc*p*NAc-(1 → 4)-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p*-(1 → 3)-β-D-Man*p*-R. The findings that GlcNAc-T VI′ and IV have similar kinetic characteristics and that hen oviduct membranes can convert methyl β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p* to methyl β-D-Glc*p*NAc-(1 → 4)-[β-D-Glc*p*NAc-(1 → 2)]-α-D-Man*p* suggest that these two activities may be due to the same enzyme. The R-group of the β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)-β-D-Man*p* (or Glc*p*)-R substrate has an important influence on GlcNAc-T VI′ enzyme activity. When R is GlcNAc or βGlc-allyl, the activity is drastically reduced. This may be due to conformational factors and may explain why hen oviduct membranes add a GlcNAc residue in (1 → 4)-β-linkage mainly to the (1 → 3)-α-D-Man arm of the bi-antennary substrate β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)-[β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 3)]-β-D-Man*p*-R to form β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)-{β-D-Glc*p*NAc-(1 → 2)-[β-D-Glc*p*NAc-(1 → 4)]-α-D-Man*p*-(1 → 3)}-β-D-Man*p*-R.

INTRODUCTION

The branches of *N*-glycans are assembled in a controlled fashion by the sequential action of *N*-acetyl-D-glucosaminyltransferases (GlcNAc-T) I to VI[1,2] (see Scheme 1). We recently described[3] an enzyme (GlcNAc-T VI) from hen oviduct that links a (1 → 4)-βGlcNAc* to the αMan residue of βGlcNAc → 6(βGlcNAc → 2)αMan → R substrates. There are at least two other (1 → 4)-βGlcNAc-Ts in hen oviduct that transfer a βGlcNAc unit to O-4 of a Man residue in *N*-glycans, i.e., GlcNAc-T IV[4] (acting on the (1 → 3)-linked arm) and GlcNAc-T III[5] (which incorporates the "bisecting" GlcNAc group linked (1 → 4) to the βMan residue). GlcNAc-T V adds a (1 → 6)-linked βGlcNAc unit to the (1 → 6)-linked arm to form βGlcNAc → 6(βGlcNAc → 2)αMan → 6R branches. These enzymes exhibit very different kinetic properties. GlcNAc-T V does not require $MnCl_2$, GlcNAc-T III and IV work optimally in the presence of 10–20 mM $MnCl_2$ and GlcNAc-T VI is optimally active under unusually high $MnCl_2$ concentrations.



Scheme 1. Mode of action of GlcNAc-transferases I–VI (VI′).

* All sugars discussed have the D configuration and are in the pyranose form.

GlcNAc-T VI is also unusual in that it can act[3] on compounds containing the "bisecting" GlcNAc group [linked (1 → 4) to the βMan unit].

We have reported[3] that hen oviduct membranes can incorporate a (1 → 4)-linked βGlcNAc unit into the αMan unit of the linear, monoantennary structure βGlcNAc → 2αMan → 6βMan (or Glc) → R; we term this activity GlcNAc-T VI′. Hen oviduct membranes cannot incorporate a (1 → 4)-linked βGlcNAc unit into the (1 → 6)-linked arm of the biantennary substrate βGlcNAc → 2αMan → 6[βGlcNAc → 2αMan → 3]βMan → R, i.e., GlcNAc-T VI′ does not appear to act on a biantennary acceptor in vitro[3,4]. The βGlcNAc → 4[βGlcNAc → 2]αMan → 6βMan → R component has, however, been described in *N*-glycans isolated from hen ovomucoid[6]. We report herein the properties and tissue distribution of GlcNAc-Ts VI′ and IV. The results suggest that the activities may be due to the same enzyme in hen oviduct. This enzyme appears to lose its branch specificity when presented with linear, monoantennary acceptors.

EXPERIMENTAL

Materials.—The following materials were purchased: AG 1-X8 and Bio-Gel P-4 (−400 mesh) from Bio-Rad; Triton X-100 and UDP-GlcNAc from Sigma. UDP-*N*-acetyl-[1-^{14}C]glucosamine (5 μCi/μmol) was synthesized as described previously[7]. The compounds obtained by chemical synthesis are listed in Table I. Compounds **5**, **6**, **7**, **8**, and **13** were synthesized as follows.

*Methyl O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 2)-α-D-mannopyranoside** (**5**).—A mixture of methyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside[15,16] (**24**, 0.2 g, 0.43 mmol), 2,4,6-trimethylpyridine (0.13 g, 1.07 mmol), silver triflate (0.28 g, 1.1 mmol), and powdered molecular sieve 4A (0.5 g) in CH_2Cl_2 (10 mL) was stirred under Ar at −40°. After 5 min, a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[17] (**25**; 0.26 g, 0.52 mmol) in CH_2Cl_2 (5 ml) was added slowly and the solution was stirred for 18 h at room temperature. The solids were filtered off, the filtrate was washed with cold water, aq M HCl, satd aq $NaHCO_3$, and water. The organic solvents were evaporated and the syrupy residue was subjected to column chromatography on silica gel to yield methyl *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1 → 2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranoside (**26**) (0.12 g, 32%). This compound was deprotected stepwise by hydrogenation in EtOH containing Pd/C (to remove benzyl group), by reflux with hydrazine hydrate in EtOH (to remove phthalimido and acetyl group), and acetylated with acetic anhydride in pyridine at room temperature. The peracetylated compound was purified by chromatography on a column of silica gel using hexane–EtOAc (3:1) and EtOAc as eluents to give methyl

* Compounds **5**, **6**, **7**, **8**, and **13** were analyzed by ^{1}H NMR spectroscopy, TLC and HPLC, amino sugar analysis, and high resolution mass spectrometry, thus ascertaining their identity. They were not subjected to elemental analysis to verify their purity (Editor).

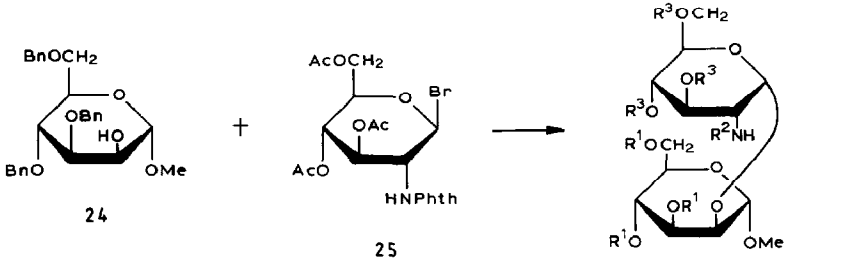
TABLE I

Oligosaccharides chemically synthesized or biochemically obtained

Cpd. No.	Structure [a]	Ref.
1	GlcNAc → 2Man → 6βMan → 4GlcNAc	14
2	GlcNAc → 2Man → 6βGlc → 4GlcNAc	14
3	GlcNAc → 2Man → 6βGlcOAll	12
4	GlcNAc → 2Man → 6βGlc → 4βGlcOAll	12
5	GlcNAc → 2ManOMe	*b*
6	GlcNAc → 6ManOMe	*b*
7	GlcNAc → 6(GlcNAc → 2)ManOMe	*b*
8	GlcNAc → 2(GlcNAc → 4)ManOMe	*b*
9	GlcNAc → 2(4Me)ManOMe	14
10	GlcNAc → 2(6Me)ManOMe	14
11	GlcNAc → 2(4,6Me)ManOMe	14
12	GlcNAc → 6(GlcNAc → 2)Man → 6β$ManOCD_3$	10
13	GlcNAc → 2Man → 6β$ManOCD_3$	*b*
14	GlcNAc → 2Man → 6βManO4NP	12
15	GlcNAc → 2Man → 6βGlcO4NP	12
16	GlcNAc → 2(4Me)Man → 6βGlcO4NP	11
17	GlcNAc → 2(6Me)Man → 6βGlcO4NP	14
18	GlcNAc → 2(4,6Me)Man → 6βGlcO4NP	14
19	GlcNAc → 6Man → 6βManOMCO	3
20	GlcNAc → 2Man → 6βManOMCO	8
21	GlcNAc → 2Man → 3βManOMCO	13
22	GlcNAc → 2Man → 6(GlcNAc → 2Man → 3)βManOMCO	3
23	GlcNAc → 6(GlcNAc → 2)Man → 6βManOMCO	9
34	^{14}C-GlcNAc → 4(GlcNAc → 2)Man → 6βManOMCO	*b*
35	^{14}C-GlcNAc → 4(GlcNac → 2)Man → 6β$ManOCD_3$	*b*
36	^{14}C-GlcNAc → 4(GlcNAc → 2)Man → 3βManOMCO	*b*

[a] Abbreviations: GlcNAc → x, β-D-Glc*p*NAc-(1 → x)-; Man → x, α-D-Man*p*-(1 → x)-; βMan → x, β-D-Man*p*-(1 → x)-; βGlc → x, β-D-Glc*p*-(1 → x); xMe, x-OMe; All, allyl, O4NP, 4-nitrophenyl glycoside; and OMCO, 8-methoxycarbonyloctyl glycoside. [b] This work.

O-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1 → 2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside. This was deacetylated with 0.1 M NaOMe solution (10 mL). The usual workup gave **5** (13.1 mg). $[\alpha]_D^{20}$ +2.2° (*c* 1.63, H_2O) {lit.[18] $[\alpha]_D^{25}$

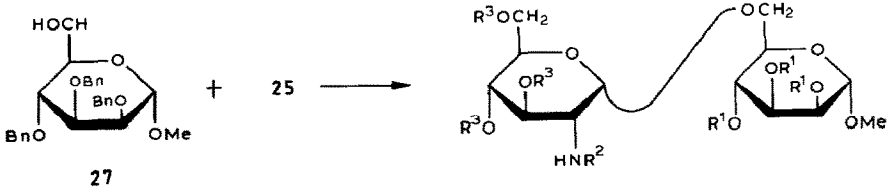


26 R^1 = Bn, R^2 = Phth, R^3 = Ac

5 R^1 = R^3 = H, R^2 = Ac

−3.3° (*c* 0.49, MeOH)}; ^{1}H NMR: δ 4.77 (1 H, *J* 1.62 Hz, H-1), 4.56 (1 H, *J* 8.4 Hz, H-1′), 4.06 (1 H, *J* 3.38 Hz, H-2), 3.38 (3 H, OCH_3), 2.06 (3 H, $NHCOCH_3$).

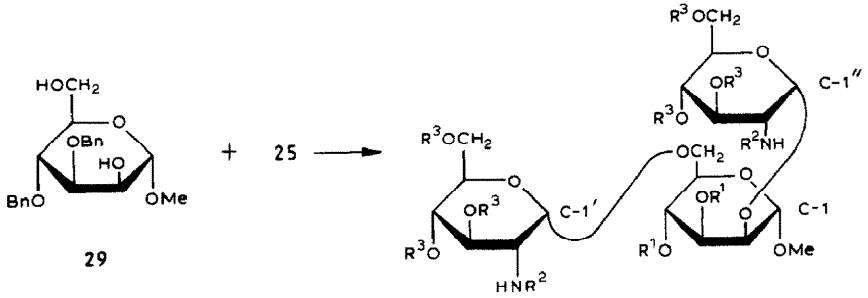
Methyl O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 6)-α-D-mannopyranoside (**6**).—A solution of methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside[19] (**27**, 0.82 g, 1.76 mmol) in CH_2Cl_2 (15 mL) was cooled to −40°, and silver triflate (1.169 g, 4.54 mmol) and 2,4,6-trimethylpyridine (0.55 g, 4.53 mmol) were added under Ar. After stirring for 5 min, a solution of bromide (**25**) (1.066 g, 2.14 mmol) in CH_2Cl_2 (10 mL) was added dropwise and the reaction mixture was stirred for 18 h at room temperature. The solids were filtered off and the filtrate was washed with cold water, aq M HCl, satd aq $NaHCO_3$, and water. The organic solvents were evaporated and the syrupy residue was subjected to chromatography on silica gel using hexane–EtOAc (5:4) to give methyl *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1 → 6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranoside (**28**; 0.91 g, 58.5%). This compound was deprotected by reflux with hydrazine hydrate in EtOH for 8 h (to remove phthalimido and acetyl group) and acetylated with acetic anhydride in pyridine at room temperature for 18 h to give methyl *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1 → 6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranoside. This was purified by column chromatography on silica gel using hexane–EtOAc (3:1) and EtOAc as eluents. The compound was deacetylated with NaOMe in MeOH, desalted in a mixed-bed resin, dried, and hydrogenolyzed in the presence of Pd/C in MeOH–EtOH–H_2O (5:3:1). The catalyst was removed by filtration, and the filtrate was de-ionized and lyophilized to give **6** (0.187 g, 45.2%) {lit.[20] $[\alpha]_D^{24}$ +11.2° (*c* 1, H_2O)}; ^{1}H NMR: δ α-Man: 4.725 (1 H, *J* 1.4 Hz, H-1), 3.914 (1 H, *J* 3.6 Hz, H-2), 3.736 (1 H, *J* 9.3 Hz, H-3), 3.596 (1 H, *J* 9.3 Hz, H-4), 3.698 (1 H, *J* 6.4 Hz, H-5), 4.182 (1 H, *J* 1.0 Hz, H-6a), 3.754 (1 H, *J* −10.1 Hz, H-6b); β-GlcNAc: 4.561 (1 H, *J* 8.6 Hz, H-1′), 3.731 (1 H, *J* 9.9 Hz, H-2′), 3.565 (1 H, *J* 10.4 Hz, H-3′), 3.44 (1 H, H-4′), 3.47 (1 H, H-5′), 3.935 (1 H, *J* 1.7, H-6′a), 3.759 (1 H, *J* −12.6 Hz, H-6′b), 2.037 (3H, $NHCOCH_3$).



28 R^1 = Bn, R^2 = Phth, R^3 = Ac

6 R^1 = R^3 = H, R^2 = Ac

Methyl 2,6-di-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside (**7**).—Methyl 3,4-di-*O*-benzyl-α-D-mannopyranoside (**29**) was obtained by treating 2,6-di-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl bromide[10] with MeOH in the presence of $Hg(CN)_2$, followed by deacetylation. Compound (**29**) (0.2 g, 0.53 mmol) was further glycosylated in CH_2Cl_2 (20 mL) with bromide (**25**) (1.52 g, 3.05 mmol) as described above by use of silver triflate (0.44 g, 1.91 mmol) in the

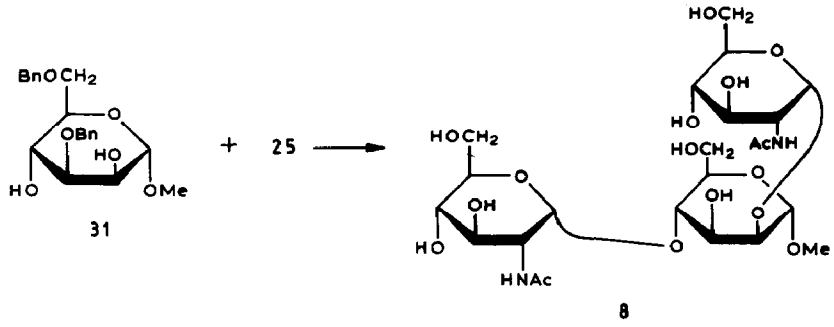


30 R^1 = Bn, R^2 = Phth, R^3 = Ac

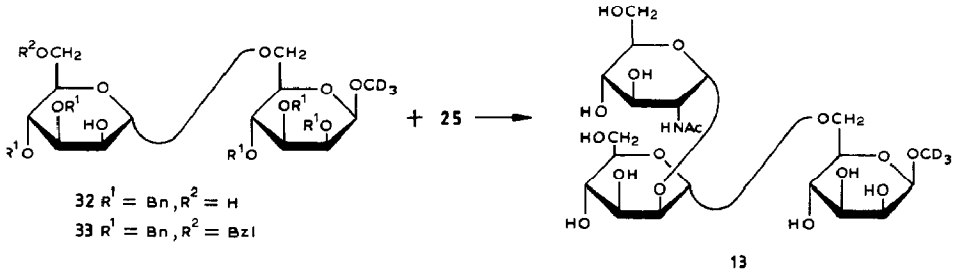
7 R^1 = R^3 = H, R^2 = Ac

presence of 2,4,6-trimethylpyridine (0.23 g, 1.89 mmol) to give methyl 3,4-di-*O*-benzyl-2,6-di-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-α-D-mannopyranoside (**30**; 0.12 g, 16.7%). The use of MeCN as a solvent in this reaction gave **30** with a 66.5% yield. Compound **30** was deprotected stepwise by the same sequence of reactions used for the synthesis of **6** to give **7**; ^{1}H NMR: δ α-Man: 4.717 (1 H, *J* 1.3 Hz, H-1), 4.036 (1 H, *J* 3.4 Hz, H-2), 3.755 (1 H, *J* 9.6 Hz, H-3), 3.415 (1 H, *J* 9.7 Hz, H-4), 3.693 (1 H, *J* 9.0 Hz, H-5), 4.193 (1 H, *J* 1.2 Hz, H-6a), 3.587 (1 H, *J* 10.8 Hz, H6b); β-GlcNAc6: 4.534 (1 H, *J* 8.4 Hz, H-1′), 3.711 (1 H, *J* 10.2 Hz, H-2′), 3.566 (1 H, H-3′), 3.44 (1 H, H-4′), 3.429 (1 H, *J* 4.5 Hz, H-5′), 3.907 (1 H, *J* 1.5 Hz, H-6′a), 3.748 (1 H, *J* −12.4 Hz, H-6′b), 2.019 (3 H, NHCOCH_3); β-GlcNAc2: 4.558 (1 H, *J* 8.3 Hz, H-1″), 3.668 (1 H, *J* 10.3 Hz, H-2″), 3.572 (1 H, H-3″), 3.43 (1 H, H-4″), 3.455 (1 H, *J* 4.5 Hz, H-5″), 3.930 (1 H, *J* 1.0 Hz, H-6″a), 3.748 (1 H, *J* −12.3 Hz, H-6″b), 2.059 (3 H, NHCOCH_3).

*Methyl 2,4-di-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside* (**8**).—The condensation was carried out as described above using methyl 3,6-di*O*-benzyl-α-D-mannopyranoside[21] (**31**; 0.47 g, 1.3 mmol) in CH_2Cl_2 (20 mL), silver triflate (0.41 g, 1.59 mmol), 2,4,6-trimethylpyridine (0.19 g, 1.56 mmol) and bromide (**25**) (1.22 g, 2.46 mmol) in CH_2Cl_2 (30 mL). Compound **8** was obtained as its blocked derivative with 1.4% yield; the use of MeCN as a solvent gave the blocked compound with a 15% yield; it was deprotected in the usual way to give **8**; $[\alpha]_D^{20}$ +3.3° (*c* 0.3, H_2O), ^{1}H NMR: δ α-Man: 4.72 (d, 1 H, *J* 2.4 Hz, H-1), 4.11 (1 H, *J* 3.6 Hz, H-2), 3.64 (1 H, *J* 9.9 Hz, H-3); β-GlcNAc4: 4.48 (1 H, *J* 8.4 Hz, H-1′), 3.68 (1 H, H-2′), 2.070 (3 H, NHCOCH_3); β-GlcNAc2: 4.53 (1 H, *J* 8.4 Hz, H-1″), 3.73 (1 H, H-2″), 2.055 (3 H, NHCOCH_3).



Trideuteriomethyl O-*(2-acetamido-2-deoxy-β-*D-*glucopyranosyl)-(1 → 2)-α-*D-*mannopyranosyl-(1 → 6)-β-*D-*mannopyranoside* (**13**).—Trideuteriomethyl *O*-(3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-2,3,4-tri-*O*-benzyl-β-D-mannopyranoside[10] (**32**; 1 g, 1.24 mmol) was selectively benzoylated with 1-benzoylbenzotriazole (0.389 g, 1.63 mmol) in CH_2Cl_2 (20 mL) containing triethylamine (0.2 mL) to give trideuteriomethyl *O*-(6-*O*-benzoyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-2,3,4-tri-*O*-benzyl-β-D-mannopyranoside (**33**; 0.55 g, 48.7%). This (0.26 g, 0.30 mmol) was glycosylated with bromide (**25**) (0.914 g, 1.83 mmol) in MeCN (10 mL) in the presence of silver triflate (0.506 g, 1.96 mmol), 2.6-di-*tert*-butyl-4-methylpyridine (0.403 g, 2.11 mmol) and molecular sieve 4A (1 g). The syrup obtained after the usual workup was purified by HPLC using a silica column and hexane–EtOAc–CH_2Cl_2 (6:3:1) as the eluant to give **13** as a blocked derivative (0.28 g, 76%) which was deblocked by conventional methods to give **13**; ^{1}H NMR: δ β-Man: 4.579 (1 H, *J* 0.8 Hz, H-1), 3.992 (1 H, *J* 2.2 Hz, H-2), 3.855 (1 H, *J* 9.68, *J* 3.43 Hz, H-3); α-Man: 4.925 (1 H, *J* 1.35 Hz, H-1′), 4.136 (1 H, *J* 3.38, *J* 1.63 Hz, H-2′); β-GlcNAc: 4.579 Hz (1 H, *J* 8.4 Hz, H-1″), 2.058 (3 H, NHCOCH_3).



All synthetic compounds were analyzed by NMR spectroscopy.

Methods.—For ^{1}H NMR spectroscopy, the samples were in D_2O with acetone as the internal standard (set at δ 2.225) as described previously[3] or were dissolved in $CDCl_3$ containing traces of Me_4Si as the internal standard (set at δ 0). Spectra were recorded at the Toronto Carbohydrate Research Centre with a 360-MHz Nicolet and a 500-MHz Bruker spectrometer. Fast-atom-bombardment mass spec-

trometry was carried out with a VG Analytical ZAB-SE double focussing mass spectrometer at the Toronto Carbohydrate Research Centre as described previously[22]. TLC was performed on silica gel 60 F254 (Merck) plates and the spots were detected by spraying with 50% H_2SO_4 in EtOH and heating at 200°. Silica gel (230–400 mesh; Toronto Research Chemicals) was used for flash chromatography. HPLC was performed as described previously[2]. Compounds were separated on a PAC column (mixed cyanoaminopropyl silica) with MeCN–water mixtures as the mobile phase. Compounds with a hydrophobic aglycon group (8-methoxycarbonyloctyl and 4-nitrophenyl glycosides) were also separated on a C-18 reverse-phase column. The compounds were detected by measuring the absorbance at 195 nm and counting the radioactivity of collected fractions. All starting materials were dried overnight under a high vacuum in the presence of KOH or P_2O_5 prior to use and the solvents were distilled from appropriate drying agents. Solutions were concentrated at 1 Pa pressure.

Methylation analysis.—Permethylation was carried out by the method of Ciucanu and Kerek[23] and purification of methylated oligosaccharides, hydrolysis, $NaBH_4$ reduction and peracetylation were carried out as described previously[22].

Protein determination.—Protein was determined by the Bio-Rad method using bovine serum albumin as the standard[24].

Amino sugar analysis.—The GlcNAc content was determined after acid hydrolysis with an amino acid analyzer in the laboratory of Dr. Max Blum, University of Toronto, as described previously[25].

Enzyme assays.—Tissue homogenates and microsomes were prepared as described previously[3]. GlcNAc-T assays were carried out as follows or as indicated in the Tables. The standard incubation mixture contained in a total volume of 40 μL, mM substrate, 0.125 M GlcNAc, 12.5 mM $MnCl_2$, 0.125 mM MES buffer (pH 7), 0.25% Triton X-100, 0.75 mM UDP-[^{14}C]GlcNAc (6133 dpm/mmol), and 10–15 μL of enzyme preparation (0.12–0.44 mg protein). The substrate for GlcNAc-T VI′ was compound **20**, for Gn-T IV compound **21** or **22**, for GlcNAc-T III compound **22**, and for GlcNAc-T V compound **20** (without $MnCl_2$). The mixtures were incubated at 37° for 1 h. The reaction was stopped with mM EDTA—20 mM Na tetraborate, pH 9 (0.4 mL), and the mixture was applied to a Pasteur pipette filled with AG 1-X8 anion-exchange resin. The column was washed with water (2.6 mL) and the eluate lyophilized. The residue was taken up in water (100 or 200 μL) and one half of the total was analyzed by HPLC.

Large-scale preparation of enzyme products.—GlcNAc-T VI′ product. βGlcNAc → 4(βGlcNAc → 2)αMan → 6βManOMCO (**34**) *from* **20**. The incubating mixture contained, in a total volume of 2.4 mL, **20** (5 μmol), hen oviduct microsomes (1.1 mL, 8.8 mg of protein), 0.125 M GlcNAc, 0.125 M MES (pH 7), 12.5 mM $MnCl_2$, 0.25% Triton X-100, and 2.3 mM UDP[^{14}C]GlcNAc (577 dpm/nmol). The mixture was incubated for 24 h at 37°. The reaction was stopped with mM EDTA–20 mM Na tetraborate, pH 9 (10 mL). The mixture was passed through a 50-mL column of AG 1-x8 anion-exchange resin; the column was washed with water (150 mL), and

the eluate was lyophilized and applied to a column (2.5 × 39 cm) of Sephadex G-25, equilibrated in water. The excluded fractions were further separated by HPLC in a PAC column eluted with MeCN–water (17:3) at a flow rate of 0.7 mL/min. The isolated product was rechromatographed in a 1.6 × 86 cm column of Bio-Gel P-4 (−400 mesh), equilibrated in water.

GlcNAc-T VI′ product. βGlcNAc → 4(βGlcNAc → 2)αMan → 6βManOCD$_3$ (**35**) *from* **13**. The incubation contained, in a total volume of 4 mL, **13** (1.5 μmol), hen oviduct microsomes (1.5 mL, 12 mg of protein), 0.125 M GlcNAc, 0.125 M MES (pH 7), 12.5 mM $MnCl_2$, 0.5% Triton X-100, and 2 mM UDP[^{14}C]GlcNAc (661 dpm/nmol). The mixture was incubated for 24 h at 37°. The reaction was stopped with mM EDTA–20 mM Na tetraborate, pH 9 (30 mL). The mixture was passed through a 50-mL column of AG 1-X8 anion-exchange resin, the column was washed with water (150 mL), and the eluate was lyophilized and applied to a column (1.6 × 86 cm) of Bio-Gel P-4 (−400 mesh), equilibrated in water. The product was analyzed by HPLC in a PAC column with MeCN–water (4:1) at a flow rate of 0.7 mL/min.

GlcNAc-T IV product. βGlcNAc → 4(βGlcNAc → 2)αMan → 3βManOMCO (**36**) *from* **21**. The incubation contained, in a total volume of 3.34 mL, **21** (6 μmol), hen oviduct microsomes (2 mL, 74 mg of protein), 0.15 M GlcNAc, 0.15 M MES (pH 7), 15 mM $MnCl_2$, 0.3% Triton X-100, and 1.35 mM UDP[^{14}C]GlcNAc (227 dpm/nmol). The mixture was incubated for 1 h at 37°. The reaction was stopped with mM EDTA—20 mM Na tetraborate, pH 9 (20 mL). The mixture was passed through a column (1.6 × 25 cm) of AG 1-X8 anion-exchange resin, the column was washed with water (200 mL), and the eluate lyophilized and applied to a column (1.6 × 84 cm) of Bio-Gel P-4 (−400 mesh), equilibrated in water. The partially excluded fractions were further separated by HPLC in a C-18 column with MeCN–water (4:21) at a flow rate of 1 mL/min. The isolated product was rechromatographed by HPLC in a PAC column with MeCN–water (21:4) at a flow rate of 0.7 mL/min.

RESULTS AND DISCUSSION

Identification of enzyme products.—GlcNAc-T VI′ product from **20**. The large-scale GlcNAc-T VI′ product from **20** was purified on Sephadex G-25 and HPLC and yielded two products, eluted from the PAC column, PA (408 nmol, 8.2% conversion to product) and PB (81 nmol, 1.6% conversion to product). Further purification on Bio-Gel P-4 showed that PB contained several components. The ^{1}H NMR spectrum of PA, **34**, compared to that of **20**, showed an additional βGlcNAc unit, as indicated by the *N*-acetyl signal at δ 2.067 and the H-1 signal at δ 4.514 with *J* 8.5 Hz (Table II).

Methylation analysis identified a 6-*O*-substituted hexose unit (derivative eluted at 11.9 min; *m/z* 102, 118, 129, 162, and 189), a terminal HexNAc unit (eluted at

TABLE II

500-MHz ^{1}H NMR chemical shift values (δ) and coupling constants (Hz), recorded at 300 K

Group or residue (H atom)	5	8	6	20	34	13	12	35	19	21	36
βGlcNAc-(1 → 2)											
H-1	4.561 (8.4)	4.53 (8.4)		4.578 (8.40)	4.564 (8.5)	4.579 (8.4)	4.6 [b]	4.56		4.544 (8.35)	4.529 (8.46)
N-acetyl	2.058	2.055		2.058	2.058 [a]	2.058	2.060	2.058		2.051	2.051 [d]
βGlcNAc-(1 → 4)											
H-1		4.48 (8.4)			4.514 (8.5)			4.52			4.529 (8.46)
N-acetyl		2.070			2.067 [a]			2.071			2.076 [d]
βGlcNAc-(1 → 6)											
H-1			4.555 (8.44)				4.550 (8.3) [b]		4.559 (8.5)		
N-acetyl			2.05				2.038		2.055		
αMan											
H-1	4.770 (1.62)	4.72 (2.4)	4.727 (1.63)	4.918 (1.40)	4.909 (0.65)	4.925 (1.35)	4.88 (< 1)	4.91	4.887	5.136 (1.65)	5.129
H-2	4.060 (3.38)	4.11 (3.6)	3.916 (3.35)	4.123 (3.45)	4.149 (1.90)	4.136 (3.38)	[c]	[c]	3.97	4.185 (3.35)	4.210
βMan											
H-1				4.662 (0.80)	4.656 (< 1)	4.579 (0.80)	4.581 (< 1)	4.57	4.662	4.667 (< 1)	4.659
H-2				3.981 (3.20)	3.973	3.992 (3.20)	[c]	[c]	3.98	4.125 (3.00)	4.096

	15	3	4	16	14	17	10	11	18	9	1	2
βGlcNAc-(1→2)												
H-1	4.516 (8.45)	4.511 (8.2)	4.518 (8.8)	4.520 (8.4)	4.544 (8.45)	4.506 (8.41)	4.552 (8.46)	4.554 (8.41)	4.512 (8.46)	4.560 (8.21)	4.553 (8.37)	4.550 (8.45)
N-acetyl	2.002	2.056	2.055	2.008	2.002	1.996	2.051	2.058	1.998	2.063	2.049	2.046
αMan												
H-1	4.860 (1.40)	4.900	4.925	4.840 (1.51)	4.880 (1.2)	4.85	4.76 [d]	4.737	[c]	[c]	4.915	4.917
H-2	4.098 (3.38)	4.124	4.114	4.083 (3.35)	4.114 (3.61)	4.103	4.065	4.057	4.090	4.041	4.092	4.089
βMan												
H-1					5.534						[c]	
H-2					4.252 (1.83)						[c]	
βGlc												
H-1	5.306 (7.45)	4.570 (7.95)	4.567 (8.4)	5.302 (7.7)		5.309 (5.39)			5.313			
βGlc-(1→4)												
H-1			4.535 (8.1)									4.541 (8.00)α 4.532 (9.40)β
α/βGlcNAc												
H-1											5.21/4.512 (8.36)	5.208/4.713
N-acetyl											2.058/2.040 [d]	2.060, 2057 [d]

[a,b] Signals may be interchanged.

[c] Not determined.

[d] Tentative assignment.

14.4 min: *m/z* 117, 129, 143, 145, 159, 161, 203, and 205), and 2,4-*O*-substituted hexose unit (eluted at 12.7 min; *m/z* 113, 130, 173, 190, and 233).

The FAB-MS of PA indicated a *m/z* of 919 for $[M + H]^+$ and *m/z* 941 for $[M + Na]^+$. The ester of the MCO group in ~ 20% of the product was hydrolyzed to the free acid, as seen in the mass-ion pair *m/z* 905 and 927. No degradation was seen in the substrate (*m/z* of 716 for $[M + H]^+$, *m/z* 738 for $[M + Na]^+$, and *m/z* 754 for $[M + K]^+$). The combined data indicated that the major GlcNAc-T VI′ product has structure **34**.

GlcNAc-T VI′ product from **13**.—The large-scale GlcNAc-T VI′ product from **13** yielded 136 nmol product PC, and a minor product peak PD (62 nmol), eluted from Bio-Gel P-4 in the partially included fractions. The ^{1}H NMR spectrum of PD indicated the presence of several components, including signals for βGlcNAc → 6(βGlcNAc → 2)-αMan → 6βManOCD$_3$. The ^{1}H NMR spectrum of PC, **35** (Table II) indicated the presence of two GlcNAc residues, in addition to αMan → 6 and βMan residues. The spectrum is different from that of synthetic **12**. HPLC analysis showed that PC was eluted earlier than βGlcNAc → 6(βGlcNAc → 2)αMan → 6βManOCD$_3$, **12** and, therefore, probably has the structure **35**, similar to PA.

GlcNAc-T IV product from **21**. The large-scale enzyme product from **21** yielded a large peak eluted from Bio-Gel P-4 in the partially included fractions (total of 543 nmol). A major product P1 (225 nmol eluted at 48 min together with **21**), and two minor products (P2, 30 nmol eluted at 70 min; and P3, 33 nmol eluted at 82 min) were isolated on HPLC in a C-18 column. The minor products were analyzed by FAB-MS and showed the presence of several components. P2 contained **21** (*m/z* 738, $[M + Na]^+$) and GlcNAc$_2$Man$_2$OMCO (*m/z* 941, $[M + Na]^+$) in a ratio of 5:2. P3 contained **21**, GlcNAc$_2$Man$_2$OMCO and GlcNAc$_3$Man$_2$OMCO (*m/z* 1144, $[M + Na]^+$) in a ratio of 3:6:10. No information could be obtained from the ^{1}H NMR spectra of P2 and P3. P1 was repurified on a PAC column and yielded two broad, radioactive peaks, P1A (114 nmol, [^{14}C]GlcNAc) and P1B (127 nmol, [^{14}C]GlcNAc), eluted at 20 and 38 min, respectively. The ^{1}H NMR spectrum of P1A indicated the presence of **21** and ~ 10–20% P1B. The FAB-MS of P1A showed GlcNAcMan$_2$OMCO as the main component. Thus, P1A may contain **21** made radioactive by the sequential actions of hexosaminidase, followed by GlcNAc-T I.

The ^{1}H NMR spectrum of P1B, **36**, compared to that of **21**, indicated the presence of an additional βGlcNAc unit (Table II) with a resonance at δ 2.076 due to the *N*-acetyl group. Both the (1 → 4)- and the (1 → 2)-linked βGlcNAc units show resonances at δ 4.529 with *J* 8.46 Hz. The H-2 signals of both αMan-(1 → 3) and βMan units shifted in the product (+0.025 and −0.029 ppm, respectively). A Man H-3 resonance appeared at δ 4.051 (shifted from < δ 3.94) and should be assigned to the αMan-(1 → 3) unit because of the attachment of the GlcNAc unit to O-4 of this Man unit (see below).

Methylation analysis identified a 3-*O*-substituted hexose unit (derivative eluted at 12.6 min; *m/z* 101, 118, 129, 161, and 234), a terminal hexNAc unit (eluted at

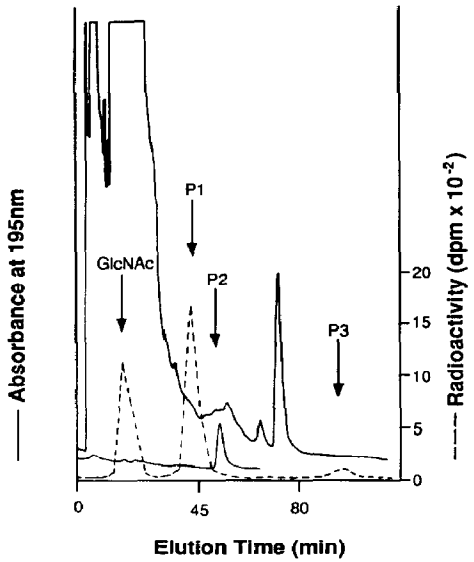


Fig. 1. GlcNAc-T VI′ assay. HPLC separation of enzyme product using **20** as the substrate. Enzyme assays were carried out as described for the standard assay in the Experimental section, by use of a PAC column at 0.7 mL/min flow rate and acetonitrile–water (41:9) as the mobile phase: P1, elution time of PA β[^{14}C]GlcNAc → 4-(βGlcNAc → 2)αMan → 6βManOMCO; P2, elution time of **23**; P3, elution time of βGlcNAc → 6(β[^{14}C]GlcNAc → 4)(βGlcNAc → 2)αMan → 6βManOMCO[3].

15.4 min; *m/z* 117, 129, 143, 145, 159, 161, 203, and 205) and a 2,4-*O*-substituted hexose unit (eluted at 13.7 min; *m/z* 113, 130, 145, 173, 190, and 233).

Therefore, the major GlcNAc-T IV product from **21** has structure **36**. However, three or more minor products were also formed.

Characterization studies.—Characterization of GlcNAc-T VI′ activity by use of **20** *as substrate.* The activity of GlcNAc-T VI′ with **13** as acceptor was linear up to at least a 2-h incubation time and 0.4 mg of protein per assay. In the absence of $MnCl_2$ the enzyme product was eluted from the HPLC PAC column at 51 min with the **23** standard, and was therefore due to GlcNAc-T V. The product of GlcNAc-T VI′ in the presence of $MnCl_2$ was eluted earlier, at 42 min. A small product peak was eluted with standard βGlcNAc → 6(βGlcNAc → 4)[βGlcNAc → 2]αMan → 6βManOMCO[3] at 98 min (Fig. 1) and is likely due to the action of GlcNAc-T V, followed by GlcNAc-T VI. Since the GlcNAc-T V product was not detectable in the presence of $MnCl_2$, it was probably converted into the GlcNAc-T VI product[3]. Consistently, for all derivatives, the 2/4-branched isomer, βGlcNAc → 4[βGlcNAc → 2]αMan → R (GlcNAc-T VI′ product), was eluted earlier from the PAC column than the 2/6-branched isomer formed in the absence of $MnCl_2$, βGlcNAc → 6[βGlcNAc → 2]αMan → R (GlcNAc-T V product). Assays were therefore performed by HPLC to distinguish between GlcNAc-T V and VI′ activities.

The apparent K_m and V_{max} values for GlcNAc-T VI′ with **20** as substrate were 4.7 mM and 24.8 nmol/h/mg, as determined by Lineweaver–Burk plots. GlcNAc-T VI′ was maximally active at pH 7, although the activity increased from pH 8 to 9

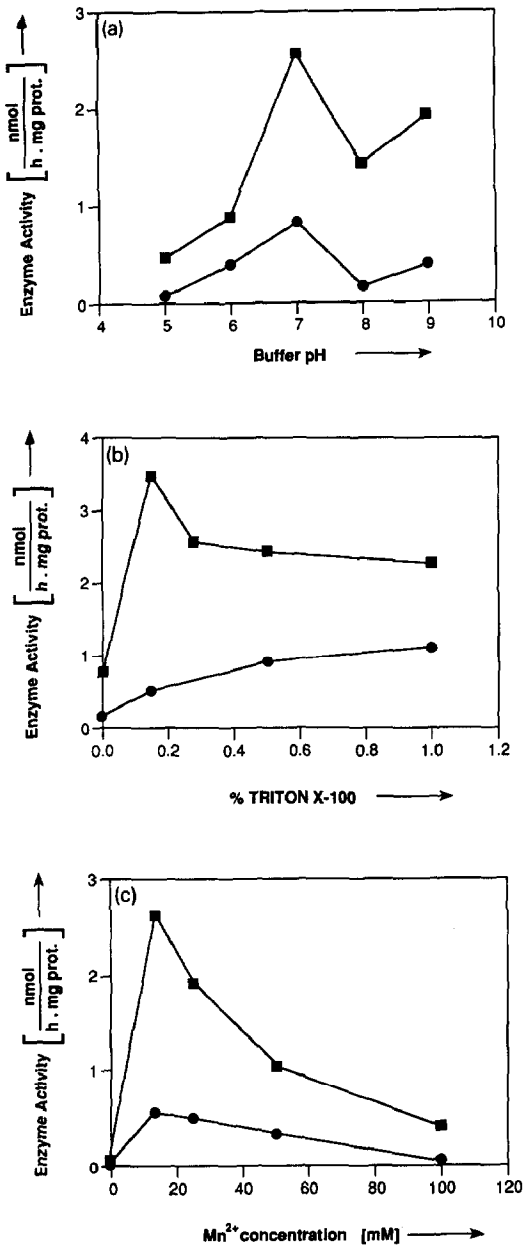


Fig. 2. Optimal assay conditions for GlcNAc-transferase VI′ and GlcNAc-transferase IV. Assays were carried out as described in the Experimental section with **20** as the substrate for GlcNAc-T VI′ (■) and **21** as the substrate for GlcNAc-T IV (●), and with 0.65 mM UDP [^{14}C]GlcNAc (1339 dpm/nmol). (a) Activity dependence on buffer pH; (b) activity dependence on Triton X-100 concentration; and (c) activity dependence on $MnCl_2$ concentration.

TABLE III

Stimulation of GlcNAc-transferase VI′ and IV activities by metal ions [a]

Cation (12.5 mM)	GlcNAc-T VI′ activity		GlcNAc-T IV activity		GlcNAc-T V activity
	nmol/h/mg	%	nmol/h/mg	%	nmol/h/mg
none	0	0	0 [b]	0	0.17
Mn^{2+}	2.64	100	0.64	100	[c]
Mg^{2+}	0.73	28	0.12	19	[c]
Ca^{2+}	0.03	1	< 0.02	< 2	0.12 [d]
Co^{2+}	0.61	23	0.03	5	[c]
Zn^{2+}	[c]	0	0	0	[c]

[a] Assays were carried out as described in the Experimental section. The substrate was **20** for GlcNAc-T VI′ and V. [b] 12.5 mM EDTA was added. [c] Not detected. [d] The substrate was **20** with 0.65 mM UDP-[^{14}C]GlcNAc (1339 dpm/nmol). Product eluted with **23** as standard.

(Fig. 2A). Triton X-100, up to 1% in the assay, stimulated the activity with maximal stimulation at 0.125% (Fig. 2B). The enzyme was not active in the absence of $MnCl_2$. The activity was maximal (100%) at 12.5 mM $MnCl_2$ in the assay, but fell off at concentrations above this value; at 100 mM $MnCl_2$, the activity was 11% (Fig. 2C). Mg^{2+} and Co^{2+}, but not Ca^{2+} and Zn^{2+}, could partially (28 and 23%, respectively) replace Mn^{2+} in the assay (Table III). The tissue distribution of GlcNAc-T VI′ activity is shown in Table IV. Only hen oviduct has appreciable GlcNAc-T VI′ activity; chicken and fetal calf liver, and pig stomach showed activities just above detectable levels. No activity was detected in rat liver and beef kidney.

The results of specificity studies are shown in Table V. Series of compounds with the general structure βGlcNAc → 2αMan → 6βMan → R and βGlcNAc → 2αMan → 6βGlc → R were tested with GlcNAc-T from hen oviduct microsomes.

TABLE IV

Tissue distribution of GlcNAc-transferases VI′, III and IV activities [a]

Tissue	Activities (nmol/h/mg) [b]			
	GlcNAc-T VI′	GlcNAc-T IV		GlcNAc-T III
	20	**21**	**22**	**22**
Hen oviduct microsomes	2.64 [c]	3.04	6.18	3.57
Hen liver homogenate	< 0.2			
Rat liver microsomes	[d]	[d]	[d]	[d]
Pig gastric microsomes	< 0.2	0.14	0.33	< 0.1
Fetal calf liver microsomes	0.11	0.54	0.96	[d]
Beef kidney microsomes	[d]	[d]	< 0.1	1.76

[a] The activities were determined by the standard assay as described in the Experimental section with 0.87 mM UDP-[^{14}C]GlcNAc (2678 dpm/nmol). [b] The substrate for GlcNAc-T VI′ was 2 mM **20**, for GlcNAc-T IV 1.2 mM **21** or mM **22**, and for GlcNAc-T III mM **22**. [c] The substrate concentration was mM **20**. [d] Not detectable.

TABLE V

Specificity of GlcNAc-transferase VI′ activity from oviduct [a]

Substrate	Conc. (mM)	Enzyme activity (nmol/h/mg)	K_m (mM)	V_{max} (nmol/h/mg)	Retention time (min)		Column/ acetonitrile concentration (%) [b]
					S [d]	P [d]	
20	2.5	4.4	4.7	24.8	22	46	PAC/84
19 [c]	2.5	< 0.1			25	36	PAC/84
		< 0.1			25	54	PAC/84
5	2.9	1.5	12.5	16.7	22	51	PAC/84
6 [c]	1.4	< 0.1			23	32	PAC/84
13	1.8	2.6	2	5.7	47	76	PAC/82
14	2.5	6.7	1.8	7.2	30	72	PAC/86
15	2.5	4.6	2.1	9.1	28	66	PAC/86
3	2.5	4.7	2.6	10.0	32	68	PAC/84
4	2.5	0.2			32	64	PAC/82
2	2.5	0.2			32	50	PAC/78
1	2.0	0.4	14.3	5.6	34	54	PAC/78
16	2.5	0.1			22	64	PAC/84
9	2.0	< 0.1					
10	2.0	0.1			13	26	PAC/81
11	2.0	< 0.1			12		PAC/84
17	2.0	0.7	1.3	1.3	22	16	C18/8
18	2.0	< 0.1			41		C18/10
	Standards:						
	βGlcNAc → 4(βGlcNAc → 2)αMan → 6βManOMCO				46		PAC/84
	βGlcNAc → 6(βGlcNAc → 2)αMan → 6βManOMCO				58		PAC/84
	βGlcNAc → 4(βGlcNAc → 2)αManOMe				53		PAC/84
	βGlcNAc → 6(βGlcNAc → 2)αManOMe				65		PAC/84
	βGlcNAc → 6(βGlcNAc → 2)αMan → 6βManOCD$_3$				86		PAC/82

[a] Assays were carried out as described in the Experimental section with 0.87 mM UDP-[^{14}C]GlcNAc (2678 dpm/nmol). [b] Percent of acetonitrile in the mobile phase; PAC (mixed propyl–amino–cyano-silica) column. In the absence of $MnCl_2$ the enzyme product was eluted with the βGlcNAc → 6(βGlcNAc → 2)αMan → 6βManOMCO standard, and was therefore due to GlcNAc-T V. In the presence of $MnCl_2$, where GlcNAc-T V product was not detectable, the product of GlcNAc-T VI′ was eluted earlier. [c] A small radioactive product peak could not be identified. [d] S, substrate; P, product.

The ^{1}H NMR spectra of these compounds (Table II) showed that the R group of βGlcNAc → 2αMan → 6βMan → R influences the chemical shift of H-1 of βMan (δ 4.579 if R = CD_3; δ 4.662 if R = MCO). An additional βGlcNAc group (1 → 6)-linked to αMan-(1 → 6) causes a small shift of H-1 of βGlcNAc-(1 → 2) and a large shift of H-1 of αMan-(1 → 6). The addition of a 4-*O*-linked βGlcNAc influences H-1 of βGlcNAc-(1 → 2) similarly, but has negligible influence on H-1 of αMan-(1 → 6). The R-groups of βGlcNAc → 2αMan → 6βGlc → R influence the chemical shifts of the protons of all sugar residues. Each of the compounds in this series has its own characteristic spectrum. Deletion of 4-OH of αMan-(1 → 6) changes the chemical shift of H-1, H-2, and H-3 of this residue only.

All compounds having the structure βGlcNAc → 2αMan → 6βMan (or Glc) → R were active as substrates for GlcNAc-T VI′, with the exception of **1**, **2**, and **4**

(Table V). The allyl group is not inhibitory since **3** is a very effective substrate. It is possible that three two inactive compounds may have unfavorable molecular folding, but conformational studies have not yet been carried out. Since **14** and **15** were both very active substrates, the 2-OH of the sugar unit at the reducing end can be either in the axial or equatorial position. In fact, the βMan residue is not required at all, since **5** is a substrate, although its apparent K_m is very high (12.5 mM). Hydrophobic aglycon groups are well tolerated by the enzyme, a phenomenon observed for many GlcNAc-transferases[3,7]. The 4-OH of the αMan-(1 → 6) residue is essential for activity since GlcNAc-T VI′ acts on this OH group. However, **16** showed some activity just above detectable levels. This may possibly be due to GlcNAc-T V activity, but no compound was available to confirm this. Substitution by 6-OMe of the αMan residue of the substrate (i.e., **10** and **17**) resulted in greatly reduced activities, indicating that this 6-OH is required for GlcNAc-T VI′ activity. GlcNAc-T VI[3], which is also present in the enzyme preparation, acts on substrates where αMan is substituted at 6-OH by GlcNAc; thus the methyl group does not substitute for GlcNAc at that position. As expected, compounds that lack 4-6-OH at αMan, **11**, and **18**, were inactive (Table V).

Compounds containing methyl group substitutions (Table V) were synthesized as potential enzyme inhibitors. None of the compounds inhibited GlcNAc-T VI′ activity; even a ten-fold excess in the assay did not reduce significantly the amount of GlcNAc incorporated into **3** or **15** (data not shown).

Characterization of GlcNAc-T IV activity by use of **21** *as substrate.* Compound **21** was used to characterize GlcNAc-T IV. The apparent K_m and V_{max} were, respectively, 3.3 mM and 20 nmol/h/mg. The activity dependence on buffer pH

TABLE VI

Competition experiment for GlcNAc-T IV and VI′ [a]

3 mM Substrate		Activities (nmol/h/mg)	
GlcNAc-T VI′	GlcNAc-T IV	Exptl.	Calcd [b]
Substrates alone:			
20		5.8	
	21	6.2	
Substrates mixed:			
20		3.4	3.6
	21	5.2	4.2

[a] Assays were carried out as described for the standard assay. Products were separated by HPLC on a C-18 column with acetonitrile–water (4:21) at a flow rate of 1 mL/min. The UDP-[^{14}C]GlcNAc concentration was 1.74 mM. [b] Enzyme activity for each individual substrate in the case of competition for the same enzyme was calculated by:

$$v = \frac{V_1 \times S_1/K_1}{1 + S_1/K_1 + S_2/K_2} \text{ and } v = \frac{V_2 \times S_2/K_2}{1 + S_1/K_1 + S_2/K_2}$$

where V_1, V_2, S_1, S_2, K_1, and K_2 are the respective apparent V_{max}, substrate concentration and apparent K_m for the individual substrates.

(Fig. 2A) and $MnCl_2$ concentration (Fig. 2C) were identical to the behavior of GlcNAc-T VI′. Triton X-100 also stimulated GlcNAc-T IV (Fig. 2B) but, in contrast to GlcNAc-T VI′, the activity appeared to increase continuously between 0 and 1% Triton in the assay. GlcNAc-T IV activity was also similar to that of GlcNAc-T VI′ in its stimulation by metal ions (Table III), and in its tissue distribution (Table IV).

Competition experiments between GlcNAc-T VI′ and GlcNAc-T IV substrates.— Competition between GlcNAc-T VI′ and IV was studied by mixing **20** and **21** in the assay. The activities found for each substrate were similar to those calculated for two competing substrates (Table VI), suggesting that the same enzyme may act on both substrates.

CONCLUSIONS

Hen oviduct has at least six GlcNAc-transferases that add GlcNAc to the core of *N*-glycans[1–3]. It is, therefore, not surprising that multiple enzyme products are found; this is due to (1) exchange of GlcNAc (removal and reincorporation), creating radioactive substances with the same structure as the substrate, (2) multiple reactions on the same substrate due to competing reactions, (3) serial reactions in which the product of the first enzyme becomes the substrate of a subsequent enzyme reaction, and (4) degradation of radioactive products. We have shown here that hen oviduct has (1 → 4)-β-D-GlcNAc-transferase activities, named GlcNAc-T VI′ and IV, that can form a βGlcNAc → 4(βGlcNAc → 2)αMan → R branch on the αMan-(1 → 6) and the αMan-(1 → 3) arms of *N*-glycans, respectively. The kinetic characteristics and tissue distributions of these two activities are similar. In addition, competition experiments and the fact that βGlcNAc → 2αMan → R is a substrate for the (1 → 4)-β-D-GlcNAc-transferase suggest that GlcNAc-T VI′ and IV activities are due to the same enzyme, but this has yet to be proven by enzyme purification. GlcNAc-T IV from tissues other than hen oviduct exhibits only very low activity[2]. Possibly, hen oviduct GlcNAc-T IV[4] is active under our assay conditions, but the enzyme from other tissues may be different and may therefore require different conditions for optimal activity. Enzyme purification will have to clarify these points.

It is interesting that the enzyme in vivo acts primarily on the αMan-(1 → 3) arm as suggested by structures from ovomucoid made in the hen oviduct[6]. When presented in vitro with partial (monoantennary) *N*-glycan structures the enzyme apparently loses this branch specificity and acts on both αMan-(1 → 3)- and -(1 → 6)- arms. It appears, however, that three of the monoantennary compounds containing an additional Glc or GlcNAc unit, linked to the βGlc βMan unit (Table V) retain features of a bi- or multi-antennary *N*-glycan which inhibit the action of GlcNAc-T VI′. This may be due to the resemblance of Glc or GlcNAc to the chitobiose core structure and a folding of the αMan-(1 → 6) arm[26] back

towards these residues preventing enzyme action. The 6-position of the αMan-(1 → 6) residue, however, is still available for the action of GlcNAc-T V[27].

REFERENCES

1 H. Schachter, *Biochem. Cell Biol.*, 64 (1986) 163–181.
2 I. Brockhausen, J.P. Carver, and H. Schachter, *Biochem. Cell. Biol.*, 66 (1988) 1134–1151.
3 I. Brockhausen, E. Hull, O. Hindsgaul, H. Schachter, R.N. Shah, S.W. Michnick, and J.P. Carver, *J. Biol. Chem.*, 264 (1989) 11211–11221.
4 P.A. Gleeson and H. Schachter, *J. Biol. Chem.*, 258 (1983) 6162–6173.
5 S. Narasimhan, *J. Biol. Chem.*, 257 (1982) 10235–10242.
6 K. Yamashita, J.P. Kamerling, and A. Kobata, *J. Biol. Chem.*, 258 (1983) 3099–3106.
7 I. Brockhausen, K.L. Matta, J. Orr, and H. Schachter, *Biochemistry*, 24 (1985) 1866–1874.
8 S.H. Tahir and O. Hindsgaul, *Can. J. Chem.*, 64 (1986) 1771–1780.
9 O. Hindsgaul, S.H. Tahir, O. Srivastava, and M. Pierce, *Carbohydr. Res.*, 173 (1988) 263–272.
10 R.N. Shah, D.A. Cumming, A.A. Grey, J.P. Carver, and J.J. Krepinsky, *Carbohydr. Res.*, 153 (1986) 155–161.
11 S.H. Khan, S.A. Abbas, and K.L. Matta, *Carbohydr. Res.*, 205 (1990) 385–397.
12 S.H. Khan, S.A. Abbas, and K.L. Matta, *Carbohydr. Res.*, 193 (1989) 125–139.
13 H. Paulsen and B. Helpap, *Carbohyr. Res.*, 216 (1991) 289–313.
14 S.H. Khan and K.L. Matta, unpublished results.
15 V.K. Handa, J.J. Barlow, and K.L. Matta, *Carbohydr. Res.*, 76 (1979) c1–c3.
16 N.E. Franks and R. Montgomery, *Carbohydr. Res.*, 6 (1968) 286–298.
17 R.U. Lemieux, T. Takeda, and B.Y. Chung, *ACS Symp. Ser.*, 39 (1976) 90.
18 T. Ogawa, K. Katano, K. Sasajima, and M. Matsui, *Tetrahedron*, 37 (1981) 2779–2786.
19 S.J. Sandheimer, R. Eby, and C. Schuerch., *Carbohydr. Res.*, 60 (1978) 187–192.
20 S.S. Rana, J.J. Barlow, and K.L. Matta, *Carbohydr. Res.*, 96 (1981) 79–85.
21 T. Ogawa, K. Katano, and M. Matsui, *Carbohydr. Res.*, 64 (1978) c3–c9.
22 I. Brockhausen, A.A. Grey, H. Pang, H. Schachter, and J.P. Carver, *Glycoconjugate J.*, 5 (1988) 419–448.
23 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
24 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
25 I. Brockhausen, G. Möller, G. Merz, K. Adermann, and H. Paulsen, *Biochemistry*, 29 (1990) 10206–10212.
26 J.-R. Brisson and J.P. Carver, *Biochemistry*, 22 (1983) 3680–3687.
27 I. Brockhausen, S. Khan, and K.L. Matta, unpublished results.