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GlcNAc Transferases and Glycoengineering in Chinese Hamster Ovary Cells

A dissertation submitted to Trinity College, University of Dublin, in
candidature for the degree of Doctor of Philosophy

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February 2011



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Declaration

I certify that none of the work presented in this thesis has been submitted for any degree at this or at any other University. I declare that this thesis is entirely my own work. I agree that the library may lend or copy this thesis upon request.



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Most of all, I dedicate this thesis to my parents, Elżbieta and Zbigniew, my sisters, Maria Antonina, Anna Maja, Aleksandra and my fiancée, Patryk Beyer (you are my hero!). Thank you for all your help and support during this PhD.

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Abstract

Glycosylation constitutes one of the most important post-translational modifications of proteins, with over 60% of proteins known to be glycosylated. GlcNAc transferases (GnTs) belong to a large subfamily of glycosyltransferases residing in the Golgi apparatus and play a key role in the synthesis of mature *N*-linked glycan structures attached to proteins. In forming multi-antennary *N*-glycan structures in Chinese hamster ovary cells (CHO), GlcNAc residues may be added to the trimannosyl core by four different GlcNAc transferases (I, II, IV and V).

At the start of this project there were no commercially available monoclonal or polyclonal antibodies against CHO GnTs and no structural or functional studies on GnTII and GnTIVB had been completed. In order to obtain abundant target proteins for generating polyclonal antibodies against CHO GnTs the prokaryotic *Escherichia coli* (*E. coli*) expression system was used. Recombinant GnTI, GnTII, GnTIVB and GnTV were cloned, expressed and purified. Ultra pure preparations served as immunogens for raising polyclonal antibodies that were further validated by Western blotting giving positive signal for purified antigen and also for GnTs that were overexpressed in CHO cells. Anti-peptide antibodies were commercially generated and used as an alternative to polyclonal antibodies against full-length proteins but failed to recognize endogenous or *myc*-tagged GnTs that were overexpressed in CHO cells.

Recombinant CHO GnTI, which was previously cloned, expressed and purified from *E. coli*, was analyzed for its activity using a spectrophotometric coupled assay. In this assay, UDP produced by GnTI was processed by pyruvate kinase (PK) and the product of PK, pyruvate was reduced by lactate dehydrogenase (LDH) to release NAD^+ from NADH, resulting in the decrease in absorbance at 340 nm. For recombinant GnTI, the K_m value for acceptor substrates, UDP-GlcNAc and $\text{Man}_5\text{GlcNAc}_2 \{M_5\}$ from ovalbumin, observed *in vitro* were 81.06 μM and 154.3 μM , respectively. The enzyme showed an absolute requirement for manganese ions (Mn^{2+}) to maintain activity. The effectiveness of other divalent cations followed the pattern: $\text{Mn}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Zn}^{2+}$. In addition, the activity of recombinant CHO GnTI was optimal when the pH of the standard reaction mixture was between 6.5 and 7.5 and the assay was carried out at 37°C.

Enzymes responsible for *N*-glycan synthesis and remodelling are common integral membrane protein constituents of Golgi membranes and are localised by cell-type-specific subcompartmentation. In most cases, enzymes catalyzing earlier steps are located in *cis* and *medial* compartments and the late-acting enzymes are distributed mainly in the *trans* Golgi. Four different GnTs (I, II, IV and V) have been characterised in wild type CHO cells, however their specific localisation in the Golgi has not been experimentally verified. Transient overexpression of *myc*-tagged GnTs in CHO cells resulted in their targeting to the Golgi apparatus; GnTI & GnTII co-localised with the *cis-medial* marker, giantin, and GnTIV & V co-localised with the *trans* marker, TGN46.

An individual glycoprotein is part of a population of glycoforms bearing different oligosaccharide structures (from single sugars to multi-branched structures) synthesized by the same cell at different times or when synthesized by different cell types. These carbohydrates may be subject to further modification such as overexpression or knockdown of enzymes that act at different stages of glycosylation. Overexpression of GnTV with the RNAi induced knockdown of β 4galactosyltransferases 3, 4, 5 & 6 significantly altered the structure of oligosaccharides on recombinant chorionic gonadotropin (HCG) by increasing the level of tri- and tetra-antennary *N*-glycan structures and increasing the level of α 2,3-sialylation. Thus, the discovery of a pathway for glycoengineering proteins with *N*-linked glycans in CHO cells is of major interest, especially as these mammalian cells are routinely used in bioprocessing and production of therapeutically valuable proteins.

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Abbreviations

aa	amino acids
2-AB	2-Aminobenzamide
ABS	Sialidase A
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Amp	Ampicillin
APS	Ammonium persulfate
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
BCP	Bromochlorophenol
BKF	Bovine kidney fucosidase
BSA	Bovine serum albumin
BTG	Bovine testes galactosidase
cDNA	complementary deoxyribonucleic acid
CGN	<i>cis</i> -Golgi network
CHO	Chinese Hamster Ovary
CMP-NeuAc	CMP-N-acetylneuraminic acid
Cys	Cysteine
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
Dol-P	Dolichol-P
Dol-P-Glc	Dolichol-P-glucose
Dol-P-Man	Dolichol-P-mannose
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EPO	Erythropoietin
ER	Endoplasmic reticulum
Fuc	Fucose
Gal	Galactose
GDP	Guanosine diphosphate

Glc	Glucose
GDP-Man	GDP-mannose
GlcNAc	<i>N</i> -Acetylglucosamine
GlcNAcT	<i>N</i> -acetylglucosaminyltransferase (GnT)
GU	Glucose unit
GUH	<i>N</i> -acetylglucosaminidase
HCG	Human chorionic gonadotropin
His	Histidine
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JBM	Jack bean mannosidase
kDa	kilodalton
LA	Luria Agar
LB	Luria Broth
L-PHA	L-phytohemagglutinin
Man	Mannose
mRNA	messenger ribonucleic acid
OD	Optical density
OST	Oligosaccharyltransferase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
polyLacNAc	<i>N</i> -linked poly- <i>N</i> -acetylactosamine
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SEM	Standard error of the mean
Ser	Serine
TAE	Tris-Acetate-EDTA
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
TGN	<i>trans</i> -Golgi network
Thr	Threonine
Tyr	Tyrosine
Tris	Tris(hydroxymethyl)aminomethane

UDP	Uridine diphosphate
UDP-Gal	UDP-galactose
UDP-Glc	UDP-glucose
UDP-GlcNAc	UDP- <i>N</i> -acetylglucosamine
UMP	Uridine monophosphate
V	Volts
Val	Valine
Vh	Volt hours
UV	Ultraviolet
v/v	volume/volume
W	Watts
w/v	weight/volume

CHAPTER 1

General Introduction

1.1 Glycosylation

1.1.1 Monosaccharide metabolism

The role of nucleoside diphosphate sugars was first discovered in 1950 by Nobel Laureate L.F. Leloir in Buenos Aires. He showed that a nucleotide triphosphate such as UTP reacts with a glycosyl-1-P to form the high-energy donor that could participate in glycoconjugate synthesis.

The biosynthesis of glycoconjugate requires the conversion of monosaccharides to sugar nucleotides and glycosyltransferases transfer the sugar moiety from activated nucleotides to various acceptors. There are several sources of such monosaccharides including external supply into the cell, recycling of degraded glycoconjugate as well as other sugars within the cell. The sugar import, interconversion, and activation occur in the cytoplasm whereas most glycosylation reactions occur in the Golgi, where the activated donors are transported via the energy independent sugar nucleotide antiporters.

The most common monosaccharides that occur within glycans in mammalian cells are glucose (Glc), mannose (Man), galactose (Gal), fucose (Fuc), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and *N*-acetylneuraminic acid (NeuAc, also Neu5Ac) (see Figure 1.1). Most of the sugars are present in nature in the D conformation with one exception for L-fucose being a mirror image of galactose with the loss of the 6'OH group.

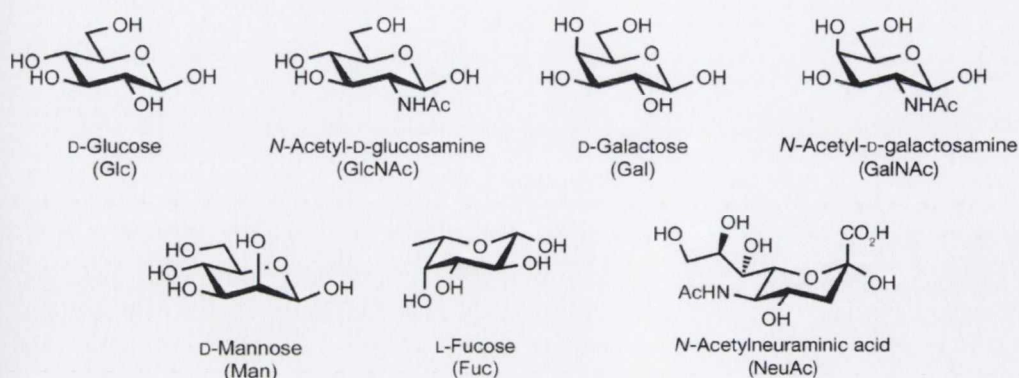
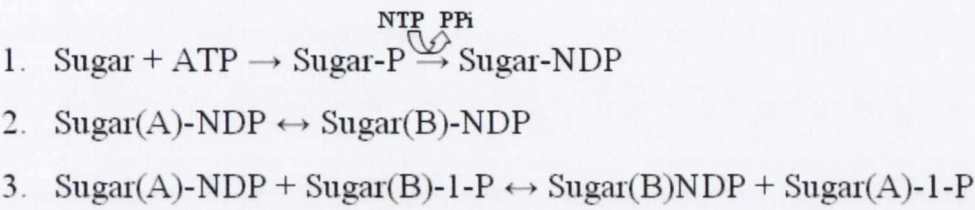


Figure 1.1 Common monosaccharides that occur in glycans.

Monosaccharides are activated to sugar nucleotides and this occurs by activation of the sugar with a kinase, followed by the transfer of the NDP (e.g. UDP, ADP) to the sugar-phosphate structure by a nucleotidyltransferase (see Figure 1.2, reaction 1). Alternatively, the activated donors can be generated from another existing activated sugar nucleotide, as illustrated in Figure 1.2 (reactions 2 and 3). The table represents the activated sugar nucleotides.



Sugar	Activated form
Glucose Galactose N-Acetylglucosamine N-Acetylgalactosamine	UDP-sugar
Mannose Fucose	GDP-sugar
Sialic acid	CMP-sugar

Figure 1.2 The formation, interconversion and activation of sugar nucleotides.

Activated sugar donors are formed by the sequential action of a kinase and nucleotidyltransferase (reaction 1). Alternatively, they can be generated from existing activated sugars, such as the interconversion of UDP-GlcNAc and UDP-GalNAc by UDP-*N*-acetylglucosamine-4-epimerase (reaction 2). Nucleotide exchange reactions can also occur to form rare sugars such as ADP-Glc, which are more common in plants and microbes (reaction 3). Table represents the activated sugar nucleotides (Varki *et al.*, 1999).

Monosaccharides are linked together with a glycosidic bond, where the hemiacetal group attached to the ring of one monosaccharide condenses with the hydroxyl group of another monosaccharide to form an acetal group. One water molecule is eliminated in this reaction, as illustrated by the β 1,4 glycosidic linkage of galactose and glucose to form lactose in Figure 1.3. Two types of stereochemistry can exist for glycosidic bonds – a β linkage is formed when bonds are made in the same plane as the monosaccharide conformation (i.e. equatorial bonds to the pyranose ring). An α linkage is formed by reaction of the axial bonds of the pyranose ring (perpendicular to the plane of the ring structure). The resulting disaccharide retains the ability to form another glycosidic linkage at the reducing end (Varki *et al.*, 1999).

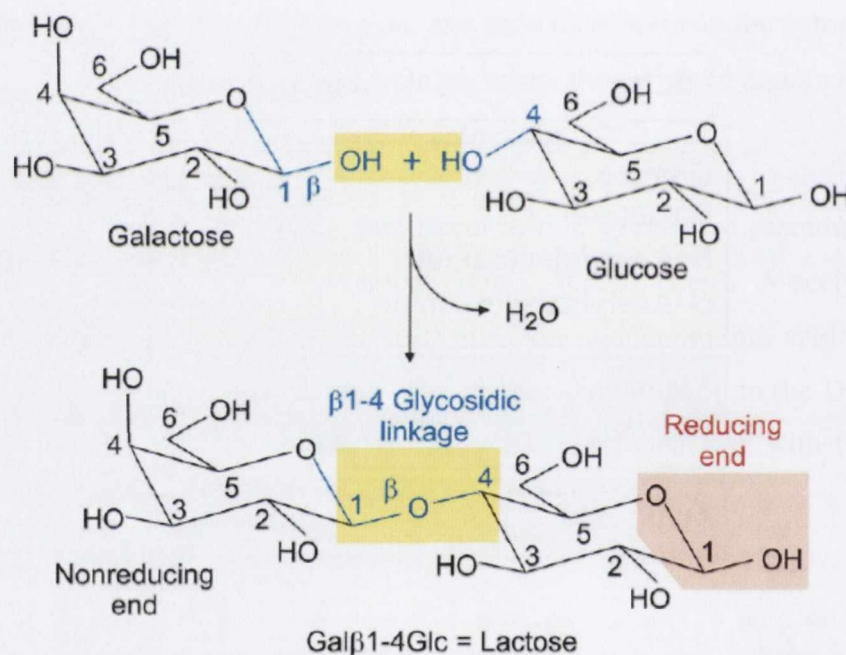


Figure 1.3 Formation of the glycosidic bond.

The glycosidic bond is formed by condensation of the hemiacetal group of galactose with the hydroxyl group of glucose to form lactose, while a reducing end is retained for the formation of further glycosidic linkages (Taylor and Drickamer, 2004).

1.1.2 O-glycosylation

O-glycosylation refers to a GalNAc monosaccharide α -linked to the hydroxyl group (the "O" linkage) of serine or threonine residues on proteins or occurs in the secretory pathway of all eukaryotic cells. However, the modification of serine and threonine residues in proteins also occurs in phosphorylation. The initiating event is the addition of the monosaccharide GalNAc (from UDP-GalNAc) to serine and threonine residues catalyzed by a polypeptide GalNAc transferase (GalNAcT). In contrast to N-glycosylation, a lipid-linked oligosaccharide precursor for transfer to protein is not required and a consensus sequence for GalNAc addition to polypeptides has not been found, however some predictions have been established. The O-glycans can be extended into long chains with variable termini and usually form biantennary structures. There is an increasing number of glycosyltransferases participating in O-glycan biosynthesis with multiple enzymes found to generate the same linkage (Varki *et al.*, 1999).

O-glycan structures include four common subtypes based on differential monosaccharide linkage reactions with the Core 1 GalT subtype (addition of galactose in a β 1,3 linkage to the GalNAc) being the most common structure (see Figure 1.4). Core 2-type O-glycans can be generated by addition of GlcNAc to the GalNAc in a β 1,6-linkage. Interestingly, Core 2 GlcNAcT appears to exhibit Core 4 GlcNAcT activity and can act on either unmodified Core 1 or Core 3 O-glycan. Core 2 O-glycan may become elongated into either a mono- or biantennary form with the presence of multiple lactosamine (Gal β 1-4GlcNAc) units and terminal linkages of fucose and sialic acid. Core 3 O-glycan production is controlled by Core 3 GlcNAcT activity which, like Core 1 GalT, uses the GalNAc α -Ser/Thr substrate therefore the formation of Core 3 O-glycan may depend on a competition between Core 1 GalT and Core 3 GalT enzymes. Core 3 O-glycan can also serve as the basis for the formation of biantennary O-glycans by virtue of Core 4 GlcNAcT activity (Varki *et al.*, 1999).

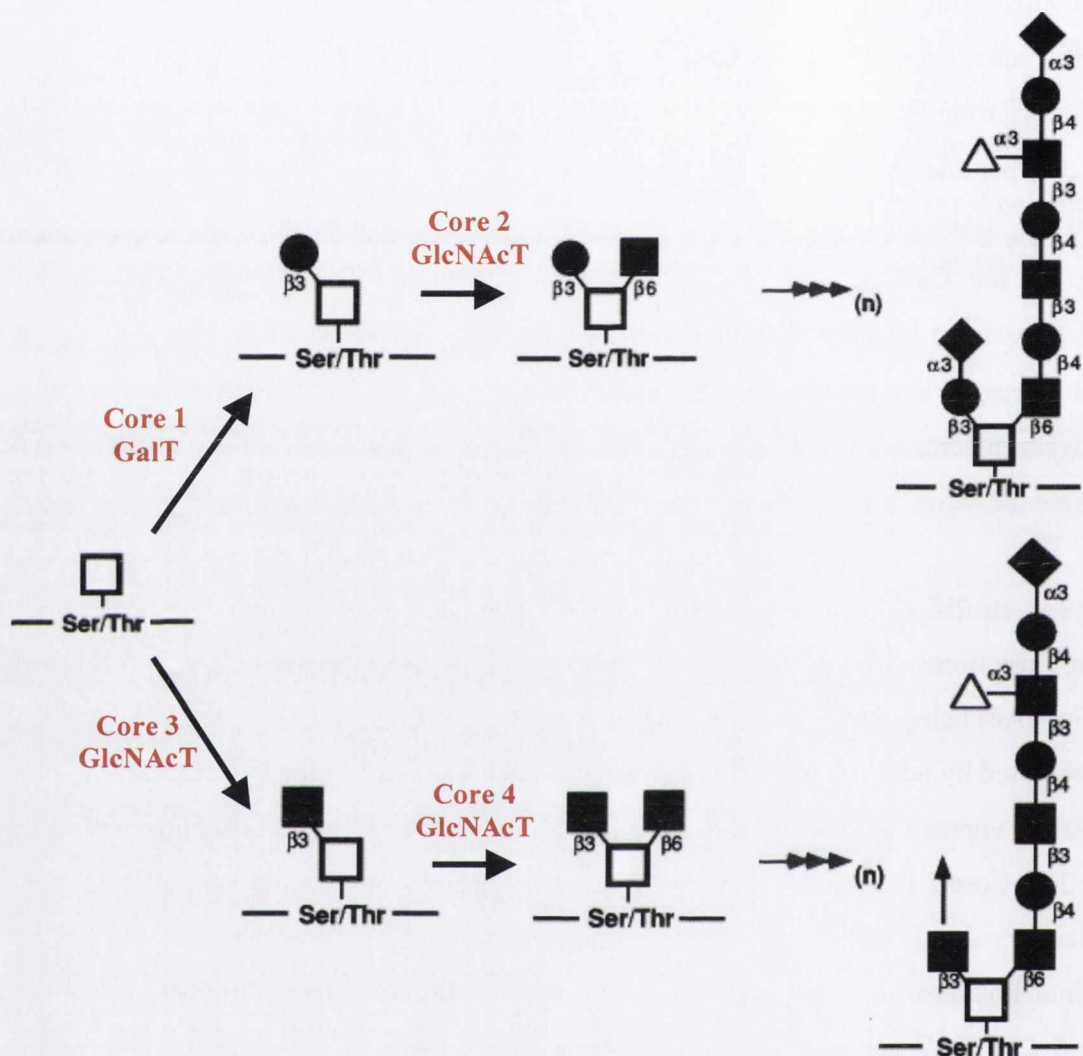


Figure 1.4 Formation of *O*-linked oligosaccharides.

Core 1 and Core 2 *O*-glycan subtype formation is controlled by the activity of Core 1 GalT and Core 2 GlcNAcT with further fucosylation and sialylation. Core 3 and Core 4 *O*-glycan subtype formation is controlled by the activity of the Core 3 GlcNAcT and Core 4 GlcNAc transferases, which can yield biantennary forms. Vertical arrow indicates additional linkages; $\square$ - GalNAc, $\blacksquare$ - GlcNAc, $\bullet$ - galactose (Gal), Δ - fucose (Fuc); $\blacklozenge$ - sialic acid; (Varki *et al.*, 1999).

O-glycosylation can result in the formation of mucin-type molecules, which are defined as cell surface or secreted glycoproteins with large numbers of clustered O-glycans. Expression of mucins is enriched in epithelial cells specialized for mucus production.

Additionally, the surface of certain circulating blood cells can be abundantly covered with O-glycans and play a role in the formation of the ABO blood group antigens. O-glycans have been reported to function in sperm binding to the egg, development and function of the hematopoietic system of vertebrates, in lymphocytes and during immune responses. Various pathogens use cell surface O-glycans as receptors for binding and infection of the host.

1.1.3 N-glycosylation

Protein N-glycosylation is an obligatory metabolic process that takes place in all eukaryotic cells. At least half of the genes functioning in this biosynthetic pathway have been conserved through evolution, including those in the earliest biosynthetic steps as well as in several subsequent reactions in the endoplasmic reticulum (ER). N-glycan biosynthesis is also responsible for structural diversification in the Golgi apparatus, including modification from high-mannose N-glycans into a large repertoire of hybrid and complex N-glycan subtypes that are either secreted or positioned at the vertebrate cell surface. This requires the presence of a large number of enzymes including glycosyltransferases and glycosidases encoded by distinct genes. The majority of these enzymes are transmembrane proteins that act in an ordered and well-orchestrated manner (Shaper and Shaper, 1992; Van den Eijnden and Joziassse, 1993). The complexity of N-glycan biosynthesis plays an essential role in diverse biological processes such as protein folding, conformation, stability, targeting to subcellular and extracellular sites, as well as cell-matrix and cell-cell interactions. In addition, most recent studies have revealed crucial roles for N-glycosylation in cell-cycle progression and cell apoptosis (Kukuruzinska and Lennon, 1998; Lis and Sharon, 1993).

1.1.3.1 Biosynthesis of the precursor oligosaccharide and transfer to protein

The first step involves the assembly of an oligosaccharide precursor structure linked to the lipid Dolichol-P (Dol-P) (see Figure 1.5) localised in the cellular compartments and the

endoplasmic reticulum (Varki *et al.*, 1999; Kornfeld and Kornfeld, 1985; Hubbard and Ivatt, 1981).

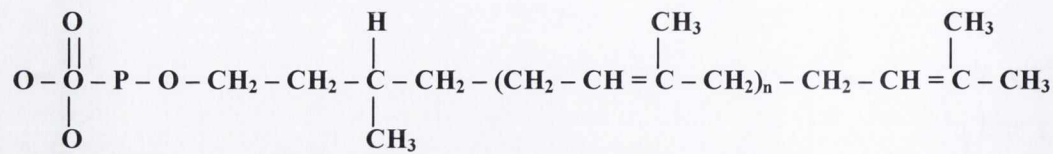


Figure 1.5 Structure of the Dol-P lipid used in constructing the dolichol oligosaccharide precursor. The number of isoprene repeats (n) varies between 15 and 19 (Varki *et al.*, 1999).

The initial linkages involving the two core *N*-acetylglucosamine (GlcNAc) monosaccharides and the first five mannose residues occur on the cytosolic side of the ER membrane and are catalyzed by glycosyltransferases. All ER glycosyltransferases are transmembrane proteins. In the first two steps GlcNAc residues are added to the Dol-P lipid by GlcNAc-1-phosphotransferase (see Figure 1.6, step I), and then by a GlcNAc-transferase (see Figure 1.6, step II). Next, five mannose (Man) residues are added sequentially by GDP-mannosyl transferase using GDP-Man as donor (see Figure 1.6, step III). Evidence suggests that the heptasaccharide is then translocated across the membrane bilayer and this process is catalyzed by flippase (not depicted). Next, four mannose units are added in rapid succession by Man-P-dolichol transferase using the Dol-P-Man donor (see Figure 1.6, step IV). The assembly of the dolichol oligosaccharide precursor is completed by Glc-P-dolichol transferase, which attaches three glucose (Glc) residues donated by Dol-P-Glc (see Figure 1.6, step V).

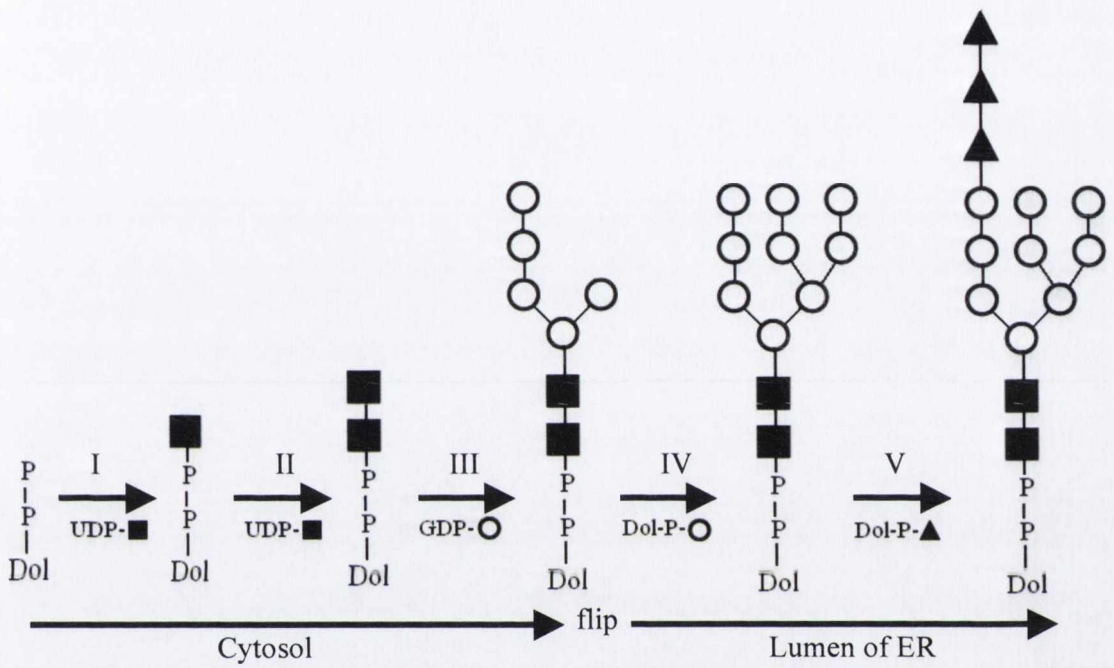


Figure 1.6 Biosynthesis of the dolichol oligosaccharide precursor.

Dol-P - dolichol-P; ■: GlcNAc; UDP-■: UDP-GlcNAc donor; ○: mannose; GDP-○: GDP mannose donor; Dol-P-○: dolichol-P mannose donor; ▲: glucose; Dol-P-▲: dolichol-P glucose donor (Varki *et al.*, 1999).

It appears that Dol-P-Man and Dol-P-Glc are also formed on the cytoplasmic side of the ER and are then flipped across the bilayer making them available to enzymes in the lumen of the ER. The completed dolichol lipid-linked oligosaccharide precursor can be transferred to asparagine residues on nascently translated proteins by a transmembrane, multisubunit protein complex called oligosaccharyltransferase (OST) as depicted in Figure 1.7. There are significant homologies among vertebrate, invertebrate, plant and fungal subunits of OST. In this mechanism, the OST complex binds to the lipid-linked oligosaccharide and transfers it to translated protein by cleavage of the high-energy GlcNAc-P bond, releasing Dol-P-P.

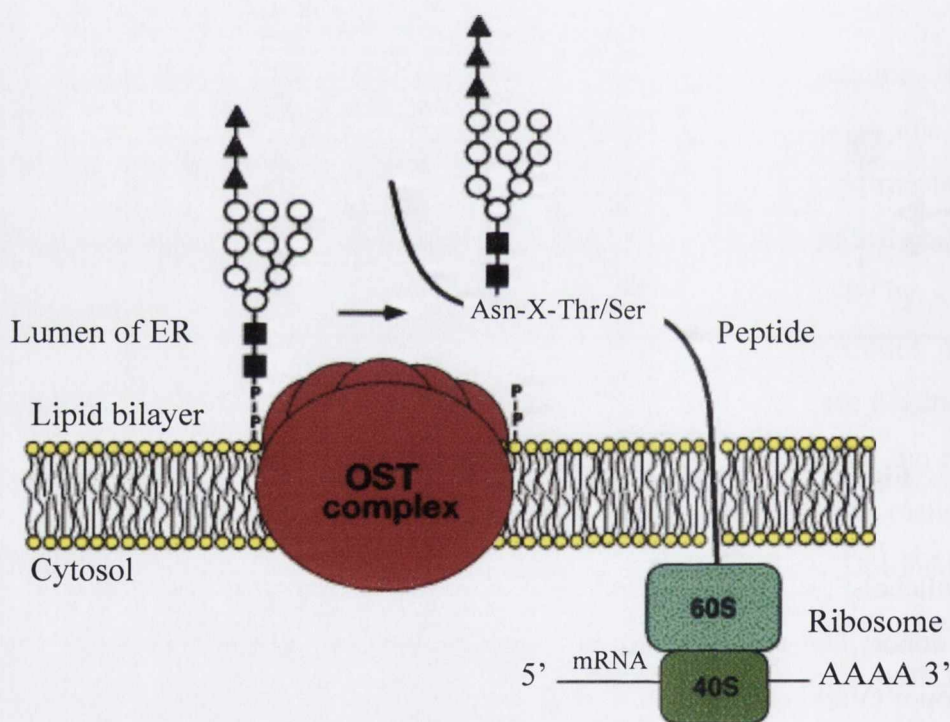


Figure 1.7 The OST complex residing in the ER membrane.

OST complex: oligosaccharyltransferase complex; ■: GlcNAc; ○: mannose; ▲: glucose; Asn: asparagine; X: any amino acid except for proline; Thr: threonine; Ser: serine; mRNA: messenger ribonucleic acid; 40S and 60S: ribosomal subunits (Varki *et al.*, 1999).

The minimal sequence requirement surrounding the acceptor asparagines is Asn-X-Thr/Ser, where X is any amino acid except for proline and the identity of this amino acid may affect the efficiency of glycosylation. It has been proven with model glycoproteins that transfer of the dolichol oligosaccharide precursor occurs approximately 30 amino acids from the active ribosome and that not all potential glycosylation sites in proteins that enter the ER are modified. Moreover, secondary structure also influences the *in vivo* efficiency of *N*-glycosylation (Baenziger, 1994; Spiro, 2002).

In the next few reactions, the oligosaccharide precursor undergoes modifications that play a key role in glycoprotein folding and lysosomal trafficking. Typically, unfolded and misfolded proteins are retained in the ER until they attain their native conformation. The initiating step, which is catalyzed by glucosidases I and II (Glcase I and II), results in gradual removal of all three glucose units (Figure 1.8). This is associated with the protein folding mechanism and contributes to the ER retention time of a given glycoprotein. Improperly folded proteins are re-glucosylated by α -glucosyltransferase (Glc-T) located in the lumen of the ER, which acts on $\text{Man}_9\text{GlcNAc}_2\text{-Asn}$. The re-glucosylated *N*-glycans are either refolded into a proper conformation or degraded. In the next processing steps, molecular chaperones such as calnexin and calreticulin selectively bind to newly translated proteins in the ER and appear to promote proper folding of the *N*-glycan (Figure 1.8). The remaining structure after the action of glucosidases and α -glucosyltransferase is subject to the action of a mannosidase yielding $\text{Man}_8\text{GlcNAc}_2\text{-Asn}$ and this *N*-glycan enters the *cis* portion of the Golgi apparatus. Some *N*-glycan precursors that have escaped into the Golgi and contain a few glucose residues are acted upon by an endo- α -mannosidase to generate the $\text{Man}_8\text{GlcNAc}_2\text{-Asn}$ structure, which can continue in the processing pathway. Though found in many mammalian cells, this activity has not been detected in CHO cells (Hiraizumi *et al.*, 1993, Hammond *et al.*, 1994).

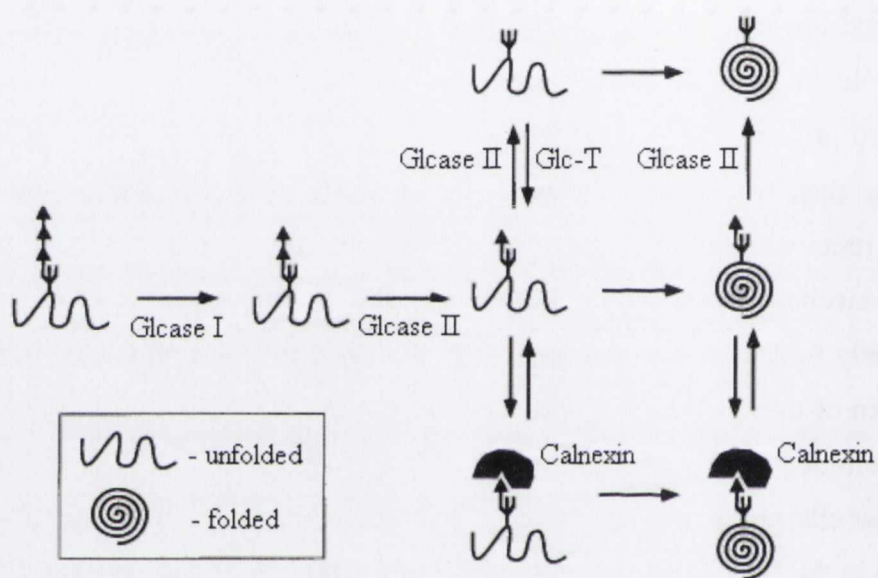


Figure 1.8 Calnexin regulates glycoprotein folding in the ER.

Glcase I and II: glucosidases I and II; Glc-T: α -glucosyltransferase; $\blacktriangle$: glucose; Ψ : $\text{Man}_8\text{GlcNAc}_2$ attached to a protein sequence (Varki *et al.*, 1999).

1.1.3.2 Synthesis of mature N-linked glycan structures

The structures of oligosaccharides in higher eukaryotes are determined by a series of processing reactions, catalyzed by glycosyltransferases and glycosidases that are initiated in the ER and then continued in the Golgi compartments (Gleeson, 1998).

Following its production in the ER, the $\text{Man}_8\text{GlcNAc}_2\text{-Asn}$ *N*-glycan is found in the *cis*-Golgi where two possible pathways exist. In most cases, the high-mannose glycans are processed by distinct α -mannosidases located in the ER and Golgi. Each α -mannosidase is capable of generating a $\text{Man}_5\text{GlcNAc}_2\text{-Asn}$ structure that serves as a substrate for the diversification of extracellular *N*-glycans.

N-glycan processing in yeast is initially similar to that in mammals, but in the case of the modification that takes place in the Golgi apparatus, mannose residues are added to the $\text{Man}_8\text{GlcNAc}_2\text{-Asn}$ to produce a high-mannose *N*-glycan of up to 15 residues. In vertebrates, these structures are generally found at low levels.

The $\text{Man}_5\text{GlcNAc}_2\text{-Asn}$ *N*-glycan serves a substrate enabling diversification into high-mannose, hybrid or complex subtypes. Most secreted and cell surface *N*-glycans are of the complex type (Kornfeld and Kornfeld, 1985) (see Figure 1.9). Core fucosylation is also a common modification that occurs after processing in the early endoplasmic reticulum. This involves the addition of fucose in an $\alpha 1,6$ -linkage to the GlcNAc that is linked to the asparagine residue (see Figure 1.9; complex type).

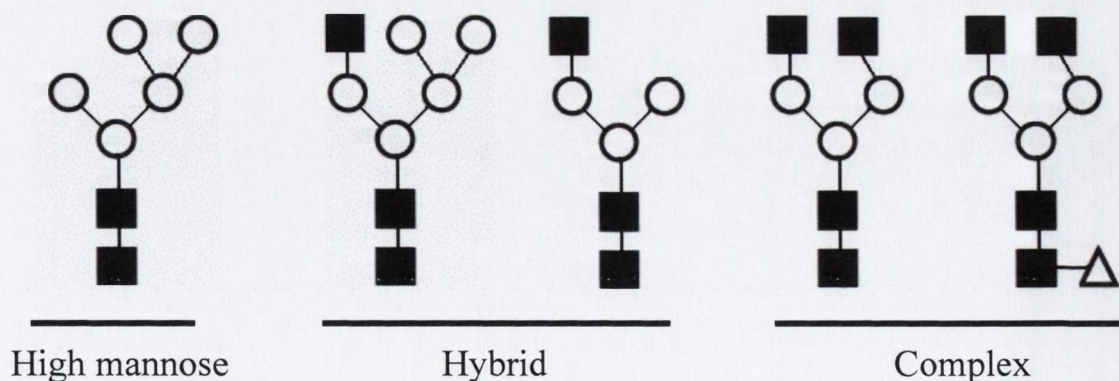


Figure 1.9 Major types of *N*-glycans (Varki *et al.*, 1999) (■: GlcNAc; O: mannose; Δ: fucose).

N-glycans of the hybrid and complex type may exist with two or more GlcNAc-bearing branches that are referred to as antennae. In forming multi-antennary *N*-glycan structures (see Figure 1.10), GlcNAc residues may be added to the trimannosyl core by six different GlcNAc transferases (I–VI). Up to six branches have been observed on *N*-glycans of some vertebrate glycoproteins. These GlcNAc residues can be further extended with other monosaccharides such as galactose or sialic acids, with the exception of those added by GlcNAc transferase III. Some reactions preclude others, so that only some linkages completed by the GlcNAc transferases can occur in any one *N*-glycan structure. This may be due to competition between enzymes for the same substrate and products may inhibit the action of other glycosyltransferases. It appears that factors other than the protein sequence may influence *N*-glycan diversification. Sugar nucleotide metabolism, transport rates in the ER and Golgi and the localisation of glycosyltransferases in the “assembly line” can also determine which enzymes encounter the glycan substrates first.

Most glycoconjugate degradation occurs in the lysosome. The process involves both endo- and exo-glycosidases, which are highly specific and act in a well-ordered manner. Monosaccharides that are eventually liberated can be reused for glycoconjugate synthesis.

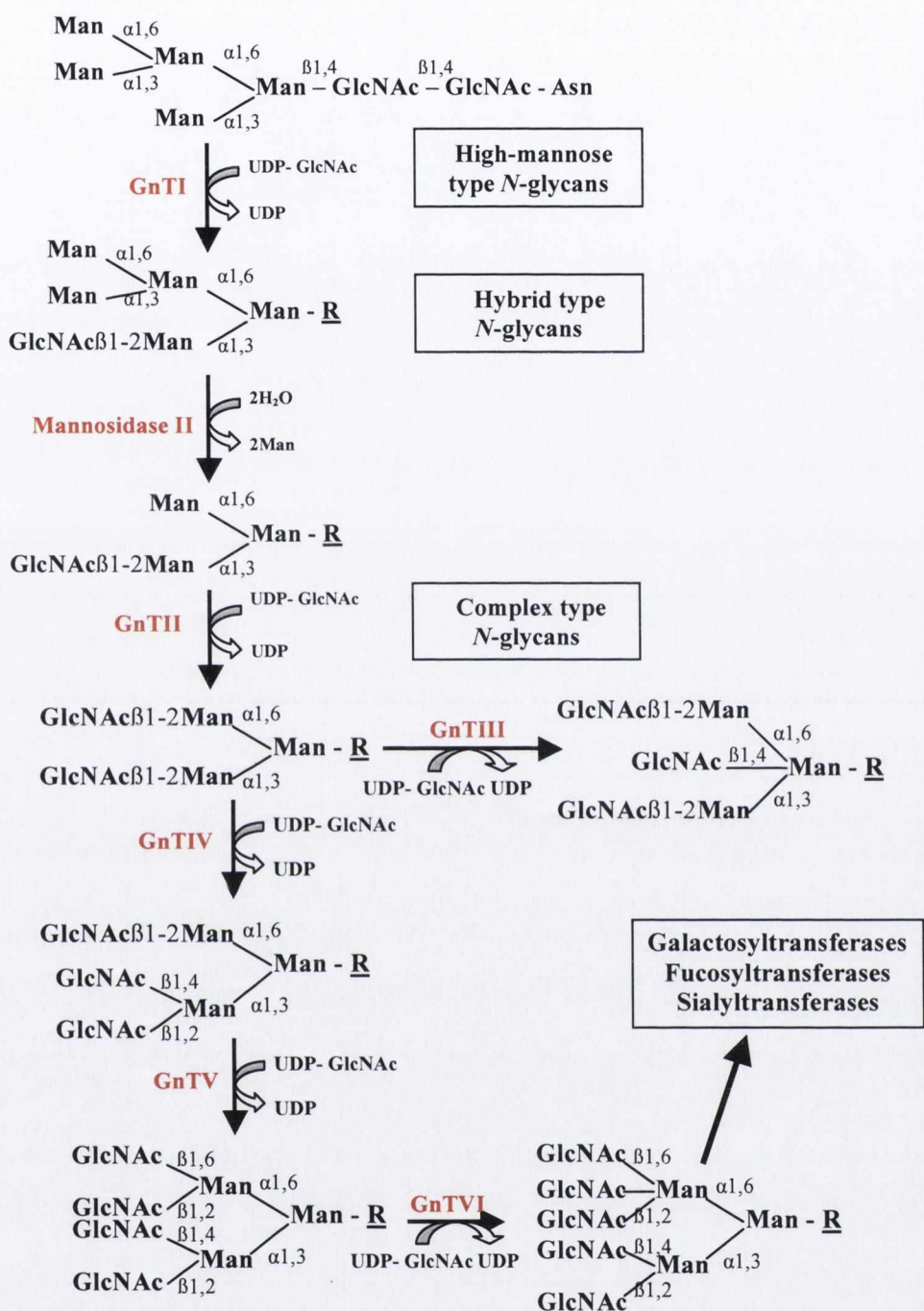


Figure 1.10 Hybrid and complex type *N*-glycan synthesis in the Golgi apparatus.

In forming multi-antennary *N*-glycan structures, GlcNAc residues may be added to the trimannosyl core by six different GlcNAc transferases (GnT). These GlcNAc residues can be further extended with other monosaccharides such as galactose, fructose or sialic acids. GnTIII is not considered an acceptor for further GlcNAc transferase action.

1.1.4 Regulation of glycosylation

Modification of proteins through glycosylation is controlled by factors that vary greatly among cell types and species, and change during development, differentiation and oncogenic transformation (Dinter and Berger, 1995). The current understanding concludes that the extent of protein glycosylation is determined by the expression level and activity of enzymes involved in this biochemical pathway (Robbins, 1991). Factors such as the distribution of glycosylation enzymes in the various ER and Golgi compartments, the availability of acceptor substrates, competition among enzymes for acceptor modification, expression levels of acceptor glycoproteins and glycolipids and rates of glycoprotein catabolism can also affect glycosylation (Breton *et al.*, 2001). Glycosylation of many proteins is also affected by hormones and cellular regulators including cAMP (Konrad and Merz, 1994). However, several lines of evidence indicate that one of the major modes of regulating cellular glycosylation is transcriptional regulation of the enzymes involved in glycan synthesis and catabolism (Comelli *et al.*, 2006; Alison *et al.*, 2008).

1.1.5 Glycosylation changes and disease

Numerous human diseases with a clear pathogenic relationship to a genetic defect in glycan structure have been now recognized. Recent remarkable progress in this area has led to the identification of several inherited biochemically and clinically heterogeneous disorders including carbohydrate-deficient glycoprotein syndromes (CDGS), leukocyte adhesion deficiency syndrome II (LADII), congenital dyserythropoietic anemia type II (HEMPAS), galactosemia, and abnormalities in proteoglycan synthesis. Individuals with CDGS, LADII, HEMPAS and galactosemia typically suffer from malfunction of multiple organ systems with severe neurological and metabolic dysfunction. Examples of human genetic alterations in glycan structure are listed in Table 1.1. Based on the studies many of the diseases are probably more genetically heterogeneous than expected and consequently, a new awareness should foster new insights into their pathogenesis and treatment.

Table 1.1 Human genetic defects in glycan formation and their clinical outcome.

Disorder	Defect
I-cell disease and pseudo-Hurler polydystrophy (mucopolidosis II and III)	Deficiency of UDP-GlcNAc: lysosomal enzyme GlcNAc-1-phosphotransferase
Carbohydrate-deficient glycoprotein syndromes (CDGS)	Several genetic defects in the assembly, transfer, and early processing of <i>N</i> -glycans
Leukocyte adhesion deficiency type II; primary defect unknown	Decreased conversion of GDP-Man to GDP-Fuc; primary effect unknown
Galactosemia defects	Defects in forming UDP-Gal, e.g., Gal-1-phosphate uridylyltransferase
Macular corneal dystrophy type I	defective keratan sulfate biosynthesis
Ehlers-Danlos syndrome with progeroid syndrome	Partial deficiency of xylosylprotein β 4 galactosyltransferase (GalT-I)
Diastrophic dysplasia, achondrogenesis type Ib, atelosteogenesis type II	Diastrophic dysplasia sulfate transporter; defect in sulfate availability
Simpson-Golabi-Behmel overgrowth syndrome	Glypican-3 proteoglycan core protein deficiency
Hereditary erythrocytic multinuclearity with positive acidified serum test (HEMPAS); also called congenital dyserythropoietic anemia type II (CDA type II)	Partial deficiency of UDP-GlcNAc: α -mannosidase GlcNAc transferase II or processing α -mannosidase II; primary genetic defect unclear
Tn syndrome	Deficiency in β 1–3 galactosyltransferase that synthesizes Core 1 <i>O</i> -glycans; affects erythrocytes
IgA nephropathy	Same as for Tn syndrome, except that IgA is affected

1.2 Golgi apparatus

1.2.1 Structure of the Golgi apparatus

In mammalian cells, the Golgi is typically located around the centrosome and can be schematically viewed as being composed of three main compartments: the *cis*-, *medial*- and *trans*-Golgi (Polishchuk and Mironov, 2004). On electron microscopy sections and its graphical image (see Figures 1.11 and 1.12), three basic structural elements can be recognized: stacks of flat disc-shaped cisternae, abundant tubular-reticular networks (*cis*-Golgi network, CGN and *trans*-Golgi network, TGN) and vesicles which together form a continuous membranous ribbon collected in a pericentriolar space (Rambourg and Clermont, 1990, Ladinsky *et al.*, 1999). Despite this architecture, the Golgi apparatus is highly dynamic and combines enormous rates of membrane flux, where newly synthesized proteins and lipids from the ER are processed and then distributed to the plasma membrane and to the endosomal/lysosomal system (Keller and Simons, 1997).

The space near the *cis* side of the Golgi stacks contains a layer of branching tubular structures and flattened saccules, potentially ER-to-Golgi carriers. These organelles exhibit membrane domains resembling budding or fusing non-coated vesicles (Ladinsky *et al.*, 1999).

The *medial* Golgi comprises all cisternae of the stack located between the CGN and the TGN and that has been referred to as the ‘central Golgi domain’. The number of cisternae forming the Golgi stacks varies from one cell type to another while the length of the cisternae frequently depends on the axis of the section. The lumen of a cisterna is usually quite narrow (10–20 nm) and may show swellings (granules) filled with a material of low electron density (Tu and Banfield, 2009). The lipid content of these different Golgi compartments also differs significantly with most of the cholesterol being concentrated at the *trans* side (Orci *et al.*, 1981).

Biochemical and TEM studies have revealed that the TGN is involved in the terminal glycosylation of proteins as well as in cargo packaging into membrane carriers destined for the plasma membrane or the endosomal/lysosomal system (Keller and Simons, 1997). The morphology and size of the TGN depend on the predominant type and amount of cargo protein departing from the Golgi complex. Interestingly, the volume and surface area of the

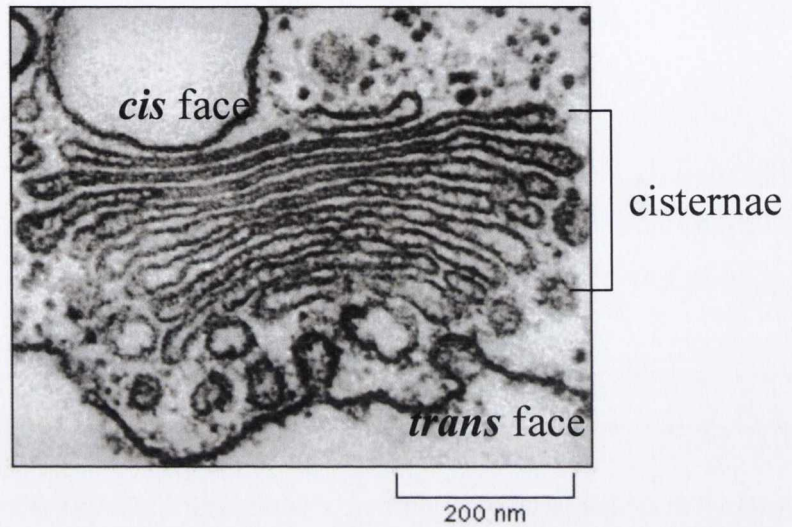


Figure 1.11 Transmission electron microscopy (TEM) image of the Golgi apparatus (Polishchuk and Mironov, 2004).

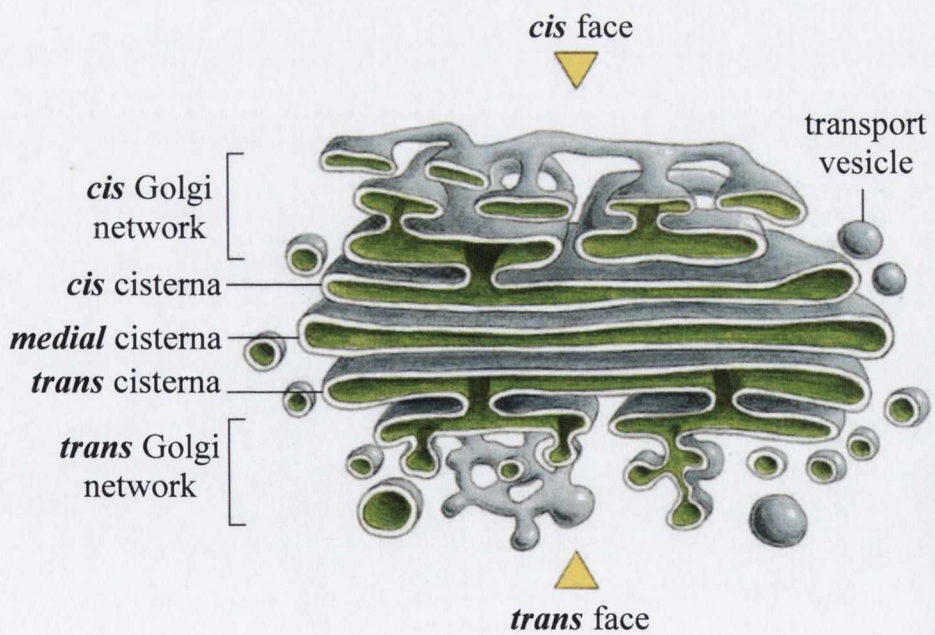


Figure 1.12 Golgi apparatus and its compartments.

TGN is seen to increase when the exit from the Golgi is blocked by a lowering of the temperature to 20°C (Griffiths *et al.*, 1989). The *trans*-most cisterna produces exclusively clathrin-coated buds, whereas the other cisternae display only non-clathrin-coated buds (Ladinsky *et al.*, 1999).

Vesicles of smaller (50–60 nm) and larger (up to 80–90 nm) diameters are considered to be important elements of the Golgi and depend on the activity of either the COPI or the clathrin machinery. Vesicles are thought to be involved either in anterograde, (Orci *et al.*, 1997) or retrograde intra-Golgi transport of cargo (Martinez-Menarguez *et al.*, 2001) as well as in the recycling of Golgi enzymes (Glick and Malhotra, 1998).

1.2.2 Golgi maturation and intra-Golgi transport

Proteins enter the secretory pathway following their translocation into the lumen of the endoplasmic reticulum (ER) and then exit the ER in transport vesicles transiting the Golgi apparatus via the *cis*, *medial*, and *trans* cisternae (Young, 2004). Forward/anterograde vesicular transport, cisternal maturation/retrograde transport and lipid partitioning are three models of *cis-trans* Golgi transport (see Figure 1.13). They are not mutually exclusive and may in fact be occurring simultaneously. Rothman and colleagues (Rothman *et al.*, 1994) have proposed that vesicle transport may provide a ‘fast track’ for cargo transport through the Golgi, whereas cisternal flow may be considerably slower in moving cargo across the stack.

The anterograde vesicular transport model considers the Golgi cisternae as a stable structure and resident proteins are specifically retained, while cargo molecules will move forward in transport vesicles which bud and fuse among static cisternae. The vesicles were found to bud from all cisternae of the stack and were associated with a specific protein coat, known as COPI (**CO**at **P**rotein **I** or coatomer) (Elsner *et al.*, 2003). However, there is now considerable evidence that COPI vesicles also mediate retrograde transport from the Golgi to the ER (Letourneur *et al.*, 1994). Another essential component of vesicular transport is the SNARE proteins, integral membrane proteins that mark both the transport vesicle and the acceptor membrane compartment, and which contribute to the specificity of vesicle docking and fusion.

The cisternal maturation/retrograde transport model (Elsner *et al.*, 2003; van Vliet *et al.*, 2003) predicts that cargo molecules move through the stack passively as the cisternae move forward to form sequentially the *cis*, *medial*, and *trans* Golgi cisternae and finally the *trans* Golgi network (TGN), while resident proteins are recycled by retrograde transport to establish differential concentrations across the stack. Retrograde transport provides a key to the generation of differences in the distribution of Golgi resident proteins (Pelham, 1998). Newly synthesized proteins, including glycosyltransferases destined to become Golgi residents, are recruited as cargo into coat protein COPII coated vesicles. The process of maturation is accomplished by the retrograde trafficking of COPI-coated vesicles, which are enriched at least three-fold in Golgi resident glycosylation enzymes as compared to anterograde cargo molecules by a process mediated by the small GTPase arf-1 (Lanoix *et al.*, 1999).

The possibility that the Golgi functions as a continuous compartment has been raised previously (Pelham, 2001; Beznoussenko and Mironov, 2002) and was further verified by Patterson *et al.*, in 2008. It was proposed that the Golgi is a continuous and dynamic structure with the clear evidence of segregation of processing enzymes into different compartments and that the differential distribution of lipids could account for the differential partitioning of these enzymes.

The conserved oligomeric Golgi complex (COG) localises peripherally to the cytoplasmic side of Golgi membranes and its role is to support the intra-Golgi retrograde transportation acting as a tether for vesicles containing recycling glycosyltransferases (Shestakova *et al.*, 2006).

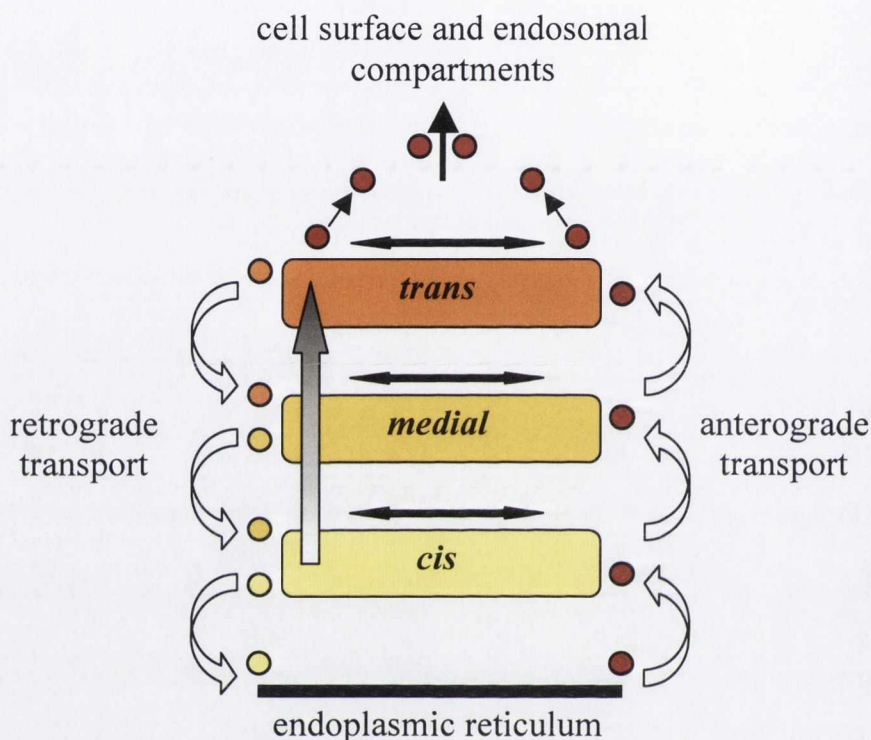


Figure 1.13 Mechanisms of protein and membrane trafficking in the Golgi.

Retrograde transport vesicles carry glycosyltransferases and transport machinery in COPI-coated vesicles. Vertical arrows indicate the direction of movement of proteins within the Golgi whereas horizontal arrows denote the lateral segregation of proteins and lipids within individual cisterna. The thicker arrow denotes the movement of proteins transiting the Golgi via cisternal maturation. The distinct protein and lipid compositions of individual cisterna may be established by transiting the Golgi apparatus in anterograde moving COPI-generated transport vesicles by rapid partitioning and/or via cisternal maturation.

1.2.3 Targeting and retaining enzymes in the Golgi

The non-uniform distribution of glycosyltransferases in the Golgi is maintained through a combination of retention and recycling mechanisms. The nature of the retention of glycosyltransferases has led to two major hypotheses for this mechanism. The first hypothesis was the lipid bilayer thickness or sorting model (Bretscher and Munro, 1993; Masibay *et al.*, 1993; Young, 2004), while the second hypothesis postulates that proteins aggregate (“kin recognition”) which leads to Golgi retention (Machamer, 1991; Nilsson *et al.*, 1993b; Young, 2004).

The lipid bilayer thickness or sorting model was based on two observations, the thickness of the lipid bilayer and the length and characteristics of the hydrophobic transmembrane domain (TMD). In mammalian cells the concentrations of cholesterol and sphingolipids increases from the ER through the Golgi to the plasma membrane which results in a thickening of the membrane bilayer. Comparisons revealed that the length of Golgi transmembrane domains were on average five amino acids shorter than those of plasma membrane proteins (Masibay *et al.*, 1993). The essence of “kin recognition” is that the interaction between the transmembrane domains and stem regions of glycosyltransferase and lipid bilayers of Golgi membranes leads to formation of homo- or hetero-oligomers and prevents delivery of enzymes to secretory vesicles resulting in their Golgi retention.

1.3 Glycosyltransferases

Glycosyltransferases (GT; EC 2.4.x.y) constitute a large and diverse family of enzymes that are involved in the biosynthesis of oligosaccharides, polysaccharides and glycoconjugates. GTs catalyze the transglycosylation reactions where the monosaccharide components from activated nucleotide donor molecules (e.g. UDP-Glc) are transferred to specific acceptor molecules (saccharide, protein, lipid or DNA) to form glycosidic bonds (see Figure 1.14) (Palcic, 1994). There are a few exceptions to this generalisation, including oligosaccharyltransferases, mannosyltransferases and glucosyltransferases (Paulson and Colley, 1989).

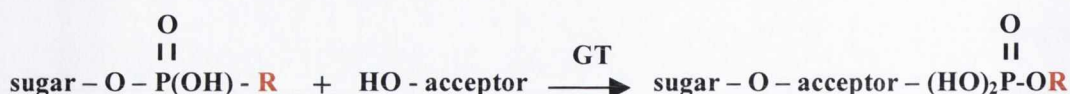


Figure 1.14 Reaction catalyzed by glycosyltransferases (Palcic, 1994).

Glycosyltransferases are resident in the ER and Golgi apparatus and belong to transmembrane type II proteins, sharing the same general structure consisting of a short cytosolic amino terminal tail, a transmembrane domain, a luminal stem region and a large globular catalytic domain facing the luminal side (see Figure 1.15) (Tu and Banfield, 2009; Burke *et al.*, 1992; Grabenhorst *et al.*, 1999). Experiments using engineered glycosyltransferase genes showed that the transmembrane domains of glycosyltransferases primarily control targeting of the enzymes to specific Golgi compartments (Weinstein *et al.*, 1996).

Glycosyltransferases have been classified into families on the basis of amino acid sequence similarities, substrate/product stereochemistry and the type of sugar they transfer (i.e. galactosyltransferases, sialyltransferases, *etc.*). To date, the database comprises of more than 15,000 known and putative GT sequences that have been divided into 92 families. However, not all GT sequences are present in the database and the number of families will likely increase with the discovery of new GT genes (Breton *et al.*, 2006). Large differences in the numbers and functions of GTs have been observed among families. Despite the fact that many GTs recognize identical donor or acceptor substrates, there is surprisingly limited sequence homology among different classes, but the enzymes share some secondary features (Ünlügil *et al.*, 2000).

Two structural folds of these enzymes, GT-A and GT-B have been identified and verified experimentally. A third glycosyltransferase fold termed GT-C is currently being under investigation using programs such as BLAST. The GT-A fold (“single domain fold”) consists of an open twisted β -sheet surrounded by α -helices on both sides, the overall architecture of the GT-A fold is reminiscent of two Rossmann-like folds, typical of nucleotide-binding proteins. Two tightly associated $\beta/\alpha/\beta$ domains, the sizes of which vary, abut closely, leading to the formation of a continuous central β -sheet (represented by SpsA

enzyme from *Bacillus subtilis* in Figure 1.16). Typical eukaryotic members of the GT-A fold have a short N-terminal cytoplasmic tail followed by the transmembrane domain and the stem region leading to the catalytic region. Additionally, most of the GT-A enzymes possess an conserved Asp-X-Asp signature (“DXD motif”), which coordinates an essential divalent cation (usually Mn^{2+}) by electrostatically stabilizing the negative charge of the nucleoside diphosphate group (Breton *et al.*, 1998). The architecture of the GT-B fold consists of two $\beta/\alpha/\beta$ Rossmann-like domains, which are less tightly associated and face each other with the active-site lying within the resulting cleft (represented by bacteriophage T4 β -glucosyltransferase Figure 1.16).

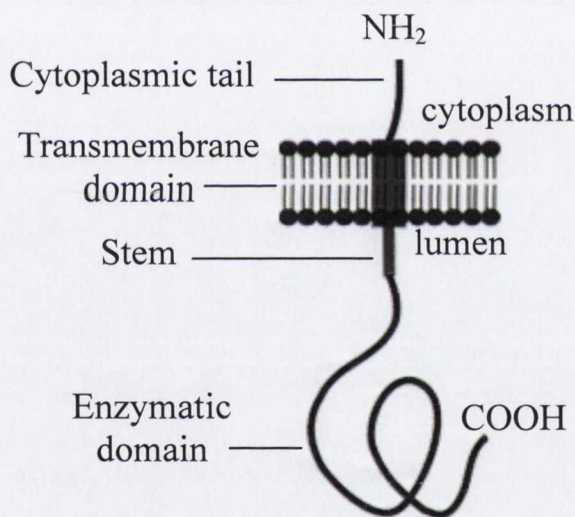
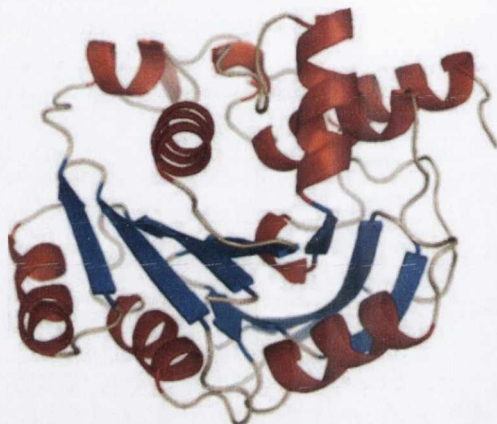


Figure 1.15 Topology of Golgi resident glycosyltransferases (Tu and Banfield, 2009).

GT-A fold



GT-B fold



Figure 1.16 Folds observed for glycosyltransferases (Lairson *et al.*, 2008).

Additionally, two stereochemical outcomes are possible in the formation of glycosidic bond: the product can either be retained or inverted with respect to the donor substrate. The inverting enzymes employ the mechanism of a direct displacement S_N2 -like reaction, which results in an inverted configuration of stereochemistry at the anomeric reaction center of the donor substrate whereas the retention of stereochemistry at the anomeric center is considered an outcome of retaining glycosyltransferase (see Figure 1.17). The proposed mechanism behind the retention of stereochemistry is a double-displacement involving a covalently bound glycosyl-enzyme intermediate with a positively charged divalent cation playing a role of the Lewis acid (Breton *et al.*, 2001; Coutinho *et al.*, 2003; Lairson *et al.*, 2008).

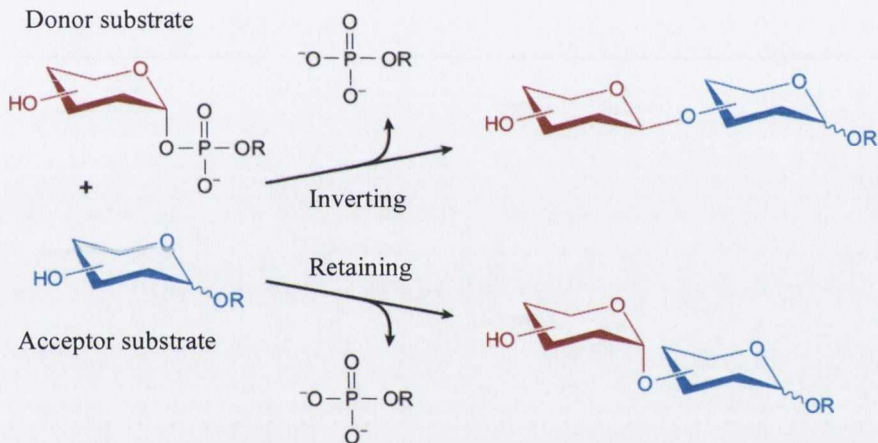


Figure 1.17 Stereochemistry of inverting and retaining catalysis.

It has been suggested that GTs acting early in protein-linked oligosaccharide biosynthesis are located in the *medial/trans*-Golgi, whereas “late” GTs are located within the *trans*-Golgi/TGN (*trans*-Golgi network). There is now evidence that most GTs show an overlapping distribution throughout the Golgi apparatus and also demonstrate cell-type specific features (Colley, 1997; Warren and Malhotra, 1998; Breton *et al.*, 2001). Many Golgi glycosyltransferases are often glycoproteins themselves. Biochemical analyses suggest that many mammalian glycosyltransferases are posttranslationally modified by glycosylation, which is required for proper folding and/or activity (Kleene and Berger, 1993).

1.3.1 GlcNAc transferases (GlcNAcTs, N-acetylglucosaminyltransferases, GnTs)

GlcNAc transferases belong to the family of inverting enzymes that catalyze the transfer of a GlcNAc residue from a nucleotide donor (UDP-GlcNAc) to specific saccharide acceptor and therefore initiate the formation of hybrid and complex-type *N*-glycans. GnTI and II are of the GT-A fold, GnTIII is predicted to adopt either the GT-A or GT-B fold and GnTIV and GnTV have not been classified yet and belong to so-called “orphan” families (Liu and Mushegian, 2003).

1.3.1.1 GlcNAc transferase I (GlcNAcT-I, GnTI; EC 2.4.1.101)

GnTI is the essential signal in the biosynthesis of hybrid and complex-type chains and transfers the first GlcNAc residue from UDP-GlcNAc to the oligomannose core (Man) structure (see Figure 1.18) (Kornfeld and Kornfeld, 1985; Sarkar *et al.*, 1991).

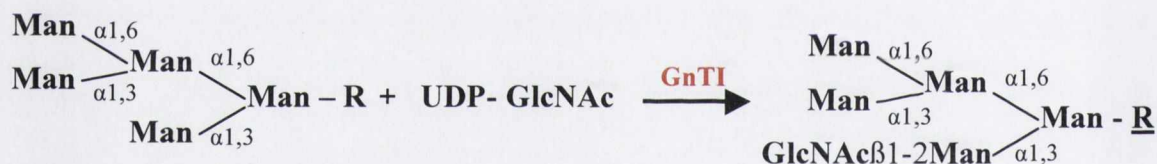


Figure 1.18 Reaction catalyzed by GnTI ($\mathbf{R} - \beta 1,4\text{GlcNAc}(\beta 1,4)\text{GlcNAc} - \text{Asn}$).

The presence of a $\beta 1,2$ -linked GlcNAc residue at the non-reducing terminus of the arm is essential for the subsequent action of several enzymes in the processing pathway.

GnTI expression has been reported in hen oviduct, baby hamster kidney cells, bovine colostrums, mammalian liver and pig trachea (Schachter *et al.*, 1989). Mutants (L-PHA resistant Lec1 CHO cells) lacking this enzyme accumulate the GlcNAc₂Man₅ structure, indicating that there are no activities compensating for this transferase (Robertson *et al.*, 1978; Kumar and Stanley, 1989; Chen and Stanley, 2003). Lec1 CHO cells have no GnTI activity whereas Lec1A cells have a partial activity of this enzyme showing an altered pH optimum and increased apparent K_m values for substrates (Stanley and Chaney, 1985;

Chaney and Stanley, 1986; Chen *et al.*, 2001). Surprisingly, removal of 106 amino acids from the N-terminus of GnTI sequence did not result in loss of enzyme activity as described by Saribas *et al* in 2007. GnTI has been shown to be essential for morphogenesis; embryos die before birth with defects in neural tube formation, vascularization and determination of body plan asymmetry (Puthalakath *et al.*, 1996, Zhang *et al.*, 2002). Disruption of the mouse locus encoding GnTI leads to embryonic death around day 10 (Ioffe and Stanley, 1994). Thus, complex *N*-linked oligosaccharides are important for aspects of morphogenesis, but not for differentiation or cell viability (Chen *et al.*, 2001).

The X-ray structure of the rabbit GnTI protein has been solved at 1.8 Å resolution (Gordon *et al.*, 2006) as shown in Figure 1.19. The structure contains an eight-stranded and a four-stranded mixed β -sheet flanked by α -helices. A 13-residue loop, which is disordered without accompanying ligands, seems to be perfectly structured in the presence of UDP-GlcNAc and Mn^{2+} ions (Ünlügil and Rini, 2000).

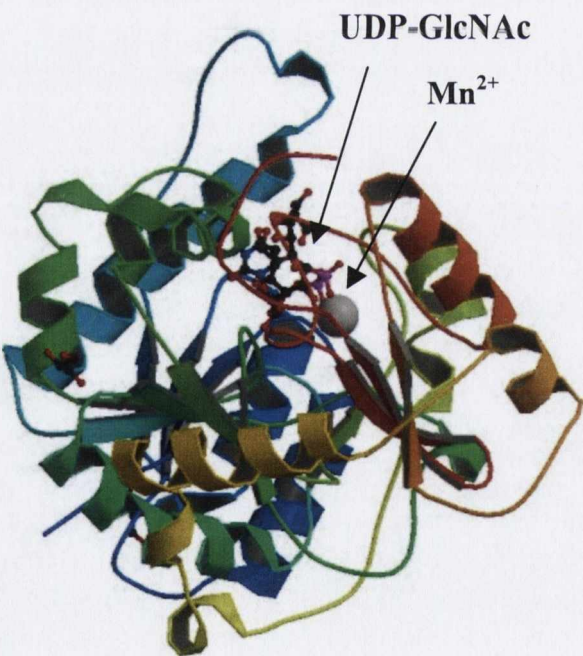


Figure 1.19 The crystal structure of rabbit GnTI.

GnTI is metal-dependent inverting GT-A fold glycosyltransferase. The image represents the 2AM3 structure of GnTI with accompanying ligands deposited in RCSB Protein Data Bank (www.rcsb.org/pdb) (Lairson *et al.*, 2008).

1.3.1.2 *GlcNAc transferase II (GlcNAcT-II, GnTII; EC 2.4.1.143)*

The oligosaccharide created by the action of mannosidase II is a highly specific substrate for GnTII that adds a GlcNAc residue in a β 1,2 linkage and creates a biantennary structure (see Figure 1.20). The acceptor molecule must have a terminal GlcNAc(β 1,2)Man(α 1,3) branch to display any activity, but does not require the reducing GlcNAc residue at the core mannose (additional residues abolish catalytic activity) (Bendiak and Schachter, 1987).

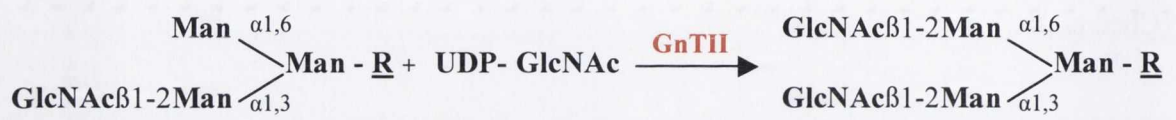


Figure 1.20 Reaction catalyzed by GnTII (**R** – β 1,4GlcNAc(β 1,4)GlcNAc -Asn).

GnTII activity has been reported in hen oviduct, CHO cells, baby hamster kidney cells, bovine colostrums, mammalian liver and pig trachea (Schachter, 1989). Mice lacking the *GnTII* gene were deficient in GnTII activity, resulting in severe gastrointestinal, hematologic and osteogenic abnormalities (Wang *et al.*, 2001).

1.3.1.3 *GlcNAc transferase III (GlcNAcT-III, GnTIII; EC 2.4.1.144)*

GnTIII can add a “bisecting” GlcNAc in a β 1,4 linkage to the β -linked mannose of the *N*-glycan core (see Figure 1.21) (Stanley, 2002).

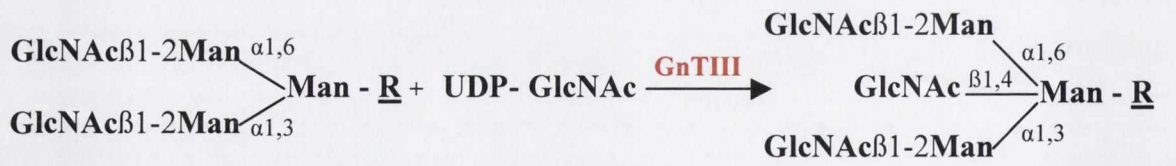


Figure 1.21 Reaction catalyzed by GnTIII (**R** – β 1,4GlcNAc(β 1,4)GlcNAc -Asn).

GnTIII requires the prior action of GnTI. The insertion of a bisecting GlcNAc prevents the actions of mannosidase II, core fucosyltransferase, GnTII, GnTIV and GnTV, thereby effectively stopping any processing of hybrid to complex structures (Narasimhan, 1982).

Tissues expressing high levels of GnTIII protein (e.g. hen oviduct) will synthesize large amounts of bisecting hybrid glycans (Schachter *et al.*, 1989).

More than one promoter may regulate *GnTIII* gene expression and the levels of GnTIII are translationally controlled during the cell cycle (Kang *et al.*, 2000). The pattern of expression of the *GnTIII* gene suggests that the action of GnTIII correlates with tissue specific functions (Bhaumik, 1995).

GnTIII expression has been reported in hen oviduct, preneoplastic hepatic nodules and in the dominant ricin-resistant mutant CHO cell line LEC 10 (Schachter *et al.*, 1989; Stanley *et al.*, 2005). There are apparent consequences of a lack of GnTIII expression in cell culture. Compared to wild-type CHO cells that do not express GnTIII, LEC10 cells manifest altered lectin binding (Campbell and Stanley, 1984; Stanley *et al.*, 2005).

The effects of overexpressing or eliminating GnTIII may vary with cell type and have different consequences for cell-cell interactions and growth control. The possession of a bisecting GlcNAc residue enhances the effector functions of recombinant IgG immunoglobulins. This may prove valuable for the development of new recombinant antibody therapeutics used for cancer therapies and passive immunology (Umaña *et al.*, 1999). Mice that have no GnTIII activity are viable and fertile, but mutation in the *GnTIII* gene leads to subtle neurological changes (Stanley, 2002). Experiments on mice lacking GnTIII activity showed enhanced liver tumour progression (Nishikawa *et al.*, 1988). The first transgenic mice overexpressing GnTIII exhibited effects on the cell morphology of hepatocytes and glycoprotein metabolism in liver (Stanley, 2002). In another example, overexpression of GnTIII in various tumour cell lines inhibited the binding of growth factors to the cell surfaces, increasing the growth rate of tumour cells (Rebbaa *et al.*, 1997). Further experiments showed that overexpression of GnTIII in different cell lines results in a loss of sensitivity to cell death by natural killer cells. This effect would undermine its use as a therapeutic strategy (Yoshimura *et al.*, 1996). Moreover, GnTIII expression was found to suppress expression of the hepatitis B virus in the human hepatoma cell line HB611, indicating the anti-viral effect of bisecting GlcNAc (Yoshimura *et al.*, 1998).

1.3.1.4 GlcNAc transferase IV (GlcNAcT-IV, GnTIV; EC 2.4.1.145)

N-acetylglucosaminyltransferase IV catalyzes the transfer of a GlcNAc residue in a β 1,4 linkage to the GlcNAc(β 1,2)Man(α 1,3) branch of an *N*-glycan to form a triantennary structure (see Figure 1.22) (Oguri *et al.*, 1997).

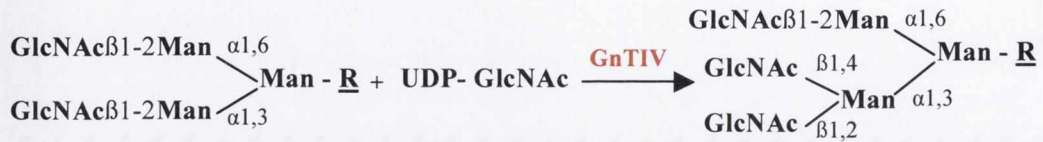


Figure 1.22 Reaction catalyzed by GnTIV ($\text{R} = \beta$ 1,4GlcNAc(β 1,4)GlcNAc-Asn).

Maximal activity requires the presence of both terminal β 1,2-linked GlcNAc residues in the substrate. Removal of the GlcNAc residue on the Man α 1-6 or both GlcNAc residues reduces activity by at least 80%. The presence of a bisecting GlcNAc prevents GnTIV action, indicating that GnTIV must act before GnTIII to form bisected multiantennary oligosaccharides (Gleeson and Schachter, 1982). Branching is inhibited by the action of GnTIII, but appears optimal on *N*-glycans bearing the GnTV branch and GnTIV requires prior branching by GnTI and GnTII (Schachter, 1989). The presence of GnTIV has been reported in hen oviduct, baby hamster kidney cells, human lymphoid and myeloid cell lines (Schachter, 1989).

Three GnTIV isozymes (IVA, IVB and IVC) were identified in human, mouse and rat and exhibit different expression profiles among tissues and cancer cell lines (Minowa *et al.*, 1998). Southern analysis showed that most animals possess both GnTIV genes. According to data obtained from experiments performed by Oguri *et al.* (2006), these three isoenzymes have the same substrate specificities, but GnTIVB seems to have a lower affinity for donors and acceptors. This may suggest that GnTIVB is more active under physiological conditions and primarily contributes to the biosynthesis of *N*-glycans. The products of GnTIV are normally found in some glycoproteins, including erythropoietin (Takeuchi *et al.*, 1988). It was found that both human chorionic gonadotropin (HCG) from choriocarcinoma patients and γ -glutamyltranspeptidase from human hepatoma tissue are additionally modified with products of GnTIV (Oguri *et al.*, 2006). The altered activity of GnTIV has been also reported in human pancreatic carcinoma (Nan *et al.*, 1998). Northern analysis of normal human tissues demonstrated that GnTIVB is widely expressed, whereas

GnTIVA and C are restricted to a few tissues, including thymus, pancreas, spleen and peripheral blood leukocytes (Yoshida *et al.*, 1999). Recent data showed that upregulation of GnTIVA gene expression is closely associated with metastatic potential in primary colorectal carcinoma (D'Arrigo *et al.*, 2006).

1.3.1.5 GlcNAc transferase V (GlcNAcT-V, GnTV; EC 2.4.1.155)

N-acetylglucosaminyltransferase V catalyzes the attachment of a GlcNAc residue in a β 1,6 linkage to the GlcNAc(β 1,2)Man(α 1,6) arm of an N-glycan to form a tetrantennary structure (Figure 1.23) (Pierce *et al.*, 1987; Shoreibach *et al.*, 1993).

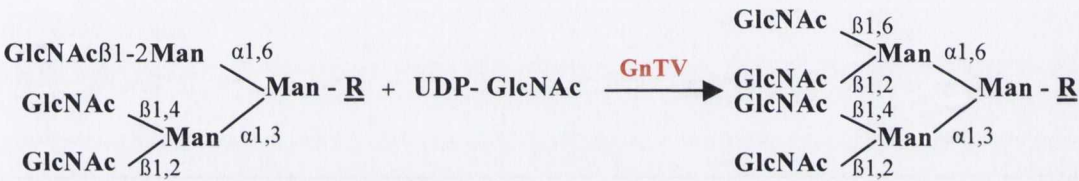


Figure 1.23 Reaction catalyzed by GnTV ($R - \beta$ 1,4GlcNAc(β 1,4)GlcNAc -Asn).

The enzyme requires the prior action of GnTII, which in turn requires the prior action of GnTI. Studies performed by Do *et al.* (1994) demonstrated that the activity of GnTV on a glycoprotein requires some structural features. Firstly, the oligosaccharide has to be a bi- or tri-antennary structure lacking galactosyl residues, with no GlcNAc residues on the reducing side. Secondly, the oligosaccharide must be accessible to GnTV. In many tissues, lack of the 1,6-branched mannose is due to inaccessibility of the enzyme (Khan *et al.*, 1993).

Molecular cloning studies revealed that GnTV is expressed at low copy numbers and the 84.6 kDa protein is one of the largest glycosyltransferases yet discovered. The amino acid and mRNA sequences of GnTV revealed its high conservation among species, exhibiting identities of 95%, 91% and 87.8% to rat, hamster and human, respectively.

GnTV displays a variable activity in many tissues and cell lines has been reported in hen oviduct, human myeloid cell lines, murine tumor cell lines, Rous sarcoma virus-infected baby hamster kidney cells, human and mouse cell lines, rat hepatoma, human lymphoid and myeloid cell lines and polyomavirus-transformed baby hamster (Schachter, 1989).

CHO mutant Lec4 and mouse lymphoma BW5147 PHAR^{2.1} cells have no detectable GnTV activity, whereas CHO Lec4A cells have the same GnTV activity as parental CHO cells, but the enzyme is not localised in Golgi membranes (Chaney *et al.*, 1989). A 2-fold increase in GnTV activity was reported in baby hamster kidney cells transformed with Rous sarcoma virus (Crawley *et al.*, 1990), but this increase was not observed for other glycosyltransferases in the pathway (Palcic *et al.*, 1990). An increased GnTV activity has also been reported in a significant population of human breast carcinomas compared to nonmalignant tissues (Dennis and Lafarté, 1989). Transcription of the *GnTV* gene may also be induced by oncogenes, which has been previously described in several publications (Chen *et al.*, 1998). Regulation of GnTV activity may be valuable in mediating cell adhesion, since this enzyme controls the expression of *N*-linked poly-*N*-acetylactosamine (polyLacNAc) sequences on cell surfaces (Shoreibach *et al.*, 1993). GnTV plays a regulatory role in the human blood system through its synthesis of complex *N*-glycans, such as Lewis antigens (Alvarez *et al.*, 2002). Activation of human T4 and T8 cell populations has also been associated with altered activity of GnTV (Do *et al.*, 1994). GnTV knockout mice showed significantly reduced metastases and increased susceptibility to autoimmune diseases due to altered T cell receptor aggregation and signalling (Alvarez *et al.*, 2002). Some studies on brain tissues showed that the level of the GnTV mRNA does not correlate with its activity, which may indicate complex regulation of this enzyme in cells.

Recent studies on human brain tissues and testis revealed high expression of a new splicing variant of GnTV protein, termed GnTVB, which shows 41% amino acid identity and 53% similarity to the original sequence (also referred to as GnTVA) (Alvarez-Manilla *et al.*, 2009). According to the experimental data, the new isoform is also well expressed in spleen, dorsal root ganglion and tumor cells, including skin melanoma and neuroblastoma. Kinetic studies on the two GnTV proteins showed some discrepancies in activity towards oligosaccharide acceptors, however, both enzymes are capable of synthesizing *N*-linked β 1,6 branched glycans (Kaneko *et al.*, 2003). GnTVB, also referred to as GnTIX, was recently identified in mouse brain tissues. According to Northern blot analysis, GnTVB transcripts were restricted to parts of the brain, including cerebrum and cerebellum, as well as thymus and testis, while no expression was observed in the liver. GnTV transcripts were expressed in all mouse tissues examined (Inamori *et al.*, 2006).

1.3.2 Galactosyltransferases

Mammalian galactosyltransferases are Golgi-localised, type II transmembrane glycoproteins that catalyze the transfer of galactose to GlcNAc, forming Gal(β1,4)GlcNAc (lactosamine) units in glycoconjugates in two anomeric configurations via α1,3, α1,4 and β1,3, β1,4 linkages following the standard reaction in Figure 1.24.



Figure 1.24 Reaction catalyzed by galactosyltransferases.

Lactosamine sequences are important for the extension of the glycan chains of glycoproteins and glycolipids and are components of carbohydrate ligands that mediate cell surface functions/cell recognition events such as cell adhesion, fertilization, apoptosis and lymphocyte trafficking, leukocyte adhesion to the endothelium and inflammation (galactosylated sialyl-Lewis X). Also, terminal galactose residues function as ligands for galactose-binding proteins such as galectins. The variety of galactosylation reactions significantly contributes to the tremendous diversity of oligosaccharide structures expressed by living organisms. Although they sometimes catalyze identical reactions and are widespread among living organisms, galactosyltransferases share only little structural similarities.

β4GalT belongs to the CAZY (Carbohydrate Active Enzymes; <http://www.cazy.org/>) family 7 of inverting glycosyltransferases which share an extensive homology. Seven human β1,4 GalT enzymes have been described to date and six of them are expressed in CHO cells (Lee *et al.*, 2003).

1.3.2.1 β4Galactosyltransferase 1 (β4GalT1)

The first mammalian GalT characterized biochemically, was also the first one to be cloned (Narimatsu *et al.*, 1986). β4GalT1 has two enzymatic activities: catalyzes the transfer of Gal from UDP-Gal to GlcNAc-terminating acceptors and, in the presence of α-lactalbumin, it changes its specificity to become lactose synthase, and transfers galactose to glucose in order to synthesize lactose (Rajput *et al.*, 1996). The lactose synthase activity is important

in mammary glands, a tissue in which the $\beta 4\text{GalT1}$ gene expression is induced during lactation (Hill *et al.*, 1968). In other tissues, the $\beta 4\text{GalT1}$ gene appears to be constitutively expressed. Additionally, the action of $\beta 4\text{GalT1}$ is required to achieve the Fringe-mediated modulation of Notch signalling (Chen *et al.*, 2001). Mice bearing an inactivated $\beta 4\text{GalT1}$ gene show retarded growth with increased lethality. The $\beta 4\text{GalT1}$ knockout mice also presented skin lesions, endocrine insufficiency, decreased fertility and absence of lactose in milk (Asano *et al.*, 1997).

1.3.2.2 $\beta 4\text{Galactosyltransferase 2}$ ($\beta 4\text{GalT2}$)

$\beta 4\text{GalT2}$ is structurally and functionally the closest to $\beta 4\text{GalT1}$ and shares 52% sequence identity with GalT1 . Like $\beta 4\text{GalT1}$, the $\beta 4\text{GalT2}$ enzyme has a lactose synthase activity in the presence of α -lactalbumin however in $\beta 4\text{GalT1}$ -null mice, the action of this enzyme is not compensated by $\beta 4\text{GalT2}$. The $\beta 4\text{GalT2}$ gene is widely expressed in human tissues but its relevance in animal development and physiology is so far unknown (Hennet, 2001).

1.3.2.3 $\beta 4\text{Galactosyltransferase 3}$ ($\beta 4\text{GalT3}$)

The identity to the $\beta 4\text{GalT1}$ protein reaches 44%. The $\beta 4\text{GalT3}$ gene is ubiquitously expressed in human tissues but its biological relevance is presently unknown (Hennet, 2001).

1.3.2.4 $\beta 4\text{Galactosyltransferase 4}$ ($\beta 4\text{GalT4}$)

At the protein level, $\beta 4\text{GalT4}$ shares about 40% identity with the other members of the $\beta 4\text{GalT}$ family. $\beta 4\text{GalT4}$ transcript is detected in all human tissues with the highest mRNA levels in placenta and pancreas. The biological significance of the $\beta 4\text{GalT4}$ enzyme *in vivo* remains to be established (Hennet, 2001).

1.3.2.5 $\beta 4\text{Galactosyltransferase 5}$ ($\beta 4\text{GalT5}$)

The $\beta 4\text{GalT5}$ gene contains nine exons unlike $\beta 4\text{GalT1}$ to 4, where the number of coding regions was six. $\beta 4\text{GalT5}$ protein shows only about 35% identity to $\beta 4\text{GalT1}$ to 4 and its biological role is currently unknown (Hennet, 2001).

1.3.2.6 β 4Galactosyltransferase 6 (β 4GalT6)

Similarly to β 4GalT5, β 4GalT6 gene consists of nine exons and their identity at the amino acid level reached 68%. β 4GalT6 plays a role in N-glycan biosynthesis but no data exists about its role in animal development and physiology (Hennet, 2001).

1.3.3 Fucosyltransferases (FucT)

A fucosyltransferase is an enzyme that transfers an L-fucose sugar from a GDP-fucose donor substrate to another sugar, galactose in an α 1,2 linkage and to GlcNAc in α 1,3, α 1,4, or α 1,6 linkages with inversion of anomeric configuration as in the N-linked glycosylation or to a protein, as in the O-linked glycosylation produced by O-fucosyltransferase, which have recently been shown to localise to the endoplasmic reticulum (Breton *et al.*, 1997; Oriol *et al.*, 1999). The vast majority of fucosyltransferases is located in the Golgi apparatus and these enzymes represent the typical domain structure of type II transmembrane proteins, consisting of a short N-terminal cytoplasmic tail, a transmembrane domain and a stem region followed by a large C-terminal catalytic domain (Joziassse, 1992; Kleene and Berger 1993). On the basis of protein sequence similarities, mammalian fucosyltransferases can be classified into distinct families including the α 2-fucosyltransferases, the α 3-fucosyltransferases and mammalian α 6-fucosyltransferases. The α 2 and α 3 and α 4FucT are involved in the last steps of the biosynthesis of ABH and Lewis related carbohydrate antigens (Henry *et al.*, 1995; Watkins, 1995; Costache *et al.*, 1997). The mammalian α 6FucT is implied in the synthesis of N-glycans (Uozumi *et al.*, 1996; Yanagidani *et al.*, 1997). Fucosylated glycoconjugates may play important roles in physiological and pathological processes such as fertilization, embryogenesis, lymphocyte trafficking, immune response, and cancer metastasis (Schenkel-Brunner, 1995; Staudacher, 1996). The presence of fucosyltransferase activities has also been demonstrated in plants, insects, snails, parasites, and bacteria (Staudacher, 1996).

1.3.4 Sialyltransferase (ST)

Sialyltransferases represent an important group of enzymes that transfer N-acetylneuraminic acid (NeuAc, also Neu5Ac) from CMP-NeuAc to various acceptor substrates. In mammals, sialic acids are usually found at the non-reducing terminal position of glycoconjugates sugar chains, α 2,3- or α 2,6-linked to a β -D-galactopyranosyl (Gal) residue, or α 2,6-linked to a β -D-N-acetylgalactosaminy (GalNAc) residue or a β -D-N-

acetylglucosaminyl (GlcNAc) residue. Additionally, α 2,8-linked sialic acid residues are found in gangliosides and in polysialic acid (PSA) observed on several glycoproteins (Tsuji, 1996; Harduin-Lepers *et al.*, 1994). Human sialyltransferases are a functional family of at least 18 different intracellular, Golgi membrane-bound glycosyltransferases with a short NH₂-terminal cytoplasmic tail and a 16–20 amino acid signal anchor domain, the highly variable in length stem region (from 20 amino acids to 200 amino acids), which is followed by a large COOH-terminal catalytic domain that resides in the lumen (Harduin-Lepers *et al.*, 2001). Sialyltransferases differ in their substrate specificity, tissue distribution and various biochemical parameters. The amino acid sequences of the human cloned sialyltransferases show very little sequence identity with the exception of the three consensus sequences called sialyl motifs L, S, and VS, whose functional significance is binding of the sugar donor and acceptor substrates as well as taking part in catalytic processes (Audry *et al.*, 2010). Finally, analysis for post-translational modifications in primary amino acid sequences of the sialyltransferases revealed several consensus sites for phosphorylation and *N*-glycosylation, which may influence the catalytic activity and intracellular traffic. In higher animals, sialylated oligosaccharide structures play crucial roles in many biological processes including *N*- and *O*-glycosylation but also in diseases, notably in microbial infection and cancer.

1.4 Human chorionic gonadotropin as a model glycoprotein

1.4.1 Structural features of HCG

Human chorionic gonadotropin (HCG) is a glycoprotein hormone from the same family as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH). HCG forms a heterodimer comprising 2 subunits, α and β joined non-covalently (Swaminathan and Bahl, 1970). The α polypeptide is composed of 92 amino acid residues and is encoded by a single gene on chromosome 6q21.1–23 (Fiddes and Goodman, 1981) whereas the polypeptide β consists of 145 amino acid residues encoded by the cluster of genes on the same location as LH gene on chromosome 19q13.3 (Policastro *et al.*, 1983). This gene cluster is composed of six gonadotropin genes, one pseudogene, and one LH gene. Many active subunit loci may serve to ensure a high level of gonadotropin production during the first trimester of pregnancy, although the expression of each chorionic gonadotropin gene is different.

HCG has a molecular weight of approximately 38 kDa and 30% of its structure is represented by carbohydrate units. HCG α and β subunits were determined to have molecular weights of 14.9 kDa and 23 kDa according to mass spectroscopy analysis (Acevedo, 2002; Stenman *et al.*, 2006).

The crystal structure of HCG was reported by Laphorn *et al.* in 1994. HCG α is structured in three loops stabilized by disulfide bonds (see Figure 1.25A). HCG β has a highly glycosylated carboxyl terminal peptide (CTP) extension, rich in serine and proline, which confers the immunological and biological specificity to the whole HCG. The HCG β molecule has two beta hairpin loops, stabilized by the disulfide bond on one side of a central cystine knot and a long loop on the other side as represented in Figure 1.25B (Laphorn *et al.*, 1994; Xing *et al.*, 2001).

HCG secretion is under the control of a large number of factors which may act by both autocrine and paracrine mechanisms (Jameson and Hollenberg, 1993). Cellular modulators interact with specific surface receptors expressed on placental trophoblastic cells and activate the expression of HCG α and HCG β by phosphorylation of a cAMP response element binding protein through the protein kinase C pathway. A second messenger is triggered in trophoblast cells at the promoter region (Johnson *et al.*, 1997). After transcription of the genetic message, the translation of the mRNA into the nascent polypeptides takes place in the membrane-bound ribosomes of the rough ER. Each translated pre-mature product is slightly larger and contains the specific sequences and the signal peptide extension, which is subsequently removed by signal peptidases. Newly synthesized polypeptides are released into RER channels and transported to the Golgi (Ruddon *et al.*, 1987), where glycosylation enzymes attach carbohydrate moieties (Kornfeld and Kornfeld, 1985) and two HCG subunits form a dimer (Hanover *et al.*, 1982).

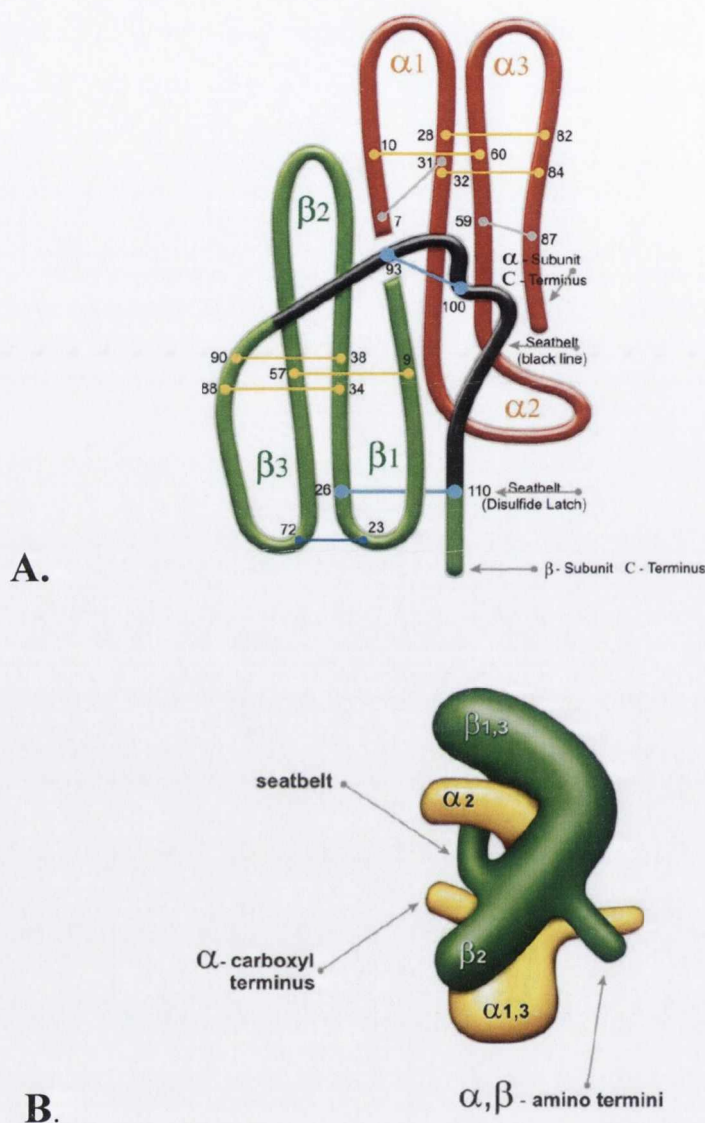


Figure 1.25 Schematic of the three dimensional structure of HCG.

Part A represents the assembly of the α (red) and β (green) subunits to form the heterodimer. The seatbelt region in the β subunit is shown in black. Yellow bars, thick blue lines and thin blue lines denote the cysteine knots on both subunits, the seatbelt disulfide bonds and the small loop disulfide bond of the β -seatbelt, respectively. Other disulfide bonds are shown with grey lines. Numbers represent amino acids. Part B of this figure shows the $\alpha\beta$ assembly stabilised by the seatbelt loop of the β subunit (de Medeiros and Norman, 2009).

1.4.2 Glycosylation of HCG

The structures of the *N*-linked sugar chains of HCG were first reported in 1979 (Kessler *et al.*, 1979). Generally, the α polypeptide of HCG has two *N*-linked oligosaccharides attached to ^{52}Asn , ^{78}Asn and its β subunit contains not only two *N*-linked oligosaccharides attached to ^{13}Asn , ^{30}Asn but also four *O*-linked oligosaccharides attached to ^{121}Ser , ^{127}Ser , ^{132}Ser , and ^{138}Asn on the carboxy terminal peptide portion (see Figure 1.26) (Kobata and Takeuchi 1999; de Medeiros and Norman, 2009).

Comparative studies of the sugar patterns of desialylated HCG α and HCG β subunits revealed that galactosylated biantennary and monoantennary structures were detected from the HCG α sample, while core fucosylated and galactosylated biantennary glycans were detected from the HCG β sample, in approximately equal amounts (Mizuochi and Kobata, 1980). Endo *et al.* in 1979 identified a hybrid structure where the α 1,3 mannose arm was substituted with a GlcNAc, Gal and NeuAc residue. Additionally, it was found that all sialic acid residues of the *N*-linked sugar chains of HCG occur as the NeuAc α 2,3Gal group.

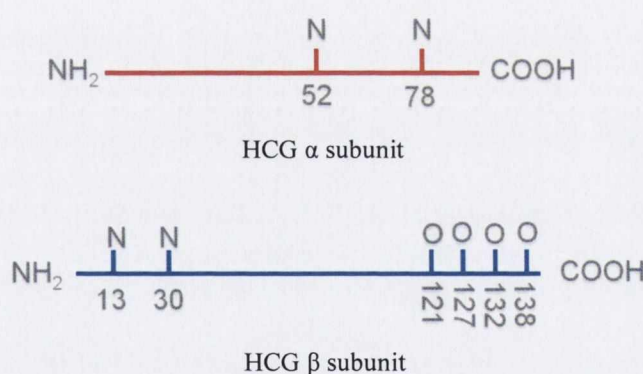


Figure 1.26 *N*- and *O*-glycosylation sites on HCG.

The analysis of *N*-glycans by the one- and two-dimensional ^1H -NMR spectroscopy performed by Weisshaar *et al.*, 1991 revealed that the main oligosaccharide attached to ^{52}Asn of HCG α was sialylated A1G1. Approximately 60% of the oligosaccharides attached to ^{78}Asn of HCG α were sialylated A2G2 and the remainder was sialylated A1G1. The FA2G2 and A2G2 structures, in the molar ratio of 1:3, were found to be linked to the ^{13}Asn

of HCG β , while mostly sialylated FA2G2 was found to be attached to the ³⁰Asn of HCG β . Amano and Kobata in 1995 confirmed their finding by investigating the behaviors of four glycopeptides obtained from HCG α and HCG β on immobilized *Aleuria aurantia* lectin and Concanavalin A columns. The same structures were confirmed with the use of more advanced techniques as described by Liu and Bowers, 1997; Kobata and Takeuchi, 1999 and Toll *et al.*, 2006 with a small proportion of tri-antennary structures with variable sialylation were identified, as well as one tri-sialylated, tetra-antennary glycan (Gervais *et al.*, 2003).

HCG exists in three main forms: regular HCG, hyperglycosylated HCG (38.5 - 40 kDa) and the free β -subunit of hyperglycosylated HCG. Each of these three molecules has different physiological functions. The hyperglycosylated HCG is secreted in the first weeks of pregnancy and in contrast to regular HCG, all the *O*-glycans on this glycoprotein have a tetrasaccharide core structure. This type of HCG is responsible for the implantation of the fetus, and its levels decline soon after implantation. The hyperglycosylated variant is also responsible for the invasive activity of the tumour cells in malignant trophoblastic diseases, such as a choriocarcinoma (de Medeiros and Norman, 2009). In serum of normal pregnancy, the concentrations of HCG β are 200 times lesser than the dimer concentration, but in urine the HCG β is one of the predominant forms (McChesney *et al.*, 2005). The concentration of the free β -subunit is systematically lower in ectopic pregnancies than in singleton normal ones (Luckas *et al.*, 1998). Stronger heterogeneity of free HCG β is found in the molecules secreted by abnormal trophoblast or other tissues, usually malignant (Butler and Iles, 2004; Roy *et al.*, 2007).

Together with regular and hyperglycosylated dimeric HCG and the free β -subunit, a very small amount of free HCG α can be detected in the urine of pregnant women. In spite of having the same amino acid sequence as the HCG α isolated from heterodimeric HCG, this free HCG α cannot bind to HCG β . The unusual *O*-glycosylation of the free HCG α was suggested to be a cause of the interrupted association with HCG β (Parsons and Pierce, 1984). Contradicting results were reported by Kawano *et al.* in 1988 and Blithe in 1990 who proposed that the larger *N*-linked sugar chains of free HCG α inhibit its binding with HCG β .

1.4.3 Role of carbohydrate chains in HCG

Many studies revealed that modification of the *N*-linked sugar chains of HCG alters its hormonal activity (Amano *et al.*, 1990; Kobata and Takeuchi, 1999). The complete removal of sialic acids from HCG enhances its binding to the target cells but reduces the hormonal activity to 50%. These results indicated that the sialic acid residues of HCG are important for the full expression of their hormonal activity *in vitro*, but the effect is independent of their linkage to the galactose residues. Removal of the whole *N*-linked sugar chains further increases the binding of HCG, but eliminates its hormonal activity almost completely. The deglycosylated HCG behaves as an antagonist to native HCG (Goverman *et al.*, 1982; Calvo and Ryan, 1985). The lack of *N*-linked sugar chains at the ⁵²Asn of HCG α significantly decreased *in vitro* signal transduction (Matzuk *et al.*, 1989). Removal of ⁷⁸Asn glycosylation in HCG α markedly reduced its assembly with HCG β (Matzuk *et al.*, 1988). Studies of HCG β showed that the *N*-glycosylation is not essential for its assembly with HCG α , but elimination of ³⁰Asn glycosylation inhibits the secretion of uncombined HCG β and the ¹³Asn branch influences mainly the assembly (Fares, 2006). The *O*-glycans attached to the β -subunit also play an important role in the half-life of HCG. Results presented by Goverman *et al.* in 1982 indicate that the *N*-linked sugar chains of HCG are important for constructing the correct conformation of each subunit and the presence of at least one *N*-acetylglucosamine residue attached at each of the four *N*-glycosylation sites is enough to keep the correct folding of the two subunits.

The HCG/LH receptor belongs to superfamily of G protein-coupled seven transmembrane domain receptors (Rousseau-Merck *et al.*, 1990) and is expressed early in life. Interaction of HCG with its receptor and subsequent transduction of a signal is greatly influenced by the glycans. Based on data indicating that sialic acid residues bind directly to the cell surface, a model of the HCG-receptor complex was constructed (see Figure 1.27) (Lapthorn *et al.*, 1994) in which a dual interaction of the peptide portion and the sialylated sugar chain of HCG with respective binding sites is essential for the signal transduction.

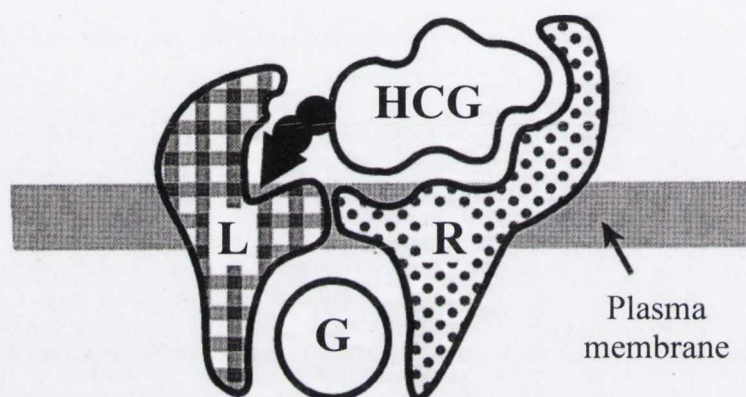


Figure 1.27 Schematic presentation of HCG-receptor complex.
G - G protein; L - lectin; R - HCG-receptor; black circles and a triangle - sugars

1.2.4 The function of HCG in the developing embryo

Human chorionic gonadotropin (HCG) is mainly a product of placental syncytiotrophoblast cells formed in developing embryo. The main function of this hormone is to maintain the activity of the corpus luteum (the region in the ovary where the last follicle was released) so that key hormone levels are sustained during the pregnancy. HCG can also be secreted by several normal non-placental tissues and trophoblastic or non-trophoblastic neoplasms (Stenman *et al.*, 2006) and can be detected in the cell membranes of embryonic, fetal and sperm cells at every stage of development. The levels of HCG decline once the placenta is formed at about 60 to 70 days gestation and remain constant up to term (Srisuparp *et al.*, 2001). Detection of HCG in urine of pregnant women is a basis of home pregnancy kits.

The strong negative charge of HCG comes from the terminal sialic acids on the oligosaccharides attached to this glycoprotein. As a result of this charge the growing embryo becomes neutral to the immune system (Acevedo, 2002).

1.4.5 HCG and its therapeutical potential

Currently, HCG is used during a wide range of assisted reproduction technologies, such as ovulation induction and *in vitro* fertilisation (IVF). In these instances, HCG induces final oocyte maturation in the ovary and causes production of testosterone in the testes. HCG treatment is a relatively inexpensive thanks to the DNA recombinant technology and the drug is now produced in a recombinant form using CHO cells (Gervais *et al.*, 2003). This recombinant form has been shown to work as effectively as the urinary form and generally, HCG is considered non-toxic with fewer physiological and tolerable side effects (Keay *et al.*, 2004).

The potential use of anti-HCG antibodies as a birth control vaccine is currently at phase II clinical trials. The antibody binds to HCG and prevents the blastocyst from implanting in the endometrium. So far, the vaccine was highly effective, fully reversible and caused no side effects but further work is required (Kaz *et al.*, 2005).

The possibilities of novel therapeutic uses of HCG include the following:

❖ Increasing pregnancy rates in assisted reproductive technologies

The increased endometrial thickness and implantation rates in estradiol/progesterone supplemented oocyte were observed in patients who received HCG (Tesarik *et al.*, 2003). The *in vivo* direct application of HCG to endometrium stimulates its morphological changes and the production of cytokines that help implantation (Filicori *et al.*, 2005). Additionally, low doses of HCG administration at the end of rFSH stimulation decreased miscarriage rate.

❖ Prevention of miscarriages

HCG treatment relaxes uterine arteries, thus increases utero-placental perfusion (Toth, 2001). In clinical testing, HCG administration prevented spontaneous, as well as habitual, miscarriages with no side effects (Toth, 2001). In addition, HCG plays an immunosuppressive role at the maternal-fetal interface (Lei and Rao, 2001).

❖ Prematurity prevention

When first tested in a mouse model, HCG was found to prevent preterm delivery and its effect was dose dependant (Kurtzman *et al.*, 1999). This suggested that HCG could be one of the hormones that contribute to maintaining pregnancy. Positive effects of HCG were also reported in women with preterm labor (Than *et al.*, 2003).

❖ Treatment of gynecological infections

Generally, infectivity of pathogens correlates with the presence of HCG or LH like molecules in their coat surface, which enables them to attach to tubal epithelial cell HCG/LH receptors (Rao, 2001) and further attack to cause damage and destruction of the cells. The above cascade of events may be prevented by preoccupying the receptors with HCG, which was demonstrated in organ cultures of Fallopian tubes with *Gonorrhoeae* organisms (Gorby *et al.*, 1991). The HCG treatment of such cultures prevented the infection. As a conclusion, simple laparoscopic delivery of HCG into Fallopian tubes to treat infectious diseases can replace the expensive antibiotics, many of which have side effects and are not well tolerated by patients.

❖ HIV/AIDS treatment

Babies born to HIV positive mothers are usually infected during the intrapartum period when they get exposed to maternal body fluids (Matheson *et al.*, 1995). This fact indicates the presence of mechanisms during the pregnancy that protect the baby from infection in utero (Casper and Fenyo, 2001). In the studies with a HIV positive transgenic mouse model, the molecule that prevented the rapid progression of disease and premature demise of homozygous pups was a dimeric HCG (De *et al.*, 1997). The mechanistic studies revealed that HCG treatment upregulates its own receptors and eliminates the virus which has its receptors in the same cells (Shapira *et al.*, 1998). One of the possible mechanisms is the upregulation of TNF- α as its serum levels are higher in untreated homozygous pups and HCG treatment decreases them (De *et al.*, 2002). However, further studies are necessary to fully establish the preventative role of HCG in HIV infection.

The potential therapeutic effects of HCG have also been reported in the treatment of the following: rheumatoid arthritis treatment (Song *et al.*, 2000), breast cancer prevention and treatment (Rao, 2001), prostate cancer treatment (Leuschner and Hansel, 2005; Bodek *et al.*, 2005; Leuschner *et al.*, 2001), Alzheimer's disease (Bowen *et al.*, 2004; Casadesus *et al.*, 2005; Meethal *et al.*, 2005; Gregory and Bowen, 2005).

1.5 Aims of thesis

❖ Generation of polyclonal antibodies against CHO GnTs

The aim of this study was to obtain purified, soluble or insoluble antigens, heterologously expressed in *E.coli*. The ultra pure preparations of the GnT proteins served as immunogens for raising antibodies. Additionally to generating antibodies against full-length or truncated proteins, raising antisera to immunogenic peptides was also attempted.

❖ Determination of K_m and V_{max} for recombinant CHO GnTI

CHO GnTI, which was previously cloned, expressed and purified from *E.coli*, was analyzed for its activity using a spectrophotometric coupled assay, in which UDP produced by GnTI was processed by pyruvate kinase (PK) and the product of PK, pyruvate was reduced by lactate dehydrogenase (LDH) to release NAD^+ from NADH, resulting in the decrease in absorbance at 340 nm. Additionally, the effects of pH, temperature, $MnCl_2$ concentration and the concentration of other divalent cations (Co^{2+} , Mg^{2+} , Ca^{2+} and Zn^{2+}) on the activity of recombinant CHO GnTI were investigated under standard assay conditions.

❖ Subcellular localisation of CHO GnTs in the Golgi apparatus

In order to provide localisation data, GlcNAc transferases (GnTs) cDNAs were cloned and transiently expressed in CHO cells. The C-terminal *myc*-epitope attached to each GnT sequence was used for detection by Western blotting, followed by immunocytochemistry (ICC) using fluorescence and confocal microscopy. CHO GnTs were co-localised to specific Golgi compartments using specific Golgi markers, giantin (*cis*- and *medial*-Golgi membranes) and TGN46 (*trans* Golgi network).

❖ Glycoengineering human chorionic gonadotropin (HCG)

Previous data confirm that overexpression or knockdown of glycosyltransferases can significantly impact the quality of oligosaccharides on recombinant glycoproteins. The aim of this Chapter was to establish a genetically modified CHO7J stable cell line, which overexpresses GnTV (forms a tetra-antennary structure) and has an RNAi-induced knockdown of $\beta 4GalTs$ (important enzymes for the extension of the glycan chains), and further investigate the effect of such genetic manipulations on the glycosylation pattern of HCG. All genetic manipulations were tested *in silico* using computer model predictions developed by Dr. McDonald (Trinity College Dublin).

CHAPTER 2

Materials and General Methods

2.1 Materials

2.1.1 General materials

All reagents were of the highest quality and purchased from Sigma-Aldrich Chemical Company, Poole, UK unless otherwise stated.

2.1.2 Cell culture

- EX-CELL® ACF CHO Medium, Blasticidin S Hydrochloride $\geq 98\%$ and DMSO - Sigma-Aldrich Chemical Company, Poole, UK
- CD CHO medium (1X), Hypoxanthine-Thymidine supplement (50X), L-glutamine (200 mM) - Invitrogen (Dun Laoghaire, Ireland)
- Puromycin (10 mg/ml)- Promega Ltd. (Southampton, UK)
- Polybrene – Millipore (Temecula, CA, USA)

2.1.3 Specialised laboratory reagents

- Exoglycosidases used in *N*-glycan release - Prozyme (San Leandro, USA)
- Bradford reagent - BioRad Laboratories (Hemel Hempstead, UK)
- Prestained Protein Marker (6-175 kDa) - New England BioLabs Inc. (Ipswich, MA, USA)
- Restriction enzymes, SpectraMulticolor Broad Range Protein Ladder and PageRuler Plus Prestained Protein Ladder – Fermentas (St. Leon-Rot, Germany)
- BCA protein assay reagent – Thermo Fischer Scientific (Surrey, UK)
- ECL Western blotting detection reagents – Millipore (Temecula, CA, USA)
- Vectashield mounting medium with DAPI – Vector Laboratories Ltd (Peterborough, UK)
- GlycoProfile™ 2-AB labelling kit - Sigma-Aldrich Chemical Company, Poole, UK
- Anode fluid 3, catode fluid 10, Servalyt Precote pI 3-10 and Serva Violet 17 – Serva Electrophoresis GmbH (Heidelberg, Germany)
- Ovitrelle, produced by Merck Serono S.A., (Geneva, Switzerland)
- 2-AB-glucose homopolymer (2-AB dextran ladder) – Ludger Ltd. (Oxford, UK)

2.1.4 Antibodies

Primary antibodies:

- monoclonal anti-Myc antibody IgG1, clone 9E10 – Clontech Laboratories (Mountain View, CA, USA)
- polyclonal anti-giantin and polyclonal anti-TGN46 antibodies – Abcam (Cambridge, UK)
- monoclonal anti-GM130 antibody - BD Transduction Labs (Erembodegem Belgium)
- monoclonal anti-His antibody, clone HIS.H8 IgG_{2b} – Millipore (Temecula, CA, USA)

Secondary antibodies:

- rabbit anti-mouse HRP conjugated IgG (H+L) - Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK)
- goat anti-rabbit HRP conjugated IgG - Millipore (Temecula, CA, USA)
- Alexa Fluor® 488 chicken anti-mouse IgG (H+L) and Alexa Fluor® 546 goat anti-rabbit IgG (H+L) - Invitrogen (Dun Laoghaire, Ireland)

2.1.5 Molecular biology

- T₄ DNA ligase, 2.5 mM dNTPs and alkaline phosphatase (BAP C75) were purchased from TaKaRa BIO Inc. (Japan)
- SOC medium, 1-Bromo-3-chloropropane (BCP), ampicillin, TRI Reagent and BSA, penol:chloroform:isoamyl alcohol (25:24:1) - Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK)
- 100 mM isopropylthio-D-galactoside (IPTG), Benzonase Nuclease (> 90%) - Novagen® (Nottingham, UK)
- The QIAquick PCR purification kit, and the QIAquick gel extraction kit, RNeasy Mini Kit, Qiashredder - Qiagen Inc. (Dorking, Surrey, UK).
- *Pfu* DNA Polymerase polymerase, 10 mM dNTPs, GoTaq® DNA Polymerase - Promega Ltd. (Southampton, UK)
- Oligonucleotides - Eurofins MWG Operon (Ebersberg, Germany)
- FavorPrep™ Plasmid DNA Purification Mini Kit, LB medium and LB agar - Favorgen (Trinity College Enterprise Centre, Dublin, Ireland)

- RNA gel loading buffer II, nuclease-free water, RNaseZAP, and TRI Reagent - Ambion Inc. (Austin, TX, USA).
- One-Shot TOP10 chemically competent cells, RT-PCR SuperScript III First-Strand Synthesis kit, PureLink™ HiPure Plasmid Filter Maxiprep Kit, pcDNA6/V5-His mammalian vector, ViraPower Packaging Mix - Invitrogen (Dun Laoghaire, Ireland)
- Ni Sepharose 6 Fast Flow resin – GE Healthcare Life Sciences (Bucks, UK)
- Cell Line Nucleofector Solution V – Lonza Group Ltd (Switzerland)
- pRNAT-U6.2/Lenti expression vector – Genscript USA Inc. (Piscataway, NJ USA)
- AffinityScript QPCR cDNA Synthesis and Quantitative Real-Time PCR – Agilent Technologies Ireland Ltd (Cork, Ireland)
- Vectors pBluescript SK+, pET15b, pcDNA3.1/myc-His were kind gifts from Dr. Vincent Kelly, Trinity College Dublin.

2.2 General methods

2.2.1 Growth and maintenance of the wild type CHO cell line

The wild type CHO cell line was obtained from the European Collection of Cell Culture (ECACC, No. 00102307). Cells were grown in suspension in a 250 ml Erlenmeyer shake flask in the Ex-Cell™ACF CHO medium supplemented with 8 mM L-glutamine. The culture was kept inside a sterile incubator at 37°C under a 5% CO₂ atmosphere. Cells were maintained between a cell density of 5 x 10⁵ and 2 x 10⁶ cells/ml by passaging every three to four days.

2.2.2 Cryopreservation of the wild type CHO cell line

The wild type CHO cells were grown to late log phase and counted to obtain an accurate value for the number of viable cells as described in Section 2.2.5. The culture medium, freezing mix containing culture medium and 20% DMSO, and cryovials were kept on ice for 30 min. Cells were centrifuged at 100 x g for 5 min at 4°C and then resuspended in the cool normal medium before merging with an equal volume of freezing mix. The final concentration of DMSO was 10% and the cell density was 1 x 10⁶ cells/ml. Cells were

immediately aliquoted into the labelled cryovials, placed in a Nalgene 5100 Cryo Freezer container and stored overnight at -80°C. Vials were transferred to the liquid nitrogen tank the following morning and stored until required.

2.2.4 Thawing the wild type CHO cell line

Medium (49 ml) was pipetted into a 250 ml Erlenmeyer shake flask and warmed to 37°C in a CO₂ incubator. A 50 ml falcon tube containing 100% ethanol was warmed to 37°C in a water bath. One cryovial of frozen stock was transferred rapidly from the liquid nitrogen and thawed by submerging in the falcon tube containing warm ethanol. Once completely thawed, the cells were immediately transferred to the shake flask and the culture was placed in the rack of an orbital shaker and maintained as described in Section 2.2.1.

2.2.5 Determination of cell numbers and cell viability

Cell numbers and viability were determined by manual counting using trypan blue dye and a bright light haemocytometer with improved Neubauer rulings. A coverslip was placed over the chamber of the haemocytometer. Approximately 10 µl of this cell suspension (either undiluted or diluted 1:1 or 1:5 with trypan blue, which selectively colour dead cells) was transferred into the space between the coverslip and the chamber of the haemocytometer. Both viable and non-viable cells in five main 1 mm² boxes were counted and the number of viable cells was calculated using the formulae below:

$$\text{Cell number (cells/ml)} = \frac{\text{No. of cells in each chamber}}{\text{No. of chambers}} \times 10^4 \times \text{Dilution factor (DF)}$$

$$\text{Cell viability} = \frac{\text{Viable cells}}{\text{Total cells}} \times 100$$

2.2.6 SDS-PAGE

A sandwich, composed of two glass plates held apart by two spacers at each vertical edge, was constructed in a vertical clamp assembly. The bottoms of each plate were sealed with a silicon gasket. The sandwich was filled with 5.5 ml of either 8% or 12% (w/v) resolving gel (see Table 2.1), overlayed with water and allowed to polymerise over 30 min. The water overlay was poured off. A 12-well comb was fitted between the two plates at the top

and the upper part of the sandwich was filled with 5% (w/v) stacking gel (Table 2.2) and, being careful to avoid the trapping of air bubbles. The stacking gel was allowed to polymerise over 30 min. The comb was removed and the wells were gently rinsed out with water. SDS-PAGE was completed in polyacrylamide resolving gels using the discontinuous buffer system described by Laemmli (1970). Protein was first concentrated by electrophoresis through a 5% (w/v) polyacrylamide stacking gel, then resolved by electrophoresis through resolving gel by virtue of the different molecular weights of each protein within the sample.

Samples for electrophoresis were diluted with equal volumes of 2X SDS-PAGE sample buffer (7.6 ml of H₂O, 0.5 ml of 1 M Tris-HCl, pH 6.8, 0.4 ml of glycerol, 0.8 ml of 10% (w/v) SDS, 0.5 ml of 1% (w/v) Bromophenol Blue and 0.2 ml of β -mercaptoethanol). Proteins were denatured at 95°C for 5 min and samples (up to 20 μ l) were loaded into the wells and subjected to SDS-PAGE. Electrophoresis was completed at constant current intensity (20 mA per gel) for approximately 1 h 30 min at room temperature in an SDS-PAGE apparatus filled with 1X running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS (w/v)). Proteins resolved by SDS-PAGE were visualized by staining the gel with Coomassie Blue stain (methanol:acetic acid:MilliQ water; 4:1:5), Coomassie Brilliant Blue R250 0.05% (w/v), which was carried out overnight on an orbital shaker at room temperature. The gel was destained for approximately 3 hours in destain (methanol:acetic acid:MilliQ water; 4:1:5).

Table 2.1 SDS-polyacrylamide resolving gel ingredients (10 ml).

8% resolving gel	12% resolving gel
4.6 ml of H ₂ O	3.3 ml of H ₂ O
2.7 ml of 30% (w/v) Acrylamide: 0.8% (w/v) bisacrylamide stock solution (37.5:1)	4 ml of 30% (w/v) Acrylamide: 0.8% (w/v) bisacrylamide stock solution (37.5:1)
2.5 ml of 1.5 M Tris-HCl, pH 8.8	2.5 ml of 1.5 M Tris-HCl, pH 8.8
100 µl of 10% (w/v) SDS	100 µl of 10% (w/v) SDS
100 µl of 10% (w/v) APS	100 µl of 10% (w/v) APS
6 µl of TEMED	4 µl of TEMED

Table 2.2 SDS-polyacrylamide stacking gel ingredients (4 ml).

5% stacking gel
2.7 ml of H ₂ O
0.67 ml of 30% (w/v) Acrylamide: 0.8% (w/v) bisacrylamide stock solution (37.5:1)
0.5 ml of 1M Tris-HCl, pH 6.8
40 µl of 10% (w/v) SDS
40 µl of 10% (w/v) APS
4 µl of TEMED

2.2.7 Protein concentration assay

Protein concentrations were determined either by the method described by Bradford in 1976 or by the bicinchoninic acid (BCA) assay for the colorimetric detection.

2.2.7.1 Bradford protein assay

Bradford reagent was diluted 1:5 with H₂O and BSA standards were made at the following concentrations in H₂O: 0 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml. Protein standards (10 µl) and test samples (10 µl) were added to individual wells of a 96-well plate. In quick succession, 200 µl of Bradford reagent were added to the protein solutions. The plate was incubated at room temperature for 5 min and then assayed at 595 nm using a microplate reader. The protein concentrations in µg/ml were determined using the BSA concentrations as a standard curve.

2.2.7.2 BCA protein assay

The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺¹) by a bicinchoninic acid containing reagent. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing standard protein (BSA) concentrations over a broad working range (20-2,000 µg/ml). A series of dilutions of known concentration were prepared from the BSA standard protein (see Table 2.2) and each unknown is determined based on the standard curve.

The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). The assay was carried out in a 96 well plate format. All samples were prepared in triplicate in a final volume of 25 µl. WR (200 µl) was added to each well and the plates were covered with thin foil followed by incubation at 37°C for 30 minutes. Absorbance was measured at 562 nm in a microplate reader.

Table 2.2 Preparation of diluted albumin (BSA) standards.

Vial	Volume of diluent (μl)	Volume and source of BSA (μl)	Final BSA (μg/ml)
A	0	300 μ l of stock	2,000
B	125	375 μ l of stock	1,500
C	325	325 μ l of stock	1,000
D	175	175 μ l of vial B	750
E	325	325 μ l of vial C	500
F	325	325 μ l of vial E	250
G	325	325 μ l of vial F	125
H	400	100 μ l of vial G	25
I	400	0	0

2.2.8 Western blotting

Protein resolved by SDS-PAGE was electrophoretically transferred on to PVDF membranes using Bio-Rad Trans-Blot apparatus. Non-specific protein binding sites on the membranes were blocked by an overnight incubation in TBST (20 mM Tris-HCl, 150 mM NaCl, 2 mM KCl, 0.1% v/v Tween, pH 7.6) containing 5% (w/v) semi-skimmed dried milk. Membranes were incubated for 1 h with a primary antibody (typically 1:1000, 1:2000 or 1:5000 dilution) in TBST containing 5% (w/v) semi-skimmed dried milk. After washing membranes for 15 min in three changes of TBST, bound primary antibody was allowed to react with a horseradish peroxidase-conjugated secondary antibody in TBST which contained 1% (w/v) semi-skimmed dried milk. Membranes were washed as before. The antibody complexes were detected by enhanced chemiluminescence (ECL) and visualised following exposure to Kodak Hyperfilm-ECL.

2.2.8.1 Stripping membrane for reprobing

The membrane was washed with two changes of fresh stripping buffer (1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% Tween 20 (v/v), pH 2.2) for 10 min each. Then washed twice in PBS for 10 min each followed by TBST for 10 min. Membranes were blocked overnight in TBST with 5% milk and reprobbed as described previously.

2.2.9 Determination of the concentration and purity of nucleic acids

The concentration of nucleic acids was determined by measuring the absorbance at 260 nm in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 50 µg of dsDNA per ml ($A_{260} = 1$, 50 µg/ml) and 40 µg of RNA per ml ($A_{260} = 1$, 40 µg/ml). This relation is valid only for measurements in water. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of nucleic acids with respect to contaminants that absorb in the UV spectrum, such as protein.

CHAPTER 3

**Cloning, expression and purification of
Chinese hamster ovary GlcNAc transferases
(GnTs) and generation of polyclonal
antibodies**

3.1 Introduction

N-glycosylation is one of the most important post-translational modifications, which takes place in the Golgi apparatus. It has been reported that the diversity of *N*-glycans on glycoproteins play roles in many natural biological processes (Kukuruzinska and Lennon, 1998; Lis and Sharon, 1993). *N*-glycosylation requires variety of specific enzymes acting at different stages of this highly conserved process. GlcNAc transferases (GnTs) catalyse the first step in the *N*-glycosylation of high-mannose type structures to hybrid and complex glycans by transferring the GlcNAc moieties from UDP-GlcNAc to the acceptor asparagines on target proteins to form a GlcNAc β -glycosidic linkage (Kornfeld and Kornfeld, 1985). To date, six GnTs have been identified in mammals and functional profiles of each member of the family have been characterized, showing that these enzymes have not only different substrate specificities, but also specific tissue expression pattern (Schachter *et al.*, 1989; Yamamoto *et al.*, 2003; Keneko *et al.*, 2003; Alvarez-Manilla *et al.*, 2009).

In forming multi-antennary *N*-glycan structures in wild-type CHO cells, GlcNAc residues may be added to the trimannosyl core by four different GnTs, I, II, IV and V (Wong *et al.*, 2010). Pamela Stanley's group showed that the *MGAT3* gene is transcriptionally silent in parental CHO cells (Bhaumik *et al.*, 1995; Raju and Stanley, 1998). Additionally, at the start of this project, there was no sequence information for CHO GnTII and three isoforms of GnTIV. The cDNAs of the unknown CHO GnTs were generated using RT-PCR primers based on the mouse and rat GnT sequences. These cDNAs were previously cloned by Dr. Tania O'Connor (unpublished, TCD).

A shortcoming of the GnT field is that the Chinese hamster ovary members have not been characterised as other mammalian members from the same family, such as those in human, rat, mouse, rabbit, *Drosophila melanogaster* or yeast (Kumar *et al.*, 1992; Wildt and Gerngross, 2005, Schachter in 2010; Takamatsu *et al.*, 2010). Surprisingly, little is known about the regulation of these enzymes at protein level therefore investigation of the GlcNAc transferase family may bring new insight into this unexplored field. Studies on biofunctions of these enzymes are limited largely due to lacking of commercial antibodies.

In order to study CHO GnTs they first had to be cloned, expressed and purified. The strategy developed for expressing cloned CHO GnT cDNAs was based on the pET System (Studier and Moffatt, 1986) as shown in Figure 3.1. Each GnT was expressed from a pET15b bacterial expression vector as a polyhistidine fusion protein that contained N-terminal six histidine residues (2.18 kDa; MGSSHHHHHHSSGLVPRGSH). BL21(DE3) *E.coli* cells are a laboratory strain that contains a chromosomal copy of bacteriophage λ DE3, which has been engineered to contain a *lacI* gene and a *lacUV5* promoter. The *lacI* gene and the *lacUV5* promoter function to repress and activate the transcription of T7 RNA polymerase, respectively. During non-induced conditions, the *lac* repressor protein binds to and inhibits the promoter driving T7 RNA polymerase expression. Within the pET15b vector, a gene encoding the *lac* repressor acts at the T7*lac* promoter to block transcription of GnTs. The addition of IPTG (isopropyl- β -D-thiogalactoside) (see Figure 3.2) to the growing culture of BL21 cells initiates a cascade of events, whereby IPTG binds to the *lac* repressor protein and relieves the transcriptional repression of the T7 RNA polymerase gene and T7*lac* promoter. T7 RNA polymerase then recognizes the T7*lac* promoter on the clone and leads to GnT gene expression.

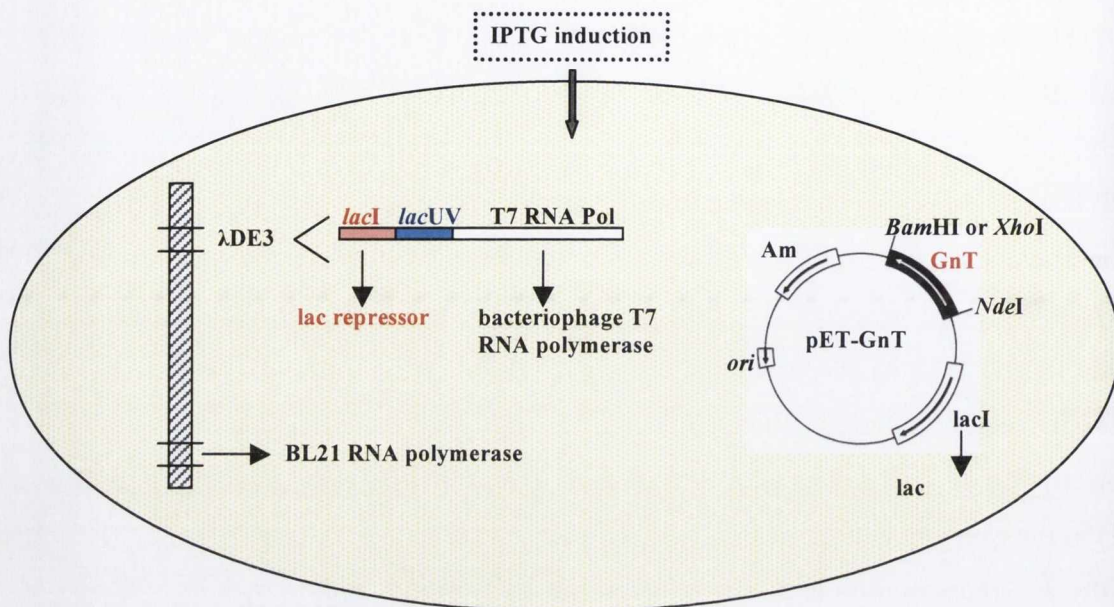


Figure 3.1 The pET expression system.

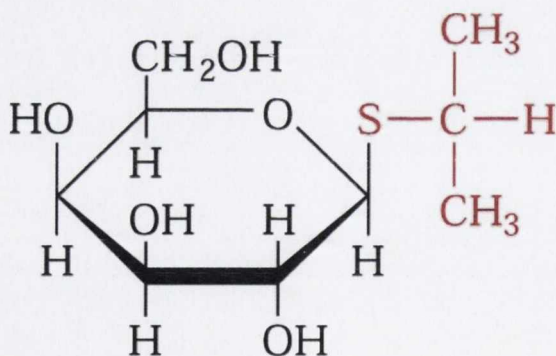


Figure 3.2 Chemical structure of IPTG (isopropyl- β -D-thiogalactoside).

3.2 Aim of Chapter

To date, there is no record of either custom made or commercially available mono- or polyclonal antibodies against CHO GnT proteins therefore the aim of this study was to obtain purified, soluble or insoluble antigens, heterologously expressed in *E.coli*, which were subsequently used for generation of polyclonal antibodies raised in rabbits.

CHO GnT cDNAs were initially amplified using a standard PCR, cloned into pBluescript SK+ and then subcloned into pET15b for high IPTG-induced expression. The BL21(DE3) *E.coli* cells used in this project were T7-promoter based and carry the lambda DE3 lysogen integrated into the bacterial chromosome. Generally, protein expression requires optimization of several factors and this focuses mainly on choosing the proper host strain and expression vector, growth temperature, cell density at time of induction and length of induction. As a result, GnTs were expressed from *E.coli* as soluble (GnTI) or insoluble proteins (GnTII, GnTIVB and GnTV), purified from bacterial pellets and used for generation of antibodies.

Additionally to generating antibodies against full-length or truncated proteins, raising antisera to immunogenic peptides was considered a good alternative. Anti-peptide antibodies were generated against GnTI, GnTII, GnTIVB and GnTV according to instructions described in this chapter.

3.3 Methods

3.3.1 Multiple sequence alignment and protein analysis

Multiple amino acid alignments were performed using on line Clustal Version 2.0 (ClustalW2) program maintained by the European Bioinformatics Institute (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The molecular size and physical parameters of each GnT protein were estimated using ExPASy ProtParam Tool (<http://www.expasy.ch/tools/protparam.html>).

3.3.2 TRI Reagent® total RNA isolation from wild type CHO cells

TRI Reagent® is a complete reagent for the isolation of total RNA from diverse biological material. The isolated RNA can be used directly for any downstream procedures, such as reverse transcriptase polymerase chain reaction (RT-PCR). CHO cells were grown as described in Section 2.2.1. The homogenate obtained from resuspending the cell pellet in 1 ml of TRI Reagent was incubated for 5 min at room temperature. This allows nucleoprotein complexes to dissociate completely. The homogenized samples could be stored at -70°C for up to one month if necessary. The homogenate was centrifuged at 12,000 g for 10 min at 4°C and the supernatant was transferred to a fresh tube. This step was required to remove insoluble material from the mixture, including proteins, polysaccharides, and extracellular material. Then, 100 µl of BCP (1-bromo-3-chloropropane) per 1 ml of TRI Reagent were added, vigorously shaken for 15 sec and incubated at room temperature for 15 min. The sample was centrifuged at 12,000 g for 15 min at 4°C and the aqueous phase was transferred to a fresh tube. RNA remained exclusively in the aqueous phase, whereas DNA and protein were in the interphase and organic phase, respectively. To wash the RNA sample, 500 µl of isopropanol per ml of TRI Reagent were added, vortexed at a moderate speed for 10 sec and incubated at room temperature for 10 min. Subsequently, the sample was centrifuged at 12,000 g for 8 min at 4°C and the supernatant was carefully removed without disturbing the pellet. Precipitated RNA formed a white pellet on the side and bottom of the tube. To wash the pellet, 1 ml of 75% ethanol was added and the sample was centrifuged again at 7,500 g for 5 min at 4°C to completely remove ethanol. The RNA pellet was allowed to air dry for 5 min. The sample was dissolved in 100 µl of nuclease-free water and stored at -70°C. The quality of RNA was assessed using denaturing agarose gel electrophoresis of RNA as described in

Section 3.3.3. The concentrations of the RNA samples were estimated as described in Section 2.2.9 and total RNA was used directly to synthesize first-strand cDNA as described in Section 3.3.5.

3.3.3 Denaturing agarose gel electrophoresis of RNA

RNA was routinely resolved and purified using denaturing agarose gel electrophoresis. A denaturing gel system was suggested, because most RNA forms extensive secondary structures via intramolecular base pairing, and this prevents it from migrating strictly according to its size. To avoid RNA degradation, the working bench area, pipettes, plasticware and gloves were treated with RNaseZAP. Gel preparation was carried out in the chemical fume hood. The gel was composed of 36 ml of autoclaved MilliQ water, 5 ml of 10X MOPS buffer (0.4 M MOPS, 0.1 M sodium acetate, and 0.01 M EDTA, pH 7.0), 9 ml of 37% (12.3 M) formaldehyde, 10 µl of 10 mg/ml ethidium bromide and 1% (w/v) agarose. The gel was allowed to set for 30-40 min. Samples containing 4 µg of RNA were adjusted to a total volume of 10 µl in RNase free water and mixed with an equal volume of gel loading buffer II (62.5% (v/v) deionized formamide, 1.14 M formaldehyde, 200 µg/ml bromophenol blue, 200 µg/ml xylene cyanole and MOPS-EDTA-sodium acetate) and vortexed briefly. To denature any secondary structures, the samples were incubated at 65°C for 5 min and cooled rapidly on ice prior to loading onto the gel using nuclease-free pipette tips. It was important to avoid any air trapped in the end of the pipette tip that might otherwise disperse the sample. Electrophoresis was carried out in 1X MOPS buffer at 5 V/cm until the blue dye front approached the end of the gel. The gel was visualized under UV light.

3.3.4 Preparation of oligonucleotides

Oligonucleotides (10 nM) were purchased from Eurofins MWG Operon. The oligonucleotides were supplied fully lyophilised. Oligonucleotide stocks of 100 pmol/µl were prepared in sterile H₂O. The final concentration of each oligonucleotide used in the polymerase chain reaction (PCR) was 50 pmol/µl.

3.3.5 SuperScript™ III First-Strand Synthesis

The SuperScript™ III First-Strand Synthesis System for RT-PCR has been optimized to synthesize first-strand cDNA from purified total RNA. The system can be used with as

little as 1 ng or as much as 5 µg of total RNA. Important factors for the synthesis of full-length cDNA are the isolation of intact RNA and prevention of RNases being introduced into the RNA preparation. A first-strand cDNA synthesis reaction was primed using an oligo(dT) primer that hybridizes to 3' poly(A) tails, which are found in the vast majority of eukaryotic mRNA. The initial reaction mixture consisted of 4 µg of total RNA extracted from CHO cells, 1 µl of 50 µM oligo(dT)₂₀, 1 µl of 10 mM dNTP mix and RNase-free water to a total of 10 µl. This was incubated at 65°C for 5 min and cooled on ice for 5 min. In the meantime, the following cDNA Synthesis Mix was prepared, with each component being added in the following order: 2 µl of 10X RT buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of RNase OUT™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl). RNase OUT™ Recombinant RNase inhibitor was added to safeguard against degradation of target RNA due to possible ribonuclease contamination. As a reducing agent, DTT loosens the secondary structure of RNA and facilitates the initiation of transcription. A volume of 10 µl of cDNA Synthesis Mix was added to the RNA/primer sample, mixed gently, collected by brief centrifugation and incubated at 50°C for 50 min. Termination of cDNA synthesis was carried out at 85°C for 5 min and the sample was chilled on ice. To degrade the template mRNA strand from the cDNA:RNA hybrid molecule, 1 µl of *E. coli* RNase H (2 U/µl) was added and incubated at 37°C for 20 min. This step results in higher yields of first-strand cDNA following PCR. The cDNA synthesis reaction was used directly for PCR or stored at -20°C.

3.3.6 DNA agarose electrophoresis

DNA was routinely resolved and purified using agarose gel electrophoresis, as described by Sambrook *et al.* (2001). The agarose gel was composed of 1 x TAE buffer (0.04 M Tris-HCl, 0.2 M sodium acetate, and 1mM EDTA, pH 8.2) containing 2 µg/ml ethidium bromide and 1% (w/v) agarose. Samples containing 100 ng to 1 µg of DNA were diluted with gel-loading dye (15% (w/v) ficoll, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 1 mM EDTA) and 20 to 50 µl was loaded into each well of the gel. Approximately 5 µl of DNA marker was also resolved on the gel to determine the size of each DNA fragment. Electrophoresis was carried out at 65 volts for 1.5 h. The gel was viewed under short wavelength UV light (320 nm). However, when purification of a DNA fragment was necessary, the gel was illuminated by long

wavelength UV light (360 nm) to minimise DNA strand nicking, the fragment of interest was excised using a scalpel and DNA was isolated using the method described in Section 3.3.7.

3.3.7 QIAquick gel extraction

The QIAquick gel extraction kit was used to extract and purify DNA of 100 bp to 10 kb from a standard TAE agarose gel. The recommended volume of 100% ethanol was added to buffer PE before use. The DNA fragment was excised from the gel with a sharp, sterile scalpel. The weight of the gel slice was calculated by weighing the slice in a pre-weighed microcentrifuge tube. Three volumes of buffer QG to one volume of gel (100 mg = 100 μ l), for example 300 μ l of buffer QG were added to each 100 mg of gel. The maximum amount of agarose per QIAquick column is 400 mg. The slice was incubated at 50°C for 10 min and mixed by inverting the tube every 2 min during incubation to help dissolve the gel. One gel volume of 100% isopropanol was added and mixed. The adsorption of DNA to the QIAquick membrane is only efficient at pH <7.5. Buffer QG contains a pH indicator that is yellow at pH <7.5 and orange or violet at higher pH values, allowing easy determination of the optimal pH for DNA binding. To bind DNA, samples were applied to the QIAquick column and centrifuged for 1 min at 16,000 g and the flow-through was discarded. As recommended in the protocol, 0.5 ml of buffer QG was added and centrifuged for 1 min at 16,000 g and the flow-through was discarded. This step removes all traces of agarose. To wash the column, 0.75 ml of buffer PE was added and centrifuged for 1 min at 16,000 g and the flow-through was discarded. Residual ethanol from buffer PE was completely removed by an additional centrifugation for 1 min. A clean 1.5 ml microcentrifuge tube was used for the final step for eluting DNA in 32 μ l of EB buffer (10 mM Tris-HCl, pH 8.5). For increased DNA concentration, elution buffer was added to the centre of the QIAquick membrane and the column was left standing for 1 min before the final centrifugation at 16,000 g for 1 min. The DNA was then stored at -20°C.

3.3.8 QIAquick PCR purification

The QIAquick PCR purification kit was used to purify double-stranded PCR products away from primers, nucleotides and polymerases. Five volumes of buffer PB were added to 1 volume of the PCR and mixed. A QIAquick spin column was placed into a 2 ml collection tube and the sample was loaded. The QIAquick column was centrifuged at

16,000 g for 1 min at room temperature. After draining the flowthrough from the tube, the QIAquick column was replaced into the collection tube. The PCR product was washed by adding 0.75 ml of buffer PE to the QIAquick column and centrifuged at 16,000 g for 1 min at room temperature. The buffer PE flowthrough was drained and the QIAquick column was centrifuged at 16,000 g for 1 min at room temperature to remove residual buffer PE. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 32 µl of 10 mM Tris-HCl, pH 8.5, to the QIAquick column and centrifuged at 16,000 g for 1 min at room temperature.

3.3.9 Bacterial strains and plasmids

The *E. coli* strains (see Table 3.1), the cloning vector pBluescript SK+ and the expression vector pET15b were kind gifts from Dr. Vincent Kelly (Trinity College Dublin, Ireland).

Table 3.1 Description of the *E. coli* strains.

<i>E. coli</i> strain	Description
TOP10	<i>F</i> ⁺ , <i>mcrA</i> , $\Delta(mrr\text{-}hsdRMS\text{-}mcrBC)$, $\phi80lacZ\Delta M15$, $\Delta lacX74$, <i>recA1</i> , <i>araD139</i> , $\Delta(ara\text{-}leu)$, 7697 <i>galU</i> , <i>galK</i> , <i>rpsL</i> (<i>Str</i> ^R), <i>endA1</i> , <i>nupG</i>
BL21(DE3)	<i>F</i> ⁺ , <i>ompT</i> , <i>hsdS_B</i> (<i>r_B</i> [−] , <i>m_B</i> [−]), <i>dcm</i> , <i>gal lon</i> (DE3)

3.3.10 Growth media and antibiotics

The Luria-Bertani broth (LB) and Luria-Bertani agar (LA) used to grow the bacterial cells were prepared as described in Table 3.2. The antibiotics were prepared as described in Table 3.3 and concentrated stocks were stored at -20°C until required.

Table 3.2 Bacterial media.

Media	Composition (1L)
Luria-Bertani broth	10 g tryptone (pancreatic digest of casein) 5 g yeast extract 5 g NaCl
Luria-Bertani agar	LB broth 15 g bacteriological agar

Table 3.3 Selective antibiotic.

Antibiotic	Stock concentration	Working Concentration
Ampicillin (Amp)	50 mg/ml in H ₂ O	100 µg/ml (w/v)

3.3.11 Preparation of competent cells

TOP10 or BL21(DE3) bacterial cells were grown overnight in 3 ml of LB at 37°C. A 1 ml aliquot of the culture was added to 100 ml of LB and grown at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.4 was reached. The culture was divided into 2 x 50 ml aliquots and placed on ice for 15 min. Cells were pelleted by centrifugation (1,800 g, 10 min, 4°C) and each pellet was resuspended in 5 ml of 75 mM CaCl₂ and incubated on ice for 20 min. Cells were pelleted again by centrifugation (1,800 g, 10 min, 4°C). Each pellet was resuspended in 2 ml of TBF2 buffer (75 mM CaCl₂, 10 mM RbCl₂, 15% (v/v) glycerol and 10 mM MOPS, pH 7.0). Cells were snap-frozen in liquid N₂ and stored in 20 x 200 µl aliquots at -70°C.

3.3.12 Favorgen plasmid mini preparations

Ultrapure preparations of pBluescript SK(+), pET15b and CHO GnT clones were made using a FavorPrep™ Plasmid DNA Purification Mini Kit. The kit provides a simple and reliable method for isolating up to 20 µg of plasmid DNA. A small scale culture of each vector was prepared by inoculating 3.5 ml of LB containing 100 µg/ml (w/v) Amp with a single colony and growing at 37°C overnight. The cell culture was centrifuged at 16,000 g for 1 min and the bacterial pellet was resuspended in 200 µl of buffer PD1 containing 100 µg/ml RNase A. To lyse the bacterial cells, 200 µl of buffer PD2 were added, mixed by 10 inversions and incubated at room temperature for 5 min. The mixture was immediately neutralised by adding 300 µl of buffer PD3 and mixing by 10 inversions, followed by centrifugation at 16,000 g for 10 min at 16°C. Supernatant was applied to the PD column and centrifuged at 16,000 g for 30 sec at room temperature. Flowthrough was discarded and the column was washed by adding 400 µl of W1 buffer, incubating for 1 min and centrifuging at 16,000 g for 1 min. After discarding the flowthrough, the column was washed once more by adding 600 µl of wash buffer, incubating for 1 min and centrifuging at 16,000 g for 1 min. An additional centrifugation step at 16,000 g for 3 min was performed to remove all traces of ethanol. DNA was eluted into a clean eppendorf tube

by allowing 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5) to adsorb onto the column membrane for 2 min and then centrifuging at 16,000 g for 2 min. The resulting preparations of pBluescript SK(+), pET15b and GnT clones were stored at -20°C. A permanent stock of each vector was prepared by adding 1 ml of overnight cell culture to 50% glycerol/50% LB to give 25% glycerol (v/v) final and mixing by inversion before storing at -70°C.

3.3.13 cDNA clones

The cDNA clones of CHO GnTII and GnTIVB in pCR4blunt-TOPO were provided by Dr. Tania O'Connor (Trinity College Dublin).

3.3.14 Restriction endonuclease digestion

Restriction enzymes were supplied as 50% glycerol stocks (10 U/µl) with the corresponding reaction buffer (10X). Typically, restriction digests were performed in a total volume of 40 µl of 1X reaction buffer containing 1 - 2.5 µg of DNA and 1-1.5 µl of the restriction enzyme. Digests were carried out at 37°C for approximately 2 h. Due to the different restriction enzyme efficiencies, digestions were carried out either as a single step or two-step reaction. In general, enzymes with compatible buffers and whose sites were more than 10 bp apart were used together in the same reaction. If one of the enzymes was a poor cutter, if the buffers were incompatible, or if the sites were separated by 10 bp or less, the digestions were performed sequentially. The digested DNA products were purified as described in Section 3.3.8 and subjected to further experiments.

It was also necessary to dephosphorylate vectors cut with two enzymes, especially when the restriction sites were close together or if one of the enzymes was a poor cutter. Alkaline phosphatase prevents self-ligation before ligation with T4 DNA ligase. The reactions were performed in a total volume of 50 µl containing 30 µl of the digested vector, 5 µl of 10X AP buffer (500 mM Tris-HCl, pH 9.0, 10 mM MgCl₂), 2 µl of alkaline phosphatase (BAP C75, 0.4 U/µl) and 13 µl of H₂O. The reaction was incubated at 55°C for 2 h. The vector was purified as described in Section 3.3.8 and used for ligation with GnT inserts.

In the cases of GnTII and GnTIVB, which were previously cloned into pCR®4Blunt-TOPO vector, it was useful to linearize the plasmids before using them as templates for

PCR. To digest GntII-pCR®4Blunt-TOPO® (605 ng/μl), the reaction was performed in a total volume of 50 μl of 1X O buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl and 0.1 mg/ml BSA) containing 605 ng of the plasmid and 0.2 units of *NotI*. The reaction was incubated at 37°C for 2 h. To digest GntIVB-pCR®4Blunt-TOPO (230 ng/μl), the reaction was performed in a total volume of 30 μl containing 460 ng of the plasmid, 3 μl of 10X O buffer, 1 μl of *NotI* and 24 μl of H₂O. The reaction was incubated at 37°C for 2 h. *NotI* restriction enzyme was inactivated by incubation at 80°C for 20 min and the linearized products were subjected to PCR.

3.3.15 Bacterial transformations

Ligation products (vectors containing Gnt-encoding inserts) were transformed into and propagated in *E. coli* TOP10 cells or BL21(DE3) for gene expression. Each transformation consisted of 5-10 μl of ligation reaction and 100 μl of competent cells. The transformation mix was placed on ice for 30 min, heat shocked by incubating at 42°C for 30 sec and immediately placed on ice for 5 min. Cells were incubated at 37°C for 1 h after adding 250 μl of SOC medium (tryptone 2% (w/v), yeast extract 0.5% (w/v), NaCl, KCl, MgSO₄ and 20 mM glucose). *E. coli* TOP10 cells with pBluescript SK+ ligations and BL21(DE3) cells with pET15b ligations were plated on LA plates containing 100 μg/ml (w/v) ampicillin. Colonies were allowed to grow overnight at 37°C.

3.3.16 Direct colony PCR screening

In order to verify ligation of the vector and insert, direct colony PCR was performed. A few single colonies were picked from the transformation plate using a sterile toothpick, streaked onto a fresh, sectioned agar plate and the same toothpick was used to inoculate 200 μl of LB containing the appropriate selection agent. The plate was incubated overnight at 37°C and the bacterial cultures were shaken overnight at 37°C. The following day, 2 μl of each culture were transferred to a fresh tube and used as a template for colony PCR. Amplification was performed in a thermal cycler in a total volume of 25 μl 1X green GoTaq® reaction buffer containing DNA template, 0.4 pmol/μl (0.4 μM) of each Gnt primer, 2 mM MgCl₂, 0.4 mM of each of the four dNTPs and 1.25 units of *GoTaq* DNA Polymerase. The PCR parameters are described in Section 3.3.18. Amplified DNA products were analyzed using agarose gel electrophoresis as described in Section 3.3.6, except no gel loading buffer was added to the sample prior to electrophoresis. The 1X

green GoTaq[®] reaction buffer contains two dyes (a blue dye and a yellow dye) that separate during electrophoresis to show the progress of migration. The buffer also contains a compound that increases sample density to aid gel loading.

3.3.17 DNA sequencing

Each GnT-pET plasmid was sent to Eurofins MWG Operon in order to verify the accuracy of amplification and cloning. Each construct was prepared according to the company requirements and sequenced using forward and reversed vector-specific oligonucleotide primers to generate sequence data. The sequence files were analyzed using ClustalW2 Alignment Tool (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The sequence file for reverse primer was converted to its reverse complement before alignment by (http://www.bioinformatics.org/sms/rev_comp.html).

3.3.18 Molecular cloning of CHO GnTs

3.3.18.1 Polymerase Chain Reaction

GnTI, III and V were amplified using oligonucleotides based on the sequence data available on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Gene	Length	Accession Number
CHO GnTI	1344 bp	U65791
CHO GnTII	1329 bp	-
CHO GnTIII	1608 bp	AY598727
CHO GnTIVB	1647 bp	-
CHO GnTV	2223 bp	U62587

The GnTII and GnTIVB cDNA clones were digested with *NotI* restriction enzyme as described in Section 3.3.14 and used directly as templates for polymerase chain reaction. PCR was performed in order to create restriction sites for sequential cloning into pBluescript SK+ and pET15b. The sequences and corresponding restriction sites of the oligonucleotides used for amplification of the coding regions of CHO GnT cDNAs are described in Table 3.4.

Table 3.4 Oligonucleotides used for amplification of CHO GnT cDNAs;
(F-forward primer; R-reverse primer).

Oligo	Sequence	Sites
G1-F	5' -GGCGAATTCATATGCTGAAGAAGCAGTCTGCA-3'	<i>EcoRI</i> <i>NdeI</i>
G1-R	5' -TATCTCGAGCCCGGGGATCCCTAAGTCCAGCTAGGATCATA-3'	<i>XhoI</i> <i>BamHI</i>
G2-F	5' -ATCTCGAGATGAGGTTCCGCATCTACAAACGG-3'	<i>XhoI</i>
G2-R	5' -AGCTCGAGGGATCCTCACTGCAGTCTTCTATAAC-3'	<i>XhoI</i> <i>BamHI</i>
G3-F	5' -ATGAGACGCTACAAGCTCTTTCTCATGTTCTGT-3'	
G3-R	5' -CTAGCCCTCCACTGTATCCAACCTGCCCCTGGC-3'	
G4B-F	5' -ATCTCGAGCATATGAGGCTCCGCAATG-3'	<i>XhoI</i> <i>NdeI</i>
G4B-R	5' -CACTCGAGCTAGTCGGCCTTTTTCAG-3	<i>XhoI</i>
G5-F	5' -GCGAATTCATATGGCTTTCTTTACTCCCTGGAAGTTGTCCT-3'	<i>EcoRI</i> <i>NdeI</i>
G5-R	5' -GCTAGTCGACCCGGGAGATCTGTGCTATAGGCAGTCTTTGCA-3'	<i>SaII</i> <i>BglII</i>

Amplification was performed in a thermal cycler in a total volume of 25 µl of 1X *Pfu* DNA Polymerase buffer (200 mM Tris-HCl, pH 8.2, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100 and 1 mg/ml nuclease-free BSA) containing 1 µl of total cDNA, 2 pmol/µl (2 µM) of each primer, 0.2 mM dNTPs and 0.625 units of *Pfu* DNA polymerase. The PCR program used for amplifying CHO GnT cDNAs is described in Table 3.5.

Table 3.5 GnT PCR program

Process		Temperature	Time
Initial denaturation		95°C	2 min
35 cycles	Denaturation	95°C	1 min
	Annealing		30 sec
	GnTI	61°C	
	GnTII	65°C	
	GnTIII	62°C	
	GnTIVB	64°C	
	GnTV	68°C	
	Elongation	72°C	
	GnTI		3 min 30 sec
	GnTII		3 min
	GnTIII		3 min 30 sec
	GnTIVB		3 min 30 sec
	GnTV		4 min 30 sec
Final elongation		72°C	7 min

The resulting amplification products were resolved by agarose gel electrophoresis as described in Section 3.3.6 and purified as described in Section 3.3.8. The DNA concentration was estimated using a spectrophotometer as described in Section 2.2.9.

3.3.18.2 Restriction digest and cloning into pBluescript SK+

The GnT products and pBluescript SK+ were digested as described in Section 3.3.14, with the restriction enzymes presented in Table 3.4. Digested products were purified using a QIAquick PCR purification kit as described in Section 3.3.8 and the DNA concentration was estimated using a spectrophotometer. The ligations were performed overnight at 16°C in a total volume of 10 µl containing a 1:3 ratio of pBluescript SK+ (60 ng) to GnT PCR product and mixed with an equal volume of Solution I (T4 DNA ligase). The ligated products were transformed into competent *E. coli* TOP10 cells as described in Sections 3.3.15. Several clones potentially containing the GnT-encoding inserts were processed as described in Section 3.3.16. The gene-positive clones were screened by linearizing 10 µl of the GnT-pBluescript SK+ plasmid preparations, followed by agarose gel electrophoresis. Favorgen plasmid mini preparation of pBluescript SK+ containing the GnT insert was used for sequencing to check that the DNA sequence was correct, as described in Section 3.3.17.

3.3.18.3 Restriction digest and cloning into pET15b

The GnT sequence was excised from pBluescript SK+ and purified from pBluescript SK+ by agarose gel electrophoresis, followed by QIAquick gel extraction. The restriction digested GnT insert was ligated to the restriction digested pET15b expression vector. Ligations and *E.coli* transformations were performed as described previously.

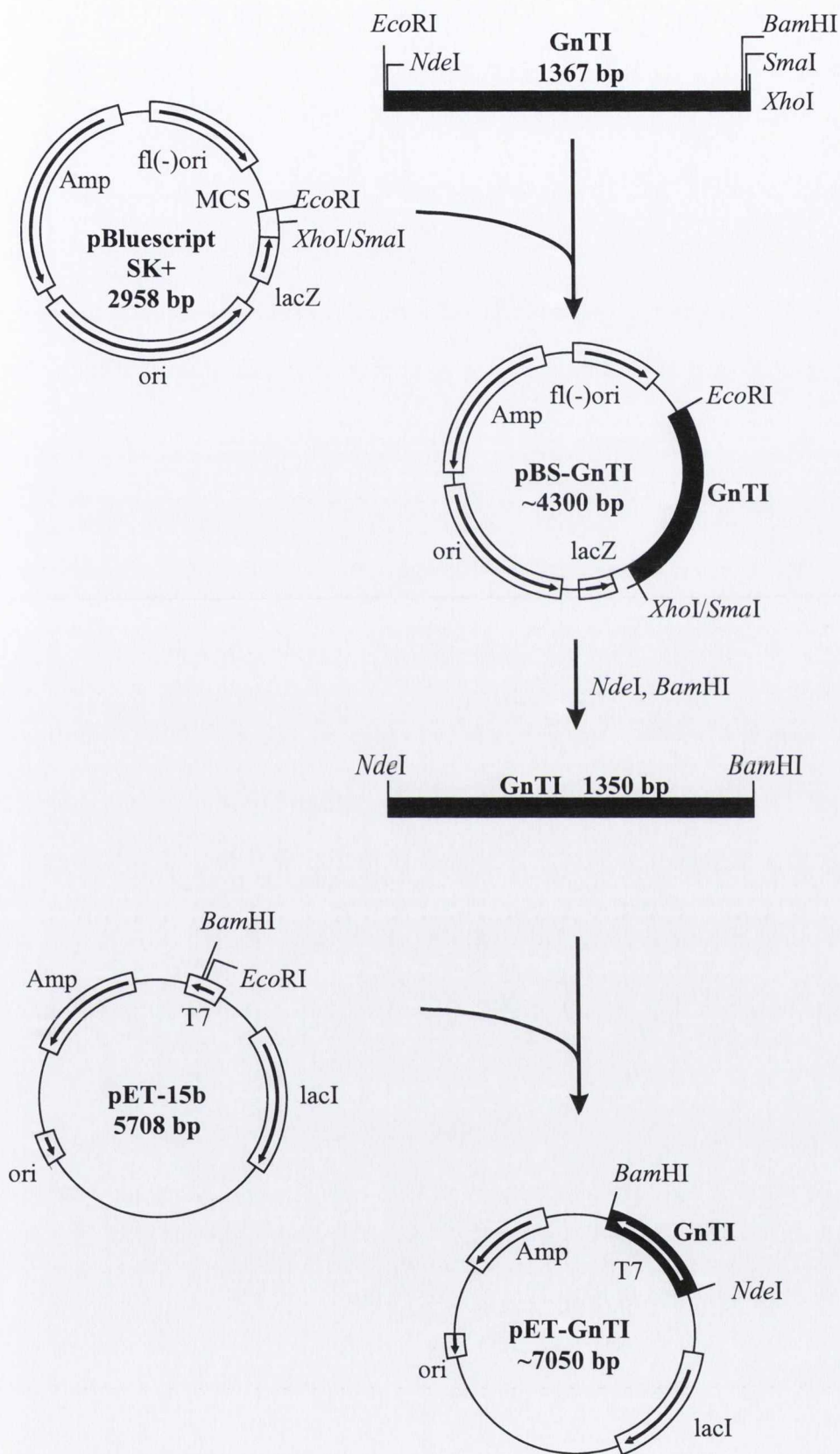


Figure 3.3 Molecular cloning of CHO GnTI.

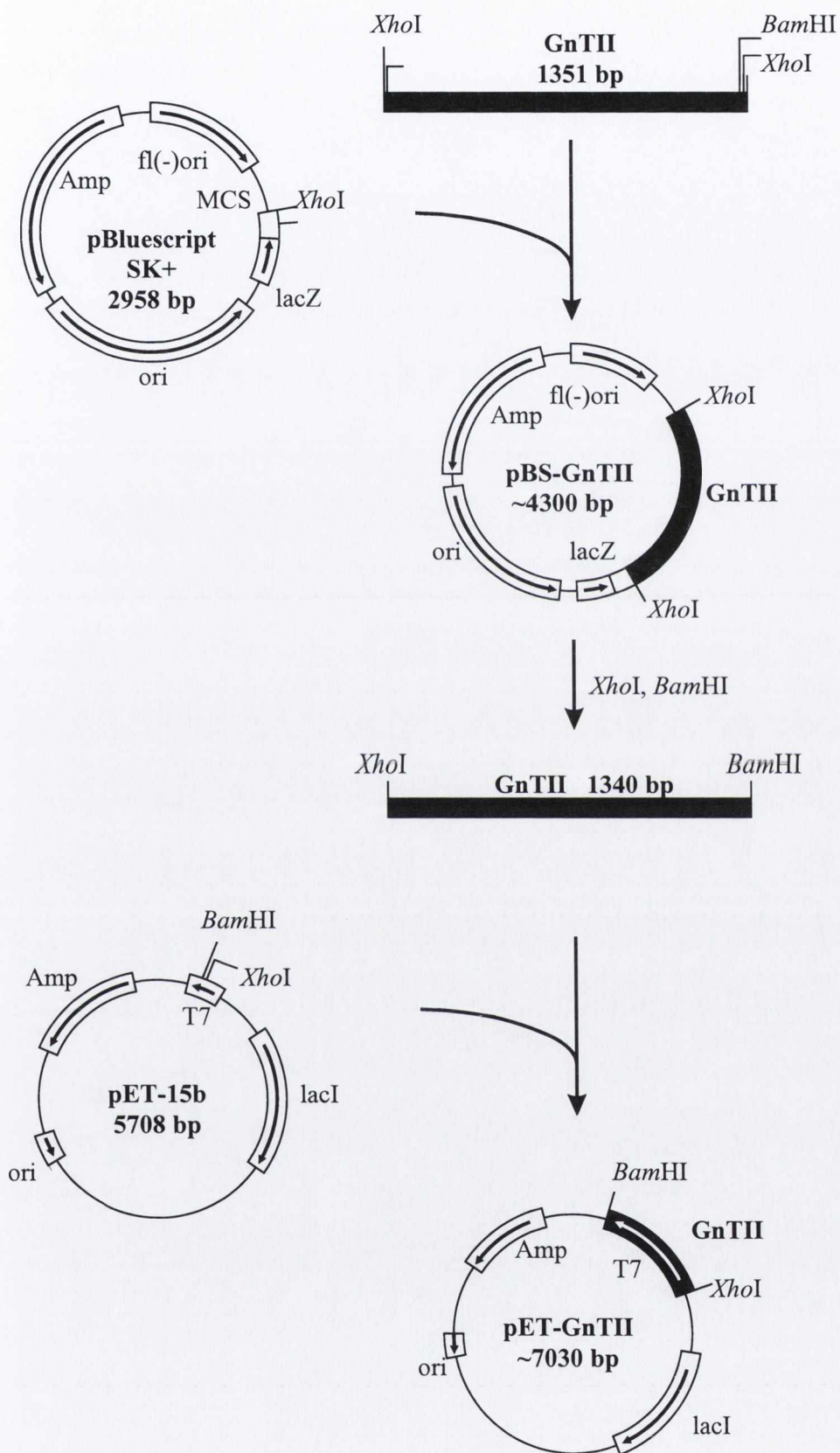


Figure 3.4 Molecular cloning of CHO GnTII.

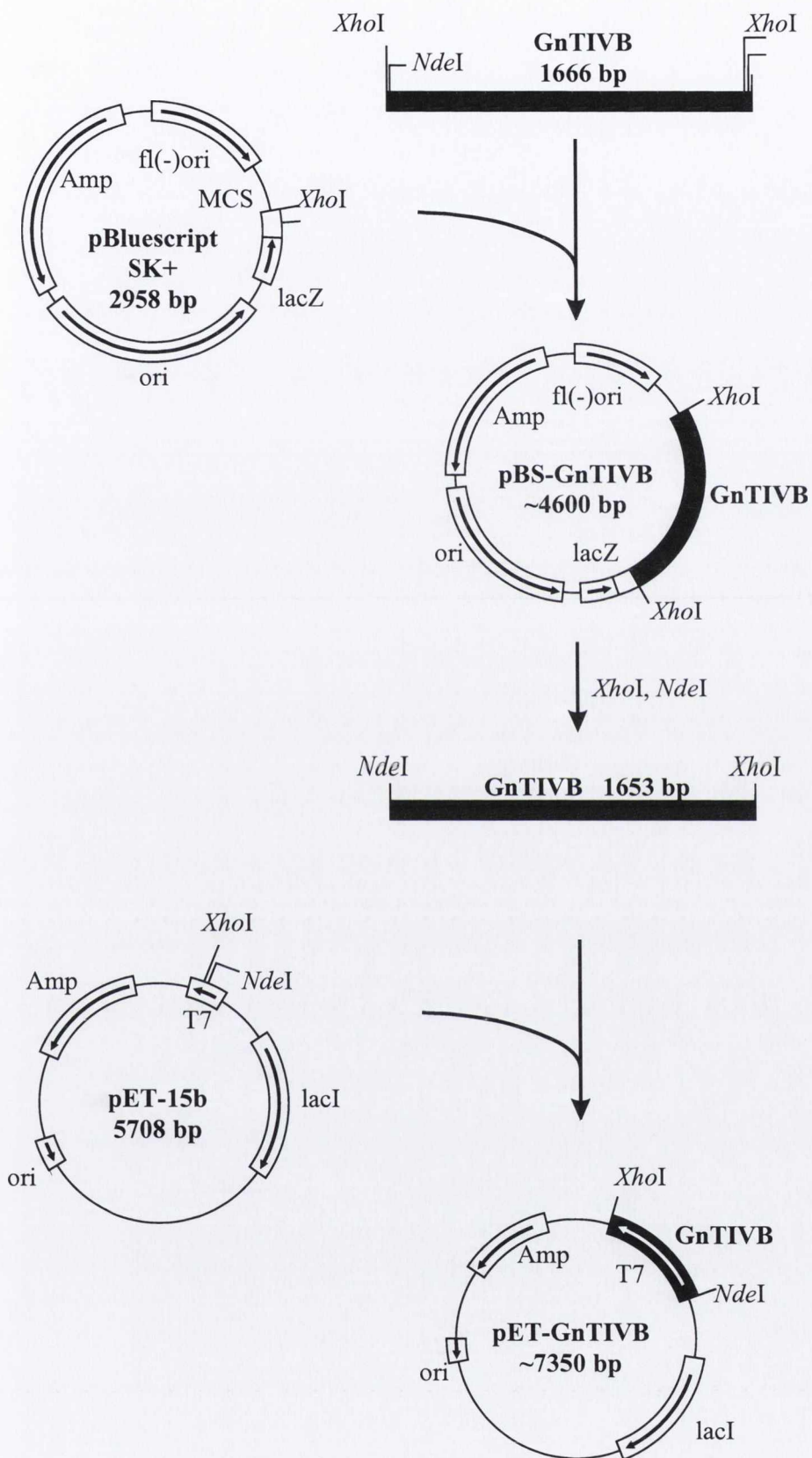


Figure 3.5 Molecular cloning of CHO GnTIVB.

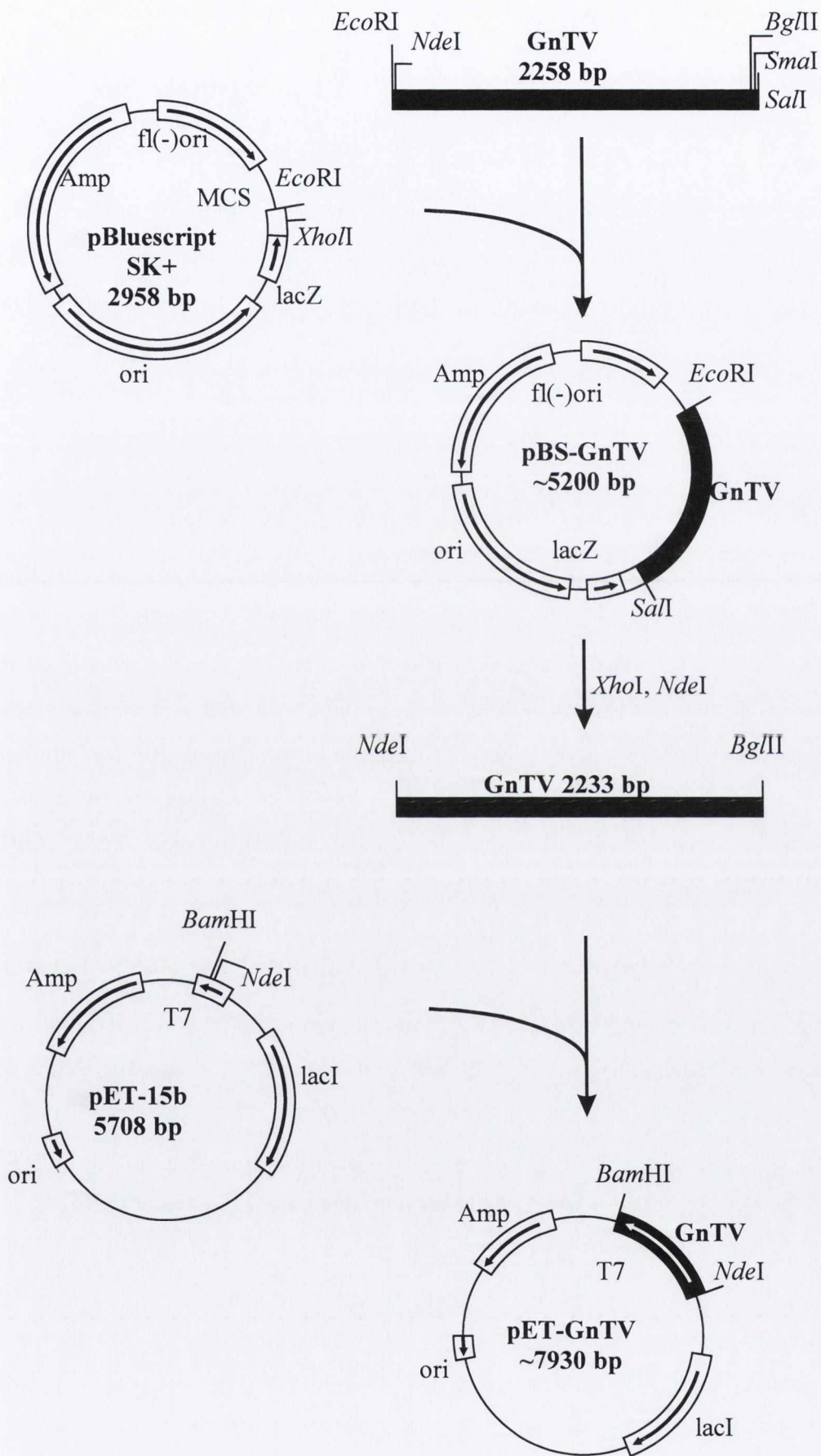


Figure 3.6 Molecular cloning of CHO GnTV.

3.3.19 Expression of recombinant CHO GnTs for determining solubility

For initial experiments designed to determine the solubility of the recombinant protein, log phase 50 ml culture was induced with 1mM IPTG for 3h at 37 °C. The cells were harvested by centrifugation at 2,000 g for 10min. The pellet was resuspended in 1ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Igepal, 1 mg/ml lysozyme, protease inhibitor mixture, 25 U/ml Benzonase). The cell suspension was sonicated on ice. The resulting cell lysate was centrifuged at 30,000 g for 45 min. The clear supernatant (soluble fraction) was collected and remaining pellet (insoluble fraction) was resuspended in the lysis buffer. Soluble and insoluble fractions were then analyzed on 12% SDS-PAGE. Total protein fractions of uninduced and induced control culture were analyzed in parallel.

3.3.20 Site-directed mutagenesis (SDM)

The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during PCR. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. DNA isolated from almost all *E.coli* strains is *dam* methylated and therefore susceptible to *DpnI* digestion which digests parental DNA. The DNA incorporating the desired mutations is then transformed into *E.coli* TOP10 competent cells and further analyzed.

Site-directed mutagenesis (SDM) was used in order to introduce new restriction sites, *NdeI* or *XhoI* and **ATG** start codon downstream to the transmembrane domain (TMD), which is placed at the N-terminus of each GnT protein. The TMHMM Server v. 2.0 (The Centre for Biological Sequence Analysis, Technical University of Denmark) makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of a database of naturally occurring transmembrane proteins (see Table 3.6).

The restriction sites used for cloning GnT cDNAs into pET15b as well as the overview of the site-directed mutagenesis procedure and the list of mutagenic primers are shown in Table 3.7, Figure 3.7 and Table 3.8, respectively.

Table 3.6 Predicted transmembrane domains in the N-terminal region of CHO GnT.

Gene	TMD region
GnTI	7-29 aa
GnTII	12-29 aa
GnTIVB	7-29 aa
GnTV	13-35 aa

Table 3.7 The restriction sites used for cloning GnT cDNAs into pET15b.

Plasmid	Restriction sites 5'→3'	Introduced restriction site
GnTI-pET15b	<i>NdeI</i> and <i>BamHI</i>	<i>NdeI</i>
GnTII-pET15b	<i>XhoI</i> and <i>BamHI</i>	<i>XhoI</i>
GnTIVB-pET15b	<i>NdeI</i> and <i>XhoI</i>	<i>NdeI</i>
GnTV-pET15b	<i>NdeI</i> and <i>BamHI</i>	<i>NdeI</i>

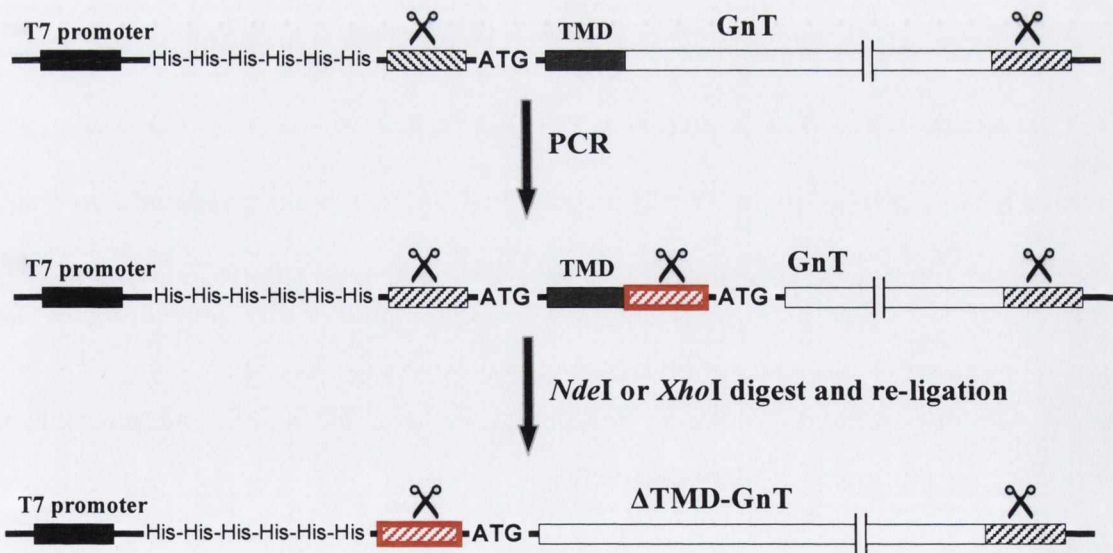


Figure 3.7 Construction of truncated GnT cDNAs and restriction sites used in the cloning procedure; {✂} - restriction sites (see Table 3.8).

Table 3.8 Mutagenic primers used in the SDM of GnT cDNAs; (**F**-forward primer; **R**-reverse primer).

Gene	Sequence (T _m >75°C)	Introduced restriction site	Δ* Base pairs	Δ* Amino acids
G1-F	5'-CTCTTCTTCTGGACACGCCAT <u>ATGCCAGCCCCTGGCAGG</u> -3'	<i>NdeI</i>	93	31
G1-R	5'-CCTGCCAGGGGCTGG <u>CATATG</u> GCGTGTCCAGAAGAAGAG-3'	<i>NdeI</i>	93	31
G2-F	5'-CAATGGGCGA <u>CTCGAGATG</u> AG GAAGAACGACGGCCT-3'	<i>XhoI</i>	93	31
G2-R	5'-AGGCCGTCGTTCTTCTC <u>CATC</u> <u>TCGAGTCGCCCATTG</u> -3'	<i>XhoI</i>	93	31
G4B-F	5'-GGCGCTCAGCGGCC <u>CATATG</u> AA AGGTGACGTGGTG-3'	<i>NdeI</i>	93	31
G4B-R	5'-CACCACGTCACCTTT <u>CATATG</u> GCCGCTGAGCGCC-3'	<i>NdeI</i>	93	31
G5-F	5'-CACTTCACCATCCAGCAGC <u>CAT</u> <u>ATGACTCAGCCTGAGAGC</u> -3'	<i>NdeI</i>	111	37
G5-R	5'-GCTCTCAGGCTGAGT <u>CATATG</u> CTGCTGGATGGTGAAGTG-3'	<i>NdeI</i>	111	37

* - (Δ) deletion of nucleotides (base pairs) or amino acids

The MWG oligos were supplied in 100 pmol/μl aliquots and the formula used to determine the optimal concentration of primers (oligos) for PCR is shown below:

$$\text{X pmoles of oligo} = \frac{125 \text{ ng of oligo}}{330 \times \text{No. of base pairs in oligo}} \times 1000$$

All primers were 5' phosphorylated so that the PCR product could be ligated into a circle and used to transform *E.coli* TOP10 cells. Amplification was completed in a thermal cycler, in a total volume of 50 μl of 1X *Pfu* DNA polymerase buffer, containing 50 ng of GnT-pET15b plasmid, 125 ng of each primer, 0.2 mM dNTPs, and 0.625 units of *Pfu* DNA polymerase. The plasmid sizes and PCR program used for amplifying plasmids are described in Table 3.9 and 3.10, respectively.

Table 3.9 GnT-pET15b parameters used for calculating the elongation time.

Plasmid	pET15b	GnT	Total	Elongation Time* (ET)	ET used in PCR
GnTI-pET15b	5708 bp	1344 bp	7052 bp	~7 min 3 sec	7 min 30 sec
GnTII-pET15b	5708 bp	1329 bp	7037 bp	~7 min 2 sec	7 min 30 sec
GnTIVB-pET15b	5708 bp	1647 bp	7355 bp	~7 min 21sec	7 min 30 sec
GnTV-pET15b	5708 bp	2223 bp	7931 bp	~7 min 55 sec	8 min

* 1 min per 1000 bp

Table 3.10 PCR program.

Process		Temperature	Time
Initial denaturation		95°C	30 sec
18 cycles	Denaturation	95°C	30 sec
	Annealing	55°C	1 min
	Elongation	68°C	
	GnTI-pET15b		7 min 30 sec
	GnTII-pET15b		7 min 30 sec
	GnTIVB-pET15b		7 min 30 sec
	GnTV-pET15b		8 min

Following the PCR, the samples were placed on ice for 2 min to cool the reaction to 37°C and the product was treated with 1 µl *DpnI* (10 U/µl) for 1 h. The *DpnI* endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and was used to digest the parental DNA template and therefore to select for mutation-containing synthesized DNA. The vectors (1 µl) containing the desired mutation were then transformed into *E.coli* TOP10 competent cells according to Section 3.3.15. Several clones, which potentially contained the mutation, were sent to MWG in order to complete the sequence analysis as described previously. In order to remove the transmembrane domains (TMD) from the GnT cDNAs, 2000 ng of GnT-pET15b with newly introduced restriction sites, were used in the restriction digest reaction with *NdeI* or *XhoI* in a total volume of 40 µl according to Section 3.3.14. The remaining restriction ends of the plasmids were then religated with T4 ligase in a total volume of 10 µl and incubated overnight at 16°C. Vectors (1 µl) containing GnT inserts lacking transmembrane domains were then transformed into *E.coli* TOP10 competent cells and single clones were analyzed by MWG as described before. Expression of truncated GnT proteins was carried in

BL21(DE3) *E.coli* cells according to Section 3.3.21 and proteins were further analyzed for solubility.

3.3.21 Expression of recombinant CHO GnTI for use as immunogen

The 6xHis- Δ 31GnTI construct was transformed into *E. coli* BL21 cells and expressed under the control of an IPTG-inducible T7 polymerase. Bacterial cultures were grown at 30°C in 3 L of LB containing a final concentration of 100 μ g/ml (w/v) ampicillin (Amp) and until an OD₆₀₀ of 0.6 was reached. At this point, IPTG was added to a final concentration of 0.5 mM and the cells were left to grow for further 15 h at 18°C. After this period of induction, the culture was cooled on ice for 30 min and the cells were harvested by centrifugation (10,000 g, 10 min, 4°C). The resulting bacterial cell pellets (each pellet was equivalent to 100 ml of cell culture) were stored at -70°C until used for purification.

3.3.22 Expression of recombinant CHO GnTII, IVB and V for use as immunogen

The GnTII-, Δ 31GnTIVB and GnTV-pET15b constructs were transformed into *E. coli* BL21 cells and expressed under the control of an IPTG-inducible T7 polymerase. Bacterial cultures were grown at 30°C in LB containing a final concentration of 100 μ g/ml (w/v) Amp until an OD₆₀₀ of 0.4 was reached. At this point, IPTG was added to a final concentration of 0.1 mM and the cells were left to grow for further 2.5 h. After this period of induction, the culture was cooled on ice for 30 min and the cells were harvested by centrifugation (10,000 g, 10 min, 4°C). The resulting bacterial cell pellets (each pellet was equivalent to 100 ml of cell culture) were stored at -70°C until used for purification.

3.3.23 Purification of recombinant Δ 31GnTI

Recombinant Δ 31GnTI protein was expressed in *E.coli* from pET15b as a polyhistidine fusion protein that contained additional six histidine residues. Recombinant protein was isolated from bacterial lysates (an equivalent of 3 L of culture) by affinity chromatography on Ni Sepharose 6 Fast Flow resin. Δ 31GnTI was routinely prepared from frozen bacterial pellets. Each cell pellet (approx. 1 g of cell paste from 500 ml of culture) was resuspended in 10 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM imidazole, 0.1% (v/v) Igepal, 1 mg/ml lysozyme, protease inhibitor mixture, 25 U/ml Benzonase, 1 mM DTT) at 37°C until suspension was clear. To ensure maximum bacterial lysis, the cells were sonicated in 30 ml aliquots at 30% capacity for 3 min each and the cell debris was removed by centrifugation (30,000 g, 45 min, 4°C). Resulting supernatant was filtered

through 0.45 μ m filter and applied to 1 ml Ni Sepharose 6 Fast Flow resin that had been pre-equilibrated with 50 ml of binding buffer (20 mM phosphate monobasic, pH 7.2, 150 mM NaCl, 20 mM imidazole, 1 mM DTT). All purification steps were performed at 4°C with the supernatant, buffers and elutions kept on ice. The column was washed with binding buffer again followed by 50 ml of wash buffer I (20 mM phosphate monobasic, pH 7.2, 150 mM NaCl, 50 mM imidazole, 1 mM DTT) and 50 ml of wash buffer II (20 mM phosphate monobasic, pH 7.2, 150 mM NaCl, 100 mM imidazole, 1 mM DTT). Finally, the Δ 31GnTI was eluted with 10 ml of elution buffer (20 mM phosphate monobasic, pH 7.2, 150 mM NaCl, 250 mM imidazole, 1 mM DTT). In order to determine the purity of protein, small aliquots of 20 μ l were taken from each elution fraction, mixed with an equal volume of 2 X LB and analyzed on SDS-PAGE gel as described in Section 2.2.6 and by Western blotting using anti-His monoclonal antibody as described in Section 2.2.8. The enzyme-containing eluate was pooled to give a final volume of 10 ml and dialyzed at 4°C against dialysis buffer (20 mM phosphate monobasic, pH 6.5, 150 mM NaCl) overnight and concentrated using a 15 ml Amicon concentrating column (30k MWCO; 2,000 g, 45 min, 4°C) to give a final volume of 1 ml. The protein content was determined by BCA protein assay as described in Section 2.2.7. Typically, 2 mg of purified Δ 31GnTI was obtained from 3 L of bacterial culture. The sample was mixed with an equal volume of 2 X LB to give a final concentration of 1 mg/ml and run on a large SDS-PAGE gel as described in Section 2.2.6.

3.3.24 Purification of recombinant CHO GnTII, GnTIVB and GnTV

Bacterial cells were harvested by centrifugation at 2,000 g for 10 min. GnTII, Δ 31GnTIVB and GnTV were routinely prepared from frozen bacterial pellets. Each cell pellet (approx. 1 g of cell paste from 100 ml of culture) was resuspended in 10 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Igepal CA 630, 1 mg/ml lysozyme, protease inhibitor mixture, 25 U/ml Benzonase) at 37°C until suspension was clear. To ensure maximum bacterial lysis, the cells were sonicated in 10 ml aliquots with 30% duty cycle for 3 min each on ice and the cell debris was removed by centrifugation (30,000 g, 30 min, 4°C). The pellet of inclusion body (insoluble fraction) was washed with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Igepal CA 630, and 1 mg/ml lysozyme) and centrifuged as before. Inclusion bodies were washed in buffers B (20mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Triton X-100), C (20mM

Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Igopal CA 630), and D (20mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Tween 20) by centrifuging at 30,000 g for 20 min at 4 °C. The final pellet was resuspended in PBS and the protein concentration was determined by BCA assay as described in Section 2.2.7.2. Protein samples were mixed with 2 X LB in order to obtain a concentration of 1 mg/ml and were resolved by a large SDS-PAGE gel as described in Section 2.2.6.

3.3.25 Generation of polyclonal antibodies against recombinant CHO GnTs

Recombinant GnT proteins were used as immunogen for the production of the antiserum. GnT proteins (1 mg/ml) were resolved by 12% SDS-PAGE as described in Section 2.2.6 with the exception for the size of the gel. In the large gel each well could hold up to 150 µg of the protein. 600 µg of each GnT were loaded into six separate wells and the gels were stained and destained as described in Section 2.2.6. The protein bands were excised from the gel and each GnT protein, in six gel slices (6 x 100 µg), was placed in separate centrifuge tubes and sent for generating antibodies. The preparation, injections, and sacrifice of female New Zealand white rabbits were performed at CovalAb UK Ltd., Cambridge, UK. Rabbits were immunised subcutaneously with 600 µg of recombinant GnTs in a total of six injections. The projects reference numbers were as follows: GnTI – CUK-1078, GnTII – CUK-1081, GnTIVB – CUK-1082, and GnTV – CUK-1085. Control pre-immune serum was prepared from 5 ml of blood obtained from the rabbit prior to immunisation. Each rabbit was first injected with 100 µg of GnT protein in Freund's complete adjuvant (1:1 ratio). Three weeks later, each rabbit was injected with 100 µg of GnT protein in Freund's incomplete adjuvant (1:1 ratio). After further three weeks, each rabbit was injected with 100 µg of GnT protein in Freund's incomplete adjuvant (1:1 ratio). A volume of 5 ml of blood was taken from each rabbit 11 days after the third injection. The boost of 100 µg of GnT protein in Freund's incomplete adjuvant was injected into each rabbit after 9 further days. A volume of 10 ml of blood was taken from each rabbit 11 days after the fourth injection. After further 10 days, each rabbit was injected with 100 µg of GnT protein in Freund's incomplete adjuvant (1:1 ratio). After two further weeks, the final boost of 100 µg of GnT protein in Freund's incomplete adjuvant was injected into each rabbit. A volume of 5 ml of blood was taken from each rabbit 11 days after the final injection. Rabbits were bled out 12 days after the final serum was collected. The antibody titer was determined from each blood sample as described in Section 3.2.26.

3.3.26 Antiserum titer determination by ELISA

Pre-immune, immune serum from day 53, 74 and 123 and purified antibodies were used to determine the antibody titer using the indirect enzyme-linked immunosorbent analysis (ELISA). The purified antigen, diluted to 10 µg/ml in 50 mM carbonate salt buffer (pH 9.6), was coated on a 96 well microtiter plate at 100 µl aliquot per well, and incubated at 4°C overnight. The wells were washed three times with PBS–Tween buffer (0.05% Tween 20 in PBS). Coated wells were blocked with 200 µl of 3% BSA for 1 h at 37°C and then incubated with 150 µl polyclonal antibodies against GnT proteins at different dilutions (from 1:500 to 1:64000). After incubation for 2 h at 37°C, the wells were incubated with 150 µl horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000) for 1 h at 37°C. Peroxidase activity on the immunoplate was detected using *o*-phenylenediamine and H₂O₂ as enzyme substrates. Colour development was stopped with 2M H₂SO₄ and the absorbance was measured at 450 nm using a microplate reader.

3.3.27 Generation of anti-peptide antibodies against CHO GnTs

The standard antibody service developed by Genosphere Biotechnologies includes: (1) custom peptide synthesis, (2) immunisation of two animals of choice (rabbits, guinea pigs or chicken), (3) titer evaluation by ELISA and (4) IgG purification.

In order to increase the chances of producing a successful antibodies pool, three peptide sequences were chosen for each CHO GnT (see Table 3.11). Each peptide was then chemically synthesized, purified by HPLC and conjugated to a large molecule called KLH (Keyhole Limpet Hemocyanin) followed by immunisation in rabbits.

Table 3.11 Peptides used for equimolar co-immunisation (KLH-peptide conjugates).

Hapten	Sequence	pI
GnTI-1	N-CKVRTNDQKELGEVR	6.25
GnTI-2	N-CRRVHLAPPQTWEGY	6.75
GnTI-3	N-CGSAVTHIRQPDLSN	6.74
GnTII-1	N-CADKVDVKTWKSTEH	6.74
GnTII-2	N-CRNVRDDGTWRPGEL	6.12
GnTII-3	N-CRKNGGWGDIRDHEL	6.75
GnTIVB-1	N-CLPHLLAKESSLQPA	6.74
GnTIVB-2	N-CDIVAKPNYLSTMKN	8.18
GnTIVB-3	N-CPPAEVSTSLKTYQH	6.74
GnTV-1	N-CEGKIKWMKDMWRSD	8.18
GnTV-2	N-CGHDIRISASLAELK	6.74
GnTV-3	N-CNIPSYVKNHGILSG	8.21

Individual antisera were tested and titer evaluated by ELISA against each peptide separately according to the company's standard protocol. Antigens were coated on EIA strips at 10 µg per well followed by washing with PBS buffer (200 µl). Diluted antisera were added on the coated strip following incubation for 2 h at 37°C. Unbound antibodies were washed with PBS-Tween buffer and anti-rabbit IgG-HRP conjugate was added. Plates were washed as before and the colour development with TMB substrate ran for 15 min. The absorbance was recorded at 405 nm. Colour intensity was directly proportional to the amount of antibodies and antibody was positive if absorbance was >2 folds over that of pre-immune serum. Total IgG fraction specific to each GnT was purified on protein A chromatography.

3.4 Results

3.4.1 Multiple sequence alignment of GnTs

Multiple amino acid alignments were performed using on line Clustal Version 2.0 (ClustalW2) program maintained by the European Bioinformatics Institute (sequence data information shown in Table 3.11). At the start of this project there was no sequence information about CHO GnTII and GnTIV. As previously reported (Oguri *et al.*, 2006; Takamatsu *et al.*, 2010), there are three isoforms of human, rat and mouse GnTIV, indicating three distinctive features of the same enzyme. GnTV has also been reported to have two isoforms with one being specific to the brain (Kaneko *et al.*, 2003; Inamori *et al.*, 2003). The sequence information of missing CHO GnTII and GnTIV isoforms could be obtained from existing data due to the high sequence identity between enzymes from different species as represented in Table 3.12. The percentage sequence identity of GnT genes from human, mouse and rat were estimated to be $\geq 84\%$, $\geq 90\%$, and $\geq 89\%$, respectively. However the sequence identity of GnT genes between each other was not greater than 6% as shown in Table 3.13. Additionally, GnTIV isoforms share only partial sequence identity with 62% between mouse isoform A and B to be the highest value (see Figure 3.14).

Table 3.11 Sequence information for CHO, mouse, rat and human GnTs.

	CHO	Mouse	Rat	Human
GnTI	1344 bp	1344 bp	1344 bp	1348 bp
GnTII	1329 bp	1329 bp	1329 bp	1344 bp
GnTIII	1608 bp	1617 bp	1611 bp	1596 bp
GnTIVA	?	1608 bp	1581 bp	1608 bp
GnTIVB	1647 bp	1647 bp	1731 bp	1647 bp
GnTIVC	?	1437 bp	1482 bp	1437 bp
GnTVA	2223 bp	2223 bp	2223 bp	2226 bp
GnTVB	*	2379 bp	2379 bp	2373 bp

(*) GnTVB is specifically expressed in the brain (Kaneko *et al.*, 2003; Inamori *et al.*, 2003).

Table 3.12 Percentage sequence identity of GnT genes.

Species	CHO GnTI	CHO GnTII	CHO GnTIII	CHO GnTIVB	CHO GnTVB
Human	85%	88%	84%	90%	86%
Mouse	92%	92%	90%	94%	92%
Rat	92%	93%	90%	95%	89%

Table 3.13 Identity of GnT genes between each other.

Species	Identity
Human GnTI, II, III, IV, V	≤ 4%
Mouse GnTI, II, III, IV, V	≤ 6%
Rat GnTI, II, III, IV, V	≤ 4%
CHO GnTI, II, III, IV, V	≤ 4%

Table 3.14 Percentage sequence identity of GnTIV isoforms.

GnTIV isoforms	Human	Mouse	Rat
GnTIVA and GnTIVB	34%	62%	44%
GnTIVA and GnTIVC	8%	12%	27%
GnTIVB and GnTIVC	11%	15%	17%

3.4.2 Molecular cloning of CHO GnTI, II, IVB and V into pET15b

Total RNA was extracted from wild type CHO cells as described in Section 3.3.2 and resolved on a denaturing agarose gel as described in Section 3.3.3. The total RNA had sharp 28S and 18S ribosomal RNA bands and the 28S RNA band was approximately twice as intense as the 18S RNA band indicating that the CHO RNA preparation was intact and of a high quality (see Figure 3.8). The CHO RNA was used in the reverse transcription reaction in which the complementary DNA (cDNA) was formed and could be used for amplification of specific regions of GnTI and GnTV. Figure 3.9 shows the PCR products from amplification of the coding regions of GnTI (1344 bp) and GnTV (2223 bp). GnTII and GnTIVB cDNAs were previously cloned into pCR®4Blunt-TOPO vector. In order to obtain the highest concentration of the cDNAs it was useful to linearize the plasmids before using them as templates for PCR. Figure 3.10 shows the PCR products from amplification of the coding regions of GnTII (1329 bp) and GnTIVB (1647 bp). Specific primer sets were used to create restriction sites in all GnT cDNAs for sequential cloning into pBluescript SK+ and then into pET15b. The restriction analysis of all GnT-pET15b constructs using either *NdeI* or *XhoI* is shown in Figure 3.11.

RT-PCR was completed in order to verify the transcriptional silence of the GnTIII gene in wild type CHO cells, as described by the Stanley *et al.* in 2002. In this cell line, the *GnTIII* gene seems to remain transcriptionally silent during the cell cycle or else the *GnTIII* mRNA is unstable. As expected, a product for GnTIII was not detected despite using numerous RT-PCR conditions (data not shown). The sequence of CHO GnTIII is available in the NCBI database and sourced from the gain-of-function CHO cell glycosylation mutant LEC10, the cell extracts of which exhibit GnTIII enzyme activity (Stanley *et al.*, 2005).

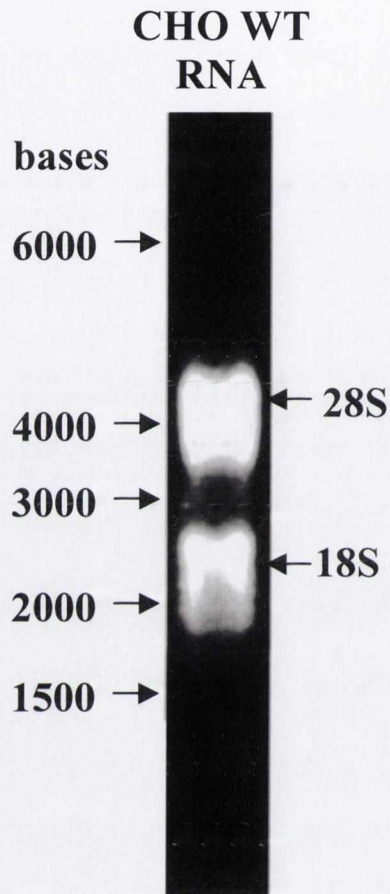


Figure 3.8 Total RNA from wild type CHO cells.

The sample containing 4 μg of RNA was adjusted to a total volume of 10 μl in RNase free water and mixed with an equal volume of gel loading buffer. The sample was heated to denature any secondary structures and resolved on a denaturing agarose gel as described in Section 3.3.3. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample.

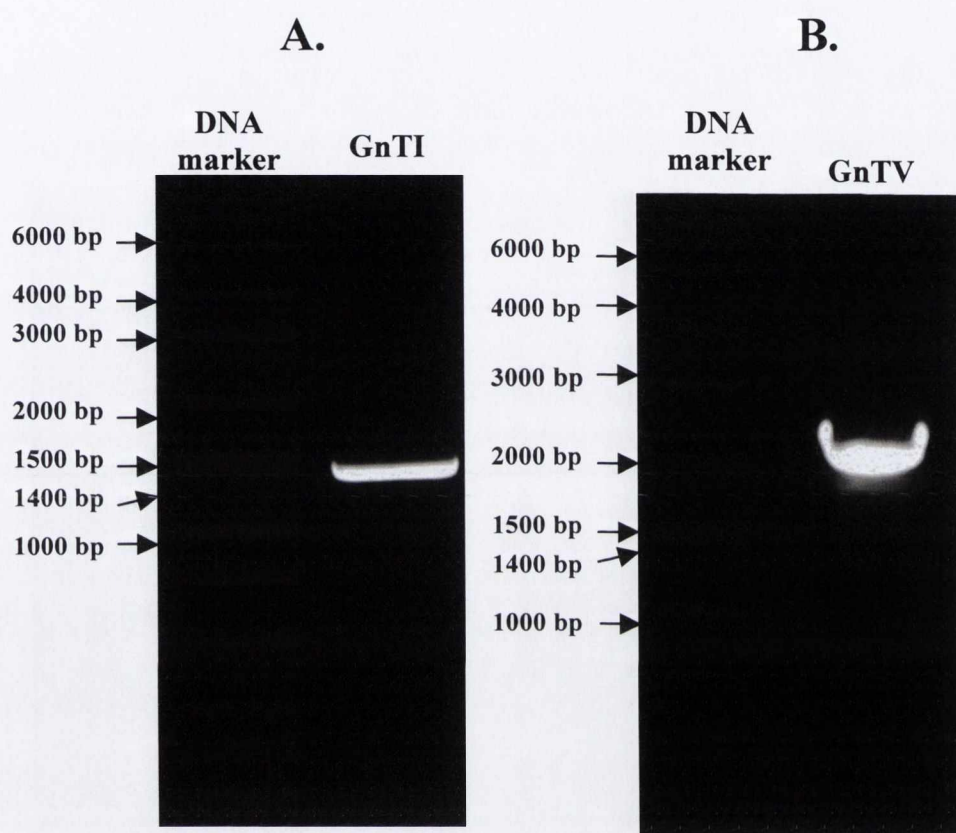


Figure 3.9 PCR products of CHO GnTI (A) and GnTV (B) cDNAs.

PCR amplification was completed for each template as described in Section 3.3.18 and the resulting amplification products were resolved by agarose gel electrophoresis as described in Section 3.3.6; agarose gel was loaded as follows: **lane 1**, DNA marker; **lane 2**, PCR product of GnT cDNA.

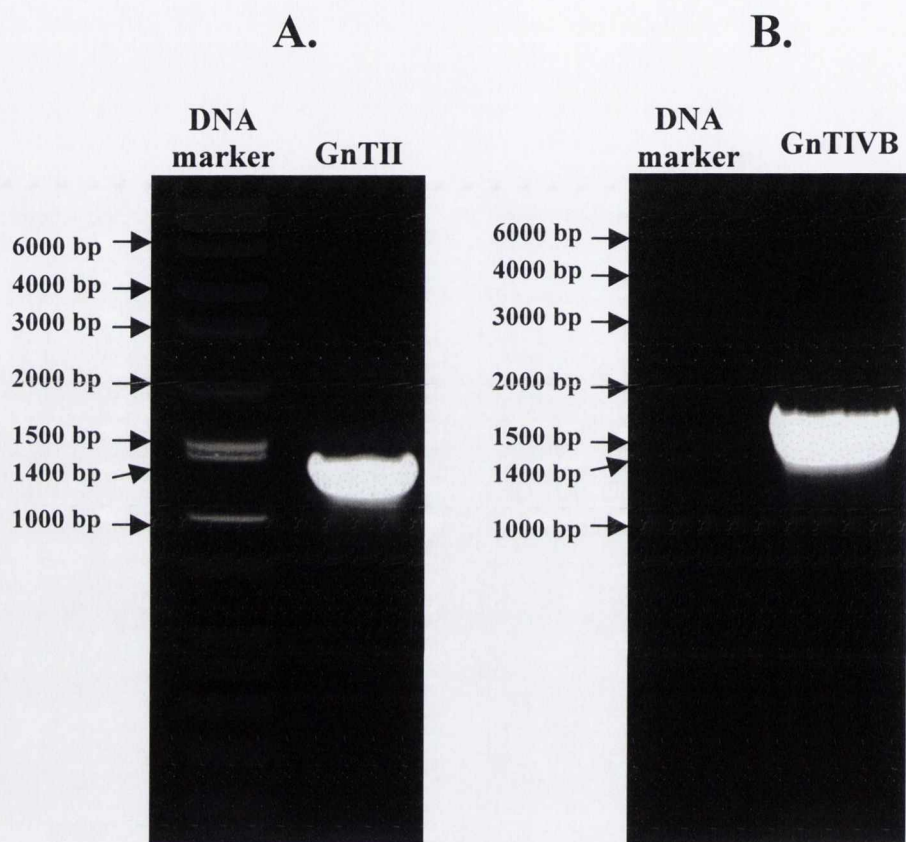


Figure 3.10 PCR products of CHO GnTII (A) and GnTIVB (B) cDNAs.

PCR amplification was completed for each template as described in Section 3.3.18 and the resulting amplification products were resolved by agarose gel electrophoresis as described in Section 3.3.6. The agarose gel was loaded with the following samples: **lane 1**, DNA marker; **lane 2**, PCR product of GnT cDNA.

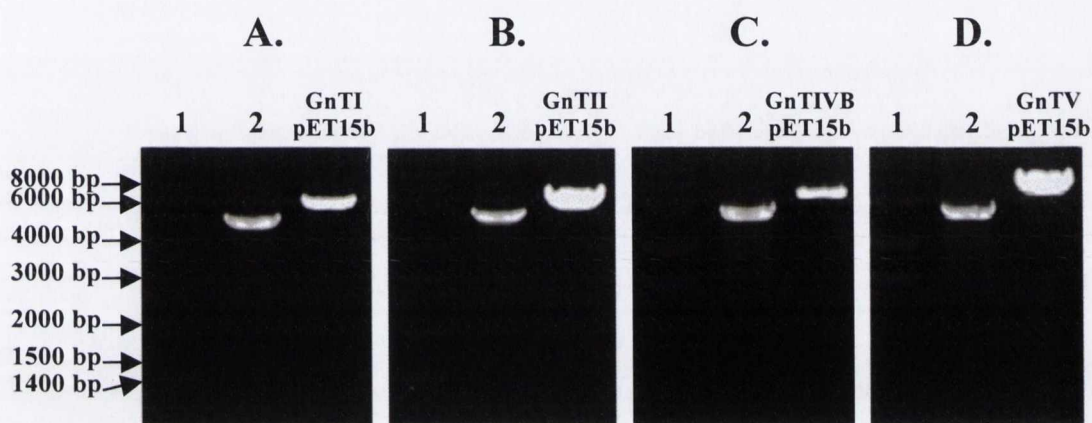


Figure 3.11 Restriction digestion of CHO GnT-pET15b constructs.

The restriction analysis of CHO **A.** GnTI- (7052 bp), **B.** GnTII- (7037 bp), **C.** GnTIVB- (7355 bp) and **D.** GnTV-pET15b (7931 bp) constructs was carried as described in Section 3.3.14. The agarose gels were loaded as follows: **lane 1**, DNA marker; **lane 2**, pET15b digested with *NdeI*; **lane 3**, GnTI-pET15b *NdeI* digest; GnTII-pET15b *XhoI* digest; GnTIVB-pET15b *NdeI* digest; GnTV-pET15b *NdeI* digest, respectively.

3.4.3 Expression of full-length recombinant GnTI, II and V from *E. coli*

The strategy developed for expressing the cloned GnT cDNAs was based on the pET system (Studier and Moffatt, 1986). Each CHO recombinant GnT was expressed in *E. coli* bacterial cells from pET15b, under the control of an IPTG-inducible T7 RNA polymerase, as a polyhistidine fusion protein that contained six histidine residues. Figure 3.12 shows that the expression of each recombinant histidine-tagged GnT protein was induced by IPTG for 2.5 h. Based on SDS-PAGE analysis, CHO GnTI, GnTII and GnTV were found in the insoluble inclusion bodies. The apparent molecular masses of polyhistidine-tagged GnTI and GnTII were estimated to be 52 kDa (6xHis-GnTI), 52 kDa (6xHis-GnTII) and 78 kDa (6xHis-GnTV). These data are in good agreement with the calculated molecular masses of 53.9 kDa, 53.4 kDa for GnTI and GnTII, respectively. The apparent molecular mass of 78 kDa for the polyhistidine-tagged GnTV protein differed from the calculated size of 86.7 kDa. Surprisingly, the full-length, histidine tagged GnTIVB was not expressed from BL21(DE3) cells induced by IPTG.

The average protein concentrations of insoluble GnTI, II and V are shown in Table 3.15. In order to confirm the presence of additional histidine residues on the N-terminus of each GnT protein, Western blotting was performed with a monoclonal anti-His antibody as shown in Figure 3.13.

The full-length GnT proteins were also expressed at 18°C overnight in 1 L culture medium. Soluble fractions were collected and loaded onto a preequilibrated column containing Ni Sepharose 6 Fast Flow resin. Analysis of the column eluate by SDS-PAGE and Western blotting showed that GnT proteins were not expressed in a soluble form (data not shown).

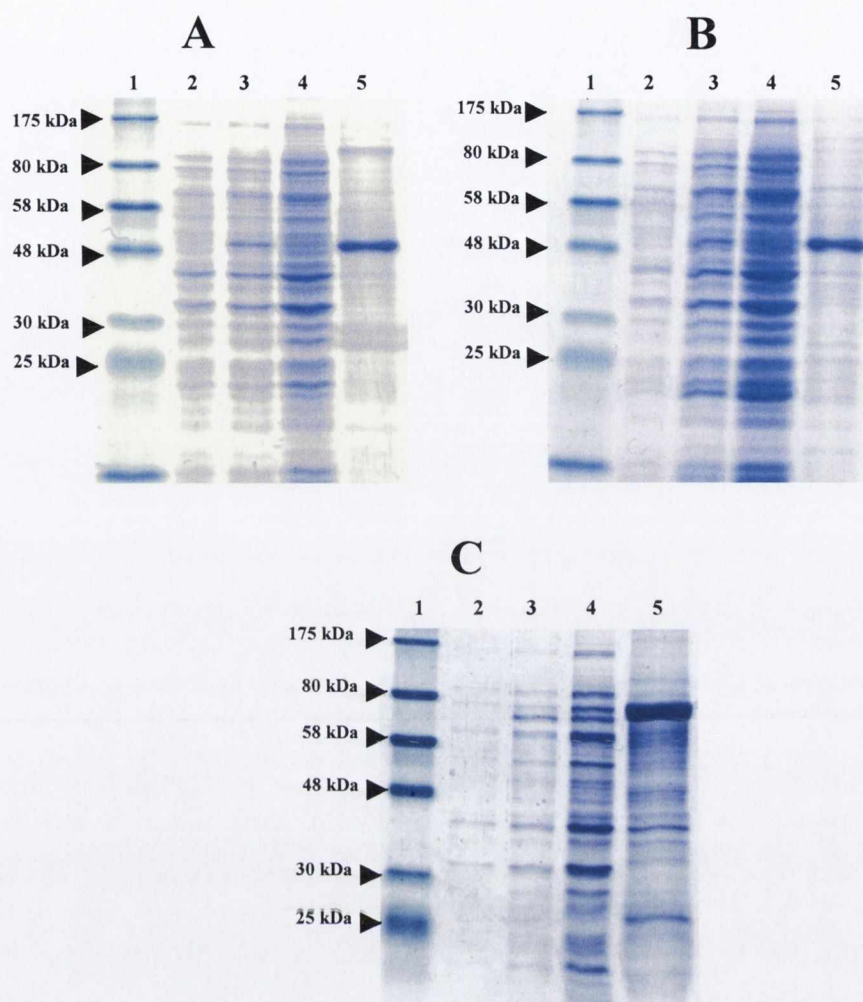


Figure 3.12 Heterologous expressions of full-length, histidine-tagged GnTI, GnTII and GnTV from BL21(DE3) *E.coli* cells.

Experiment details are described in Section 3.3.19. The aliquots of all protein fractions were mixed with an equal volume of 2X SDS sample buffer and heated immediately for 5 min at 95°C. The SDS-PAGE gels for GnTI (A), GnTII (B) and GnTV (C) were loaded with the following samples: **lane 1**, protein marker; **lane 2**, 5 µl of total cell protein fraction (TCP) taken just before IPTG induction; **lane 3**, 5 µl of total cell protein fraction (TCP) taken at time point 2.5 h after IPTG induction; **lane 4**, 5 µl of the soluble protein fraction taken at time point 2.5 h after IPTG induction; **lane 5**, 10 µl of the insoluble, partially purified protein fraction (inclusion bodies) taken at time point 2.5 h after IPTG induction.

Table 3.15 Yield of each GnT from the 50 ml culture volume.

Protein	Form	Volume	Concentration (mg/ml)
GnTI	Insoluble	50 ml	0.37
GnTII	Insoluble	50 ml	0.41
GnTIVB	-	50 ml	-
GnTV	Insoluble	50 ml	0.47

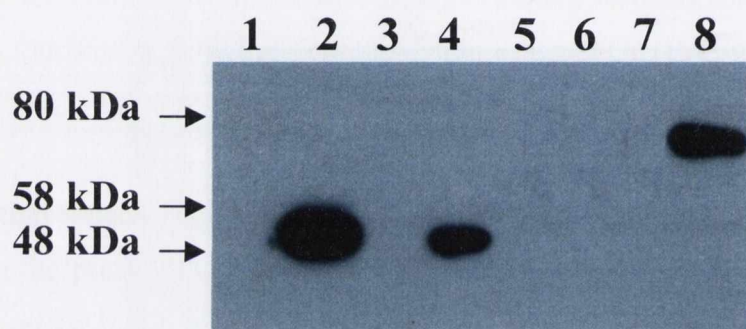


Figure 3.13 Detection of histidine tagged GnTs by Western blotting with anti-His monoclonal antibody.

Equal protein aliquots were mixed with 2X SDS sample buffer and boiled at 95°C for 5 min. GnT proteins were resolved on a 12% SDS-PAGE gel prior to transfer onto PVDF membranes as in Section 2.2.7 and 2.2.8. Blots were probed with mouse anti-His, 1:5000 and rabbit anti-mouse HRP, 1:5000. The SDS-PAGE gel was loaded with the following samples: **lane 1**, GnTI soluble fraction, **lane 2**, GnTI insoluble fraction; **lane 3**, GnTII soluble fraction; **lane 4**, GnTII insoluble fraction; **lane 5**, GnTIVB soluble fraction; **lane 6**, GnTIVB insoluble fraction, **lane 7**, GnTV soluble fraction; **lane 8**, GnTV insoluble fraction.

3.4.4 Expression of truncated GnTI, II, IVB and V from *E. coli*

All Golgi glycosyltransferases described to date have a single transmembrane domain flanked by a short amino-terminal domain and a longer carboxy-terminal domain. This structure is characteristic to so-called type II transmembrane proteins, whose single amino-terminal membrane-spanning domain functions as a signal-anchor sequence, placing the short amino-terminal segment within the cytosol while directing the larger carboxy-terminal domain to the Golgi lumen. The literature search showed very limited data of successfully expressed, active transmembrane proteins using procaryotic system. In most cases the overall expression was very low or the proteins were found exclusively in the insoluble fraction. Previous attempts to refold mammalian glycosyltransferases from insoluble inclusion bodies were also unsuccessful (Saribas *et al.*, 2007). Therefore, in order to generate soluble, possibly active forms of GnTs, the transmembrane domains were removed in the process called site-directed mutagenesis. Table 3.16 shows the alignment of transmembrane regions of GnTs based on computer predictions using the TMHMM Server v2.0. In each case, the new restriction site, either *NdeI* or *XhoI*, was introduced downstream to the transmembrane domain localised within the first 100 base pairs. The short DNA fragments were then removed from the newly formed plasmids and the sticky ends were religated to form circular constructs.

Each truncated GnT (Δ GnT) was expressed in *E.coli* from pET15b as described previously. Figure 3.14 and 3.15 represent the expression of each recombinant histidine-tagged GnT protein that was induced by IPTG for 2.5 h. Based on SDS-PAGE analysis histidine-tagged CHO Δ 31GnTI, Δ 31GnTII, Δ 31GnTIVB and Δ 37GnTV proteins were found in the insoluble inclusion bodies. The apparent molecular masses of polyhistidine-tagged truncated GnT proteins were in good agreement with the calculated molecular masses. Western blotting of purified inclusion bodies confirmed the presence of a histidine tail on each GnT protein (see Figure 3.16). Truncated GnTs were expressed overnight at 18°C and then purified from 1 L of bacterial supernatant by affinity chromatography using Ni Sepharose 6 Fast Flow resin as described previously. Analysis of the column eluate by SDS-PAGE showed that GnTII, GnTIVB and GnTV were not expressed in a soluble form and could not be detected by Western blotting with the anti-His monoclonal antibody. Unlike the other GnTs, truncated GnTI was expressed overnight at 18°C as described in Section 3.3.21 and 3.3.23, respectively. Analysis of the elution fractions by SDS-PAGE

showed that, following application of 250 mM imidazole buffer, $\Delta 31\text{GnTI}$ eluted from the purification column mostly in the first 6 ml (4 x 1.5 ml fractions) (see Figure 3.17).

GnT proteins were resolved by large SDS-PAGE and gel slices containing approximately 100 μg (6 x 100 μg each) were sent to CovalAb (Villeurbanne, France). The final yield of each GnT protein from the purification step is shown in Table 3.19.

Table 3.16 Localisation of transmembrane domains in GnT proteins in hamster, human, rat and mouse. Predictions are based on TMHMM v2.0.

GnTI

Species	Accession number	N-terminal protein sequence	TMD
<i>Cricetulus griseus</i>	AAC52872.1	MLKKQS AGLVLWGAILFVGWNAL LLFFFW TRPAPG...	7-29
<i>Homo sapiens</i>	AAA75523.1	MLKKQS AGLVLWGAILFVAWNAL LLFFFW TRPAPG...	7-29
<i>Mus musculus</i>	NP_034924.1	MLKKQT AGLVLWGAIIFVGWNAL LLFFFW TRPAPG...	7-29
<i>Rattus norvegicus</i>	AAH74010.1	MLKKQS AGLVLWGAIIFVGWNAL LLFFFW TRPAPG...	7-29

GnTII

Species	Accession number	N-terminal protein sequence	TMD
<i>Cricetulus griseus</i>	-	MRFRYIKRKVL ILTLVVAACGFV LWSSNGR QRKND...	12-29
<i>Homo sapiens</i>	AAH06390.1	MRFRYIKRKVL ILTLVVAACGFV LWSSNGR QRKNE...	12-29
<i>Mus musculus</i>	AAH10583.1	MRFRYIKRKVL ILTLVVAACGFV LWSSNGR QRKSD...	12-29
<i>Rattus norvegicus</i>	AAA86721.1	MRFRYIKRKVL ILTLVVAACGFV LWSSNGR QRKND...	12-29

GnTIVB

Species	Accession number	N-terminal protein sequence	TMD
<i>Cricetulus griseus</i>	-	MRLRNG TFLTLLLFCLCAFLSLS WYAALS GQKGD...	7-29
<i>Homo sapiens</i>	NM_014275	MRLRNG TFLTLLLFCLCAFLSLS WYAALS GQKGD...	7-29
<i>Mus musculus</i>	BAC55019.1	MRLRNG TFLTLLLFCLCAFLSLS WYAALS GQKGD...	7-29
<i>Rattus norvegicus</i>	NM_001127533.1	MRLRNG TFLTLLLFCLCAFLSLS WYAALS GQKGD...	7-29

GnTV

Species	Accession number	N-terminal protein sequence	TMD
<i>Cricetulus griseus</i>	U62587	MAFFT TPWKLSSQKLGFFLVTFGF IWGMMLLHFTIQ QRTQPESS	13-35
<i>Homo sapiens</i>	AAD22449.1	MAFFT TPWKLSSQKLGFFLVTFGF IWGMMLLHFTIQ QRTQPESS	13-35
<i>Mus musculus</i>	BAC87707.1	MITVNP DGKIMVRRCLVTLRPFR LFVLGIGFFTL CFLMT SLGG QSA RRLG	20-43
<i>Rattus norvegicus</i>	AAA41665.1	MAFF SPWKLSSQKLGFFLVTFGF IWGMMLLHFTIQ QRTQPESS	13-35

Table 3.17 Number of amino acids that were removed from the N-terminal sequence and molecular sizes of GnT proteins estimated by ExPASy ProtParam Tool as described in Section 3.3.1.

Gene	Full-length protein (kDa)*	Removed amino acids	Truncated protein (kDa)*
GnTI	53.9	31	50.5
GnTII	53.4	31	50.0
GnTIVB	65.5	31	62.1
GnTIV	86.7	37	82.3

* Final molecular weight of protein including a polyhistidine tag (~2.2 kDa)

A.



B.

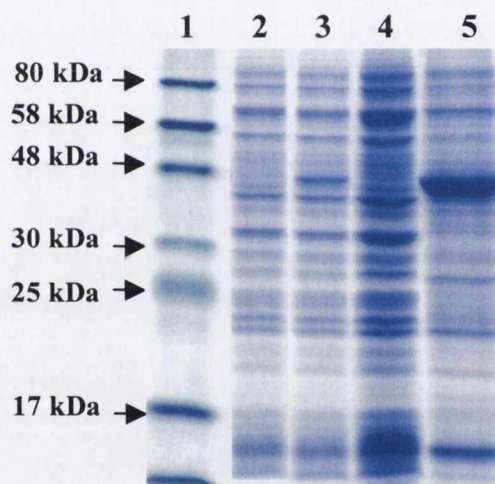
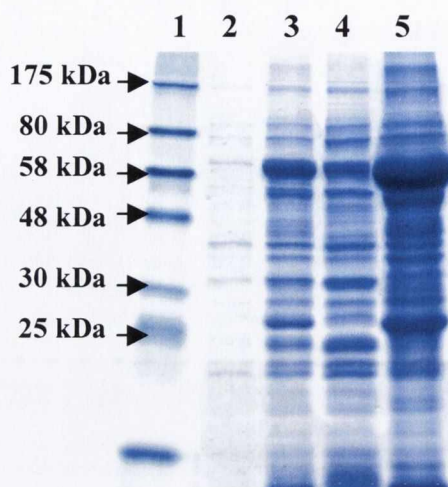


Figure 3.14 Heterologous expression of $\Delta 31\text{GnTI}$ and $\Delta 31\text{GnTII}$ proteins from BL21(DE3) *E. coli* cells.

Experiment details are described in Section 3.3.19. The aliquots of all protein fractions for $\Delta 31\text{GnTI}$ (A) and $\Delta 31\text{GnTII}$ (B) were mixed with an equal volume of 2X SDS sample buffer and heated immediately for 5 min at 95°C. The SDS-PAGE gels were loaded as with the following samples: **lane 1**, protein marker; **lane 2**, 5 μl of total cell protein fraction (TCP) taken just before IPTG induction; **lane 3**, 5 μl of total cell protein fraction (TCP) taken at time point 2.5 h after IPTG induction; **lane 4**, 5 μl of the soluble protein fraction taken at time point 2.5 h after IPTG induction; **lane 5**, 10 μl of the insoluble, partially purified protein fraction (inclusion bodies) taken at time point 2.5 h after IPTG induction.

A.



B.

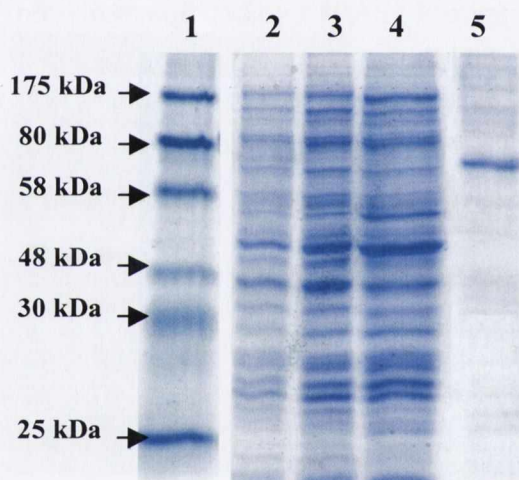


Figure 3.15 Heterologous expression of $\Delta 31\text{GnTIVB}$ and $\Delta 37\text{GnTV}$ from BL21(DE3) *E.coli* cells.

Experiment details are described in Section 3.3.19. The aliquots of all protein fractions for $\Delta 31\text{GnTIVB}$ (A) and $\Delta 37\text{GnTV}$ (B) were mixed with an equal volume of 2X SDS sample buffer and heated immediately for 5 min at 95°C. The SDS-PAGE gels were loaded with the following samples: **lane 1**, protein marker; **lane 2**, 5 μl of total cell protein fraction (TCP) taken just before IPTG induction; **lane 3**, 5 μl of total cell protein fraction (TCP) taken at time point 2.5 h after IPTG induction; **lane 4**, 5 μl of the soluble protein fraction taken at time point 2.5 h after IPTG induction; **lane 5**, 10 μl of the insoluble, partially purified protein fraction (inclusion bodies) taken at time point 2.5 h after IPTG induction.

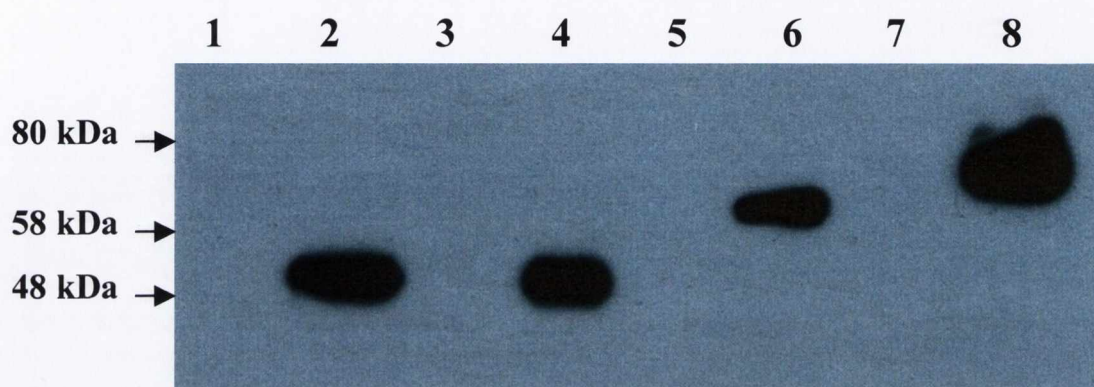


Figure 3.16 Detection of histidine tagged GnTs by Western blotting with anti-His monoclonal antibody.

Equal protein aliquots were mixed with 2X SDS sample buffer and boiled at 95°C for 5 min. GnT proteins were separated on a 12% SDS-PAGE gel prior to transfer onto PVDF membranes as in Section 2.2.7 and 2.2.8. Blots were probed with mouse anti-His, 1:5000 and rabbit anti-mouse HRP, 1:5000. The SDS-PAGE gel was loaded with the following samples: **lane 1**, GnTI soluble fraction, **lane 2**, GnTI insoluble fraction; **lane 3**, GnTII soluble fraction; **lane 4**, GnTII insoluble fraction; **lane 5**, GnTIVB soluble fraction; **lane 6**, GnTIVB insoluble fraction, **lane 7**, GnTV soluble fraction; **lane 8**, GnTV insoluble fraction.

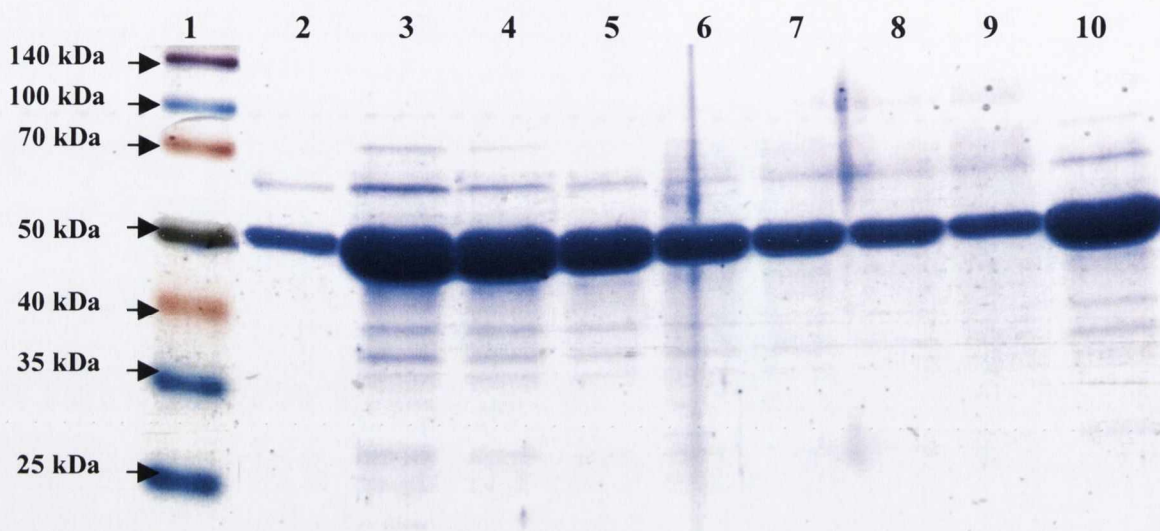


Figure 3.17 Purification profile of truncated GnTI from Ni Sepharose 6 Fast Flow.

Experimental procedure is described in Section 3.3.23. Aliquots from the elution fraction (10 μ l) were resuspended in an equal volume of 2 x LB and used for SDS-PAGE to determine the efficiency of the purification system. The SDS-PAGE was loaded with the following samples: **lane 1**, protein marker; **lanes 2-9**, elution fractions; **lane 10**, pooled fraction of $\Delta 31$ GnTI.

Table 3.18 Yield of GnTI from the purification process.

Purification step	Volume (ml)	Protein concentration (mg/ml)*	Total protein (mg)*
1.Supernatant	50	14.1	705
2. IMAC	10	0.43	4.3
3. Dialysis	10	0.21	2.1
4. Final	1	2.05	2.05

* These values represent the average value taken from two separate purifications.

Table 3.19 Final concentrations of GnTs used for generating antibodies.

Protein	Form	Volume	Concentration (mg/ml)
Δ 31GnTI	Soluble	3 L	2.05
Δ 31GnTII	Insoluble	100 ml	1.05
Δ 31GnTIVB	Insoluble	100 ml	1.45
Δ 37GnTV	Insoluble	100 ml	0.79

3.4.5 Generation of polyclonal antibodies against CHO GnT proteins.

Full-length GnTII and GnTV and truncated GnTI and GnTIVB were used to generate polyclonal antibodies raised in rabbits. Proteins were resolved by SDS-PAGE and gel slices containing approximately 100 µg each (600 µg total for each protein) were sent to CovalAb (France). IgG fractions against each CHO GnT was purified from rabbit antiserum by Protein A Sepharose chromatography. Fractions containing the antibody were pooled and the final concentration of immunopurified antibody was determined by reading the optical density at 280 nm. The final concentration of 100 ng/ml was considered a good yield of specific and immunoreactive antibody. Final yields of purified antibodies are represented in Table 3.20. Individual fractions were tested and titer evaluated by ELISA (see Table 3.21). Antibodies at different dilutions (from 1:500 to 1:64000) reacted with the purified antigen (10 µg/ml), coated on a 96 well microtiter plate. The pre-immunized rabbit serum was used as a negative control. The titer was determined by the lowest concentration of antibody giving the optical density reading at 450 nm ≥ 1 . Antibody titers for all GnT proteins were considered good and the approximate values were at least ≥ 4000 . The specificity of the purified anti-GnT polyclonal antibodies was further tested by Western blotting analysis.

Initially, the polyclonal anti-GnT antibodies were tested against purified antigen. Pre-immune serum was used as a negative control for purified antibodies and no reaction was observed (data not shown). Only GnTV antibody seemed not specific to its antigen giving very high background and resulting in the blot impossible to analyze. In the following step, the antibodies were verified against endogenous enzymes that are localised in the Golgi apparatus. CHO lysates were prepared from wild-type cells und run on the standard gel (10 µg) as described previously. CHO lysates were also run on a large gel which can hold up to 100 µg of protein in a single well. Western blotting analysis with polyclonal antibodies against CHO GnT proteins was completed with the purified antibody at a dilution range from 1:100 to 1:2000. Unfortunately, the signal was detected neither for the mini nor the large gel, indicating that the amount of GnT proteins in cells is very limited or the epitopes are not efficiently exposed. To verify this hypothesis, CHO GnT proteins were transiently overexpressed as fusion proteins with a C-terminal *myc*-epitope in wild-type CHO cells as described in Chapter 5. Western blotting analysis with polyclonal antibodies against CHO GnT proteins was completed with the purified antibody

at a dilution of 1:1000 and the results are represented in Figure 3.18A. Expected bands of ~50 kDa, ~50 kDa and ~64 kDa were detected for positive controls, GnTI, GnTII and GnTIVB, respectively. Bands of approximately 54 kDa, 55 kDa and 75 kDa were displayed in the CHO lysates harbouring *myc*-tagged GnT proteins. There was an additional band (~78 kDa) in the CHO lysate transfected with GnTIVB indicating non-specific binding of the anti-GnTIVB antibody. In order to verify the specific bands that appeared for each GnT protein in CHO lysates, the membrane was stripped and reprobed with an anti-*myc* monoclonal antibody at a dilution of 1:5000 and the specific bands were shown for *myc*-tagged GnT proteins (see Figure 3.18B) indicating that custom made anti-GnTI, II and IVB antibodies can serve as a good tool for further research.

Table 3.20 Affinity purification of anti-GnT antibodies.

Antigen	Total IgG	Immunopurified antibody
GnTI	1.728 mg/ml	100 µg/ml
GnTII	3.131 mg/ml	1.25 µg/ml
GnTIVB	2.849 µg/ml	630 ng/ml
GnTV	3.382 µg/ml	630 ng/ml

Table 3.21 Antiserum titer determination by ELISA.

Antigen	Day	Rabbit No.1	Rabbit No. 2	Immunoreactivity
GnTI	53	500	<500	Low
	74	<2000	2000	Low
	123	4000	4000	Good
GnTII	53	<500	<500	Non
	74	<500	<500	Non
	123	4000	2000	Good
GnTIVB	53	1000	500	Low
	74	500	<500	Low
	123	2000	8000	Good
GnTV	53	1000	2000	Low
	74	500	2000	Low
	123	4000	4000	Good

Interpretation of ELISA titer:

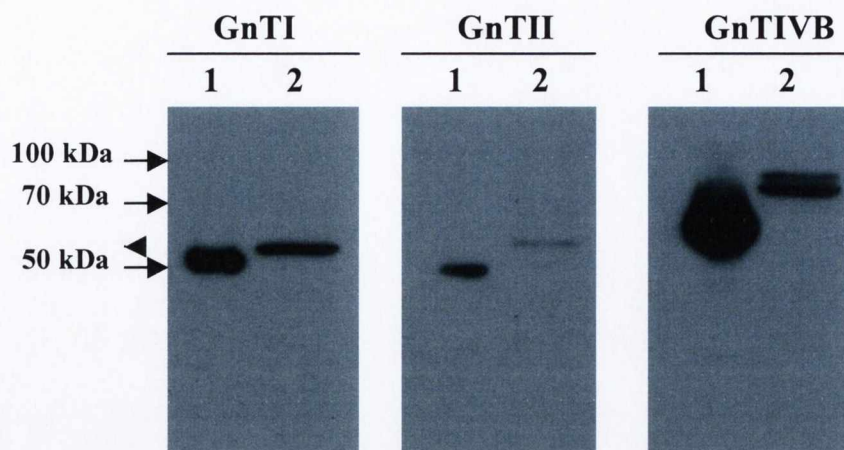
Titer < 500: non immunoreactivity

500 ≤ Titer < 2000: low immunoreactivity

2000 ≤ Titer < 8000: good immunoreactivity

Titer ≥ 8000: very good immunoreactivity

A.



B.

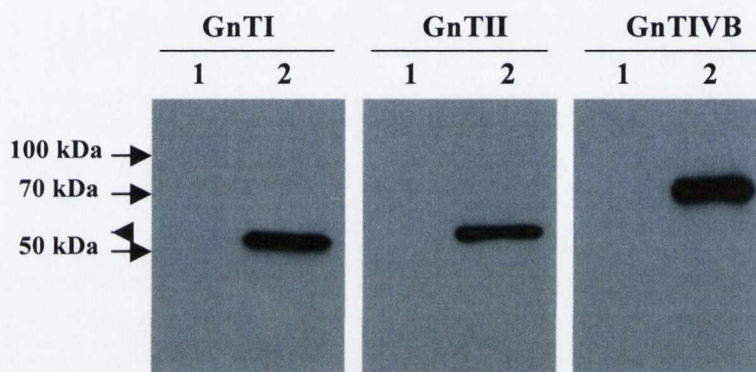


Figure 3.18 Validation of polyclonal antibodies specificity towards full-length and truncated GnT proteins.

Protein aliquots were mixed with 2X SDS sample buffer, boiled at 95°C for 5 min and were separated on a 12% SDS-PAGE gel prior to transfer onto PVDF membranes as in Section 2.2.7 and 2.2.8. Blots were probed initially with rabbit anti-GnTI, anti-GnTII and anti-GnTIVB at 1:1000 and goat anti-rabbit HRP, 1:2000 (A) then re-probed with anti-*myc* monoclonal antibody at 1:5000 and rabbit anti-mouse HRP, 1:10000 (B). The SDS-PAGE gel was loaded with the following samples: **GnTI**, lane 1, GnTI positive control (1 µg), lane 2, CHO lysate with overexpressed *myc*GnTI protein; **GnTII**, lane 1, GnTII positive control (1 µg), lane 2, CHO lysate with overexpressed *myc*GnTII protein; **GnTIVB**, lane 1, GnTIVB positive control (3 µg), lane 2, CHO lysate with overexpressed *myc*GnTIVB protein.

3.4.6 Generation of anti-peptide polyclonal antibodies

As an alternative to custom made polyclonal antibodies against full-length or truncated GnT proteins, anti-peptide polyclonal antibodies were generated by Genosphere Biotechnology (Paris, France).

When evaluating a protein sequence for antigenic determinants, it was critical to choose regions that are antigenic, hydrophilic, and with secondary structures that allows good solvent accessibility. In order to improve the chances of producing a successful antibodies pool, several target peptides were chosen within the same protein sequences. Each peptide was then synthesized, purified and conjugated to a large molecule called KLH. The resulting conjugate was used as immunogen. Individual antisera were tested and titer evaluated by ELISA against each peptide separately (see Table 3.22). Antibody was positive if absorbance was >2 folds over that of pre-immune serum. The final yields of specific IgG fraction purified by protein A chromatography are represented in Table 3.23.

Anti-peptide antibodies were tested against purified antigens – recombinant CHO GnTs, resulting in detection of GnTI and GnTII at ~50 kDa and ~50 kDa, respectively (see Figure 3.19). Unfortunately, those antibodies were unable to detect the endogenous or overexpressed GnT proteins from CHO cell lysates. Anti-GnTIVB and GnTV antibodies were unable to detect their antigens and were disregarded from further experiments.

Table 3.22 Antiserum titer determination by ELISA.

Coated Antigen	GnTI				
Antibody Dilution	Rabbit No.1		Rabbit No.2		
	Pre-immune	Bleed	Pre-immune	Bleed	
	1:1000	0.143	1.82	0.142	1.81
	1:10000	0.042	1.46	0.041	1.47
	1:100000	0.031	0.78	0.022	0.80
	GnTII				
	Rabbit No.1		Rabbit No.2		
	Pre-immune	Bleed	Pre-immune	Bleed	
	1:1000	0.151	1.85	0.154	1.84
	1:10000	0.043	1.48	0.043	1.45
	1:100000	0.013	0.81	0.033	0.78
		GnTIVB			
Rabbit No.1		Rabbit No.2			
Pre-immune		Bleed	Pre-immune	Bleed	
1:1000		0.152	1.82	0.155	1.86
1:10000		0.047	1.47	0.043	1.49
1:100000		0.035	0.80	0.031	0.84
		GnTV			
	Rabbit No.1		Rabbit No.2		
	Pre-immune	Bleed	Pre-immune	Bleed	
	1:1000	0.146	1.82	0.147	1.84
	1:10000	0.042	1.41	0.043	1.44
	1:100000	0.033	0.80	0.031	0.79

Interpretation of ELISA titer:

< 1:1000 = Low

1:1000 = Average

1:10000 = Good

1:10000 – 100000 = Excellent.

Table 3.23 Purification of peptide antibodies on Protein A completed in Genosphere Biotechnologies (Paris, France).

Antigen	Total IgG fraction in PBS (mg/ml)	
	Rabbit No 1	Rabbit No. 2
GnTI	9.1	9.0
GnTII	9.2	9.1
GnTIVB	8.4	8.5
GnTV	8.6	8.7

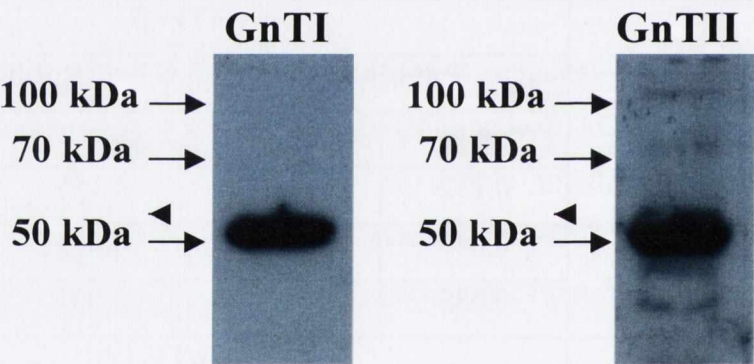


Figure 3.19 Western blotting of polyclonal anti-peptide antibodies against GnTI and GnTII.

Purified protein antigens (1 µg) were mixed with 2X SDS sample buffer, boiled at 95°C for 5 min and were separated on a 12% SDS-PAGE gel prior to transfer onto PVDF membranes as in Section 2.2.8. Blots were probed with rabbit anti-GnTI and anti-GnTII peptide antibodies at 1:1000 and goat anti-rabbit HRP, 1:2000. The SDS-PAGE gel was loaded with the following samples: **GnTI**, GnTI positive control (1 µg); **GnTII**, GnTII positive control (1 µg).

3.5 Discussion

3.5.1 Multiple sequence alignment

At the start of this project there was no sequence information about CHO GnTII and GnTIV. In addition, several publications report three isoforms of human, rat and mouse GnTIV, which indicates three distinctive features of the same enzyme (Oguri *et al.*, 2006; Takamatsu *et al.*, 2010). The sequence information of missing CHO GnTII and GnTIV isoforms were obtained using existing data due to the high sequence identity between enzymes from different species. According to Dr. O'Connor's (Trinity College Dublin) experiments no specific RT-PCR product was obtained for CHO GnTIVA or GnTIVC. GNTIVB seems to be widely expressed in normal human tissues, whereas GnTIVA and IVC are restricted to a few tissues, including thymus, pancreas, spleen, leukocytes and small intestine (Yoshida *et al.*, 1998; Oguri *et al.*, 2006; Ide *et al.*, 2006). Human and mouse GnTIVC sequences published on NCBI are described as “putative”. Rat GnTIVC is described as “predicted by automated computational analysis using the gene prediction method GNOMON”. Similarly to GnTIII, it is probable that GnTIVA and IVC are specifically expressed in other tissues and are not expressed in CHO cells (data not published).

GnTV has also been reported to have two isoforms (Kaneko *et al.*, 2003; Inamori *et al.*, 2003) and its expression has been reported in most human and rodent tissues while GnTVB (IX) expression is limited mainly to neural tissue and testes. Both enzymes show high degrees of amino acid sequence identity and similarity (about 41% identical and 53% similar), particularly in their catalytic regions. However, the catalytic activities they encode are significantly different (Alvarez-Manilla *et al.*, 2009).

To summarize, high sequence identity between CHO GnT genes and other species including human, mouse and rat ($\geq 84\%$) indicates the fact that these enzymes play a fundamental role in cell metabolism and therefore have been highly conserved through the evolution. However the low sequence identity of GnT genes between each other ($\leq 6\%$) is evidence of distinctive enzymatic features and specificities.

3.5.2 Molecular cloning and expression of CHO GnT cDNAs

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. Since no single set of conditions can be applied to all PCR amplifications, the concentrations of reaction components and the time and temperature parameters had to be optimized for efficient amplification of specific GnT gene sequences. Lack of knowledge on the molecular cloning of glycosyltransferases may be explained by the fact that these enzymes are difficult to express in a fully active form. Also, the purification step may be a challenge for those who need quantities sufficient to employ reliable functional assays. *Escherichia coli* (*E. coli*) is one of the most widely used hosts for the production of heterologous protein. It has the ability to grow rapidly at high densities and has been well characterized genetically. Although there are many advantages to using this system, there is no guarantee that a recombinant protein will accumulate in *E. coli* at high levels in a full and active form. Previous attempts to express and purify mammalian GnTs have been reported (Nishiu *et al.*, 1995; Fujiyama *et al.*, 2001). However, the expression of foreign proteins in *E. coli*, in most cases, results in the formation of cytoplasmic granules or inclusion bodies composed of insoluble protein aggregates (Swartz, 2001; Jana and Deb, 2005, Ventura and Villaverde, 2006).

In the preliminary experiments polyhistidine-tagged, full-length CHO GnTI, GnTII, GnTIVB and GnTV were introduced into BL21(DE3) *E. coli* cells and expressed at 37°C to verify the solubility. Recombinant CHO GnTI, GnTII and GnTV were highly and rapidly expressed in *E. coli* cells, whereas polyhistidine-tagged GnTIVB was not detected by standard staining methods or Western blotting. In order to increase the solubility of GnTs, different types of *E. coli* strains (RIL and GroEL), media (LB, M9), IPTG concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) and different temperatures (20, 25, 30, and 37 °C) were tried. Unfortunately, none of these methods improved the final solubility and heterogously expressed GnTs were still found in the inclusion bodies.

GnTs are transmembrane proteins, which have their short, N-terminal tail facing the cytoplasm and large catalytic domain localised in the Golgi lumen. The single transmembrane domain, which is anchored in the Golgi membrane is not considered an antigenic determinant and can be removed from the protein sequence along with the short tail, leaving the entire catalytic part. The expression and purification of truncated

glycosyltransferases has been previously reported (Wu *et al.*, 2007; Zhang *et al.*, 2009; Skretas *et al.*, 2009; Nishiu *et al.*, 1995; Saribas *et al.*, 2007; Fujiyama *et al.*, 2001). The most efficient method of changing or removing unwanted fragments of DNA is the site-directed mutagenesis. In this procedure, the DNA fragment is altered by introducing mutations to the original sequence via primers using a simple PCR. In this case, the idea was to introduce a new restriction site (*NdeI* or *XhoI*) downstream to the transmembrane domain which was localised by completing computer predictions and comparison between species including *Cricetulus griseus*, *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*. Due to high sequence identity of GnTs, the transmembrane domains were predicted to be localised in the same regions with one exception for GnTV in a mouse sequence. The new restriction sites were introduced at 31st amino acid for GnTI, II and IVB and at 37th amino acid for GnTV.

The truncation of the membrane spanning region allowed for high-level expression of GnTIVB. Unfortunately, only GnTI was expressed in a soluble form, which can be associated with the fact that this enzyme is lacking potential glycosylation sites. Post-translational modifications of proteins are required for proper folding and activity. Therefore GnTII, IVB and V, which are heavily glycosylated, are accumulated in insoluble inclusion bodies. Although the formation of these structures in a cell can be used as a means to purify the active form of a protein, it may not always result in high yields (Singh and Panda, 2005).

3.5.3 Purification of CHO GnTs

Recombinant CHO GnTI was the only protein expressed in a soluble form and could be further purified on the ion-metal affinity column. During the initial cell lysis, the *E. coli* cell pellets were resuspended in buffer containing 0.1% Igepal, thawed slowly at room temperature, and sonicated before centrifugation step. This method was modified to maximise the percentage of cell lysis, thereby increasing the amount of cytosolic protein present in the lysate. It was found that the following alternative steps increased cell lysis by enhancing cell membrane damage: the addition of lysozyme to the buffer, rapid thawing at 37°C and sonication in small aliquots. Additionally, cell lysate was centrifuged to increase separation of soluble GnTI protein from cell debris and resulted in a more homogeneous supernatant that was loaded onto an affinity column. SDS-PAGE showed that an increasing imidazole concentration effectively washed protein impurities off the column,

leaving GnTI protein still bound. Typically, GnTI protein was found to elute predominantly in 250 mM imidazole. However, it was necessary to include the 50 mM and 100 mM washes to elute any remaining protein impurities before eluting the more specifically, tightly bound GnTI protein.

Since inclusion bodies are considered relatively pure (>90%), recombinant CHO GnTII, IVB and V were purified from cell lysates by high-speed centrifugation and several washing steps with detergent-containing buffers accompanied by sonication.

3.5.4 Generation of polyclonal antibodies against CHO GnTs

Polyclonal antibodies were generated against recombinant CHO GnTs recovered from SDS-PAGE gel slices, containing approximately 100 µg of protein each per injection. The antibody titers for all GnTs were considered good and the approximate values were ≥ 4000 . The specificity of antibodies was further tested by Western blotting and gave positive signal for purified GnTI, II and IVB, and also for *myc*-tagged GnTs that were overexpressed in CHO cells. However antibodies were not successful in recognizing endogenous GnTs from CHO cell lysates probably due to their low expression levels.

The polyclonal anti-GnTV antibody was disregarded as it appeared to be not specific to its antigen giving very high background despite numerous attempts to optimize the Western blotting procedure.

3.5.5 Generation of anti-peptide antibodies against CHO GnTs

Generation of anti-peptide antibodies against CHO GnT proteins was an alternative to custom made polyclonal antibodies against full-length or truncated proteins. There are many advantages of using peptides over whole proteins for antibody production including the fact that only the amino acid sequence is necessary not the purified antigen. It eliminates or minimizes potential cross-reactivity between structurally homologous proteins and also enables the production of different antibodies for different epitopes of the same protein. Peptides can be conveniently purified on affinity column and, most importantly, the procedure is time-saving and cost-effective. Highly-specific anti-peptide antibody can serve as a good tool for further research.

There are several rules for selecting peptide sequences for chemical synthesis. Firstly, the peptide length should be between 10-20 residues, preferably around 15 residues. Secondly, most antigenic sequences are long stretches of hydrophilic residues that are more likely to reside on the surface of native proteins. Additionally, the inclusion of unstable amino acids or peptide sequences (Cys, Asp-Gly, Asn-Gly, Asp-Pro, N-terminal Gln, or N-terminal Asn) should be minimized, if not totally eliminated. Thirdly, common sequence motifs such as the helix-loop-helix and sequences that are associated with some biological activities should be avoided. Finally, single peptides are conjugated to a larger protein carrier, KLH, since they are too small to elicit a satisfactory immune response in animals. In addition, it is critical for further successful immunization that the peptide-hapten to protein-carrier molar ratio is very high, and that all epitopes on the peptides are properly oriented in order to induce high titer antibodies immune response with fine-tuned specificities.

In order to improve the chances of producing a successful antibodies pool, several target peptides were chosen within the same protein sequence. Having several epitopes derived from a single protein mathematically increases the chances of obtaining a pool of antibodies that will recognize the target protein. Unfortunately, despite numerous attempts the anti-peptide antibodies failed to recognize endogenous or *myc*-tagged GnTs that were overexpressed in CHO lysates.

3.5.6 Summary

Ideally, generation of antibodies should be based on the endogenous, native forms of antigen, purified from the tissues or cells. Such approach guarantees fully folded and post-translationally modified protein. However, purification procedures often require time-consuming optimisation and the final protein recovery may be very low thus preventing further antibody generation. With the advent of molecular cloning and heterologous expression the number of specific antibodies increased significantly thanks to the large-scale production of recombinant proteins. There are two main systems used for expression of fully or partially post-translationally modified proteins, mammalian or insect cells. Despite numerous advantages of these systems, the final yield of highly purified protein may be very low. In contrast to previous expression systems, *E.coli* cells offer high level production of proteins from various sources. Unfortunately, bacterial cells lack the post-

translation modification machinery and many heterologously expressed proteins usually form misfolded aggregates, whose epitopes are not effectively exposed to the immune system.

The advantage of full-length over anti-peptide polyclonal antibodies in the case of CHO GnTs was demonstrated initially by Western blotting using GnT antigens as a positive control. There might be a couple of reasons why the anti-peptide antibodies do not recognize their antigens. Firstly, the synthetic peptides sequences that were used for generation of polyclonal antibodies may not be part of an epitope. Secondly, most protein epitopes are composed of different parts of the polypeptide chain that are brought into spatial proximity by the folding of the protein. These epitopes are called discontinuous and comprise >90% of all epitopes. The linear or continuous epitopes are the remaining 10% and, in this case, corresponding antibodies cross-react with a linear peptide fragment that is mainly composed of a single stretch of the polypeptide chain (Larsen *et al.*, 2006; Andersen *et al.*, 2006).

The chances for mammalian enzymes of having continuous epitopes are very limited. Most of these proteins are post-translationally modified including glycosylation, phosphorylation, acetylation etc. that have a direct impact on folding and activity. So far, the most informative and accurate method for identification of discontinuous epitopes is determination of antigen-antibody interaction by X-ray crystallography with the three-dimensional structure. Unfortunately, the data on such interactions is much reduced due to still limited number of known crystallographic structures. Even though, the linear epitopes are of limited relevance, identification of such linear fragments may be the initial step in the search for antigenic determinants.

CHAPTER 4

Determination of kinetic parameters of recombinant CHO GlcNAc transferase I (GnTI)

4.1 Introduction

GlcNAc transferase I (GnTI; EC 2.4.1.101) belongs to the family of inverting enzymes of the GT-A fold that transfers the first GlcNAc residue from UDP-GlcNAc to the oligomannose core structure and plays an important role in the biosynthesis of hybrid and complex-type chains (Lairson *et al.*, 2008, Kornfeld and Kornfeld, 1985; Sarkar *et al.*, 1991). With regard to kinetic properties, experiments have shown that GnTI follows a sequential *Bi Bi* kinetic mechanism, where the enzyme first binds to both metal cofactor and UDP-GlcNAc and subsequently to the oligosaccharide acceptor. The product is then released, followed by UDP (Kozmon and Tvaroška, 2006).

In general, the activity of GnTs has been previously determined by employing several methods based on different labelling, purification and detection techniques, including radioactive or fluorescent labelled substrates (Chen *et al.*, 2001 and Oguri *et al.*, 2006). In these assays, product formation was quantitated by measuring the amount of transfer after product isolation, usually by chromatographic methods. Immunologically based assays have also been reported (Crawley *et al.*, 1990). All the above methods, although very sensitive, require sophisticated and expensive materials and equipment and the methods involve time-consuming steps to separate unreacted products. Also, none of these methods allow for the continuous measurement of enzyme activity.

Like many other enzymes, GnTI was directly assayed from the cell extracts as described by Chen *et al.* in 2001 with the use of radiolabeled sugar nucleotides and range of natural or synthetic acceptors. GnTI was also purified from different organs, e.g. liver and further analyzed for activity towards oligosaccharide from hen ovalbumin and radioactive sugar nucleotide (Schachter *et al.*, 1989 and 2003). Some groups report functional assays of recombinant human, rat or *Nicotiana tabacum* GnTI expressed as a fusion protein from *E.coli* using pyramidalated (PA) sugar acceptors. The products of such reactions are further identified by HPLC (Nishiu *et al.*, 1995; Fujiyama *et al.*, 2001; Dohi *et al.*, 2010). The baculovirus and mammalian expression systems have also been successfully used for producing fully active GnTI (Sarkar *et al.*, 1987; Roni *et al.*, 2006).

In the spectrophotometric coupled assay used to determine kinetic constants of CHO GnTI (see Figure 4.1), chicken egg albumin (ovalbumin) was an acceptor molecule. Ovalbumin

is the main secretory phosphorylated-glycoprotein found in egg white, making up to 60-65% of the total protein and its function is still unknown (Huntington and Stein, 2001). Ovalbumin has a single glycosylation site at ²⁹²Asn with a second potential glycosylation at ³¹⁷⁻³¹⁹Asn. The carbohydrate chain is heterogeneous but different glycopeptides share a common core structure of high-mannose-type (Tai *et al.*, 1999). Ovalbumin has also two potential phosphorylation sites at ⁶⁹Ser and ³⁴⁵Ser. The carbohydrate and phosphate portions account for an additional weight, giving a total molecular size of approximately 45 kDa (Tai *et al.*, 1977). Carbohydrates of high-mannose type attached to ovalbumin are the most physiological acceptors in the GnTI-catalyzed reaction and usually have the highest $V_{\max}/K_m$ ratio as described previously by Schachter *et al.* in 2003. Synthetic analogs of Man₃-R (e.g. Man₃-octyl) are also highly effective, which indicates that for full enzyme activity, the protein moiety is not essential. Uridine diphosphate *N*-acetylglucosamine or UDP-GlcNAc (see Figure 4.2) is a nucleotide sugar used by GnTI to transfer *N*-acetylglucosamine residues to core mannoses attached to ovalbumin.

Two more enzymes taking part in the coupled enzyme assay are pyruvate kinase (PK; EC 2.7.1.40) and lactate dehydrogenase (LDH; EC 1.1.1.27). PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to UDP, yielding one molecule of pyruvate and one molecule of UTP. This process also requires a manganese ion (Mn²⁺). LDH catalyzes the interconversion of pyruvate to lactate with accompanying interconversion of β -NADH to β -NAD⁺ (see Figure 4.3). β -NADH is a reduced form of pyridine nucleotide and biologically active form of nicotinic acid that absorbs strongly at 340 nm unlike β -NAD⁺. In this case, the decrease in absorbance at 340 nm is the basis for measurement of the enzyme activity.

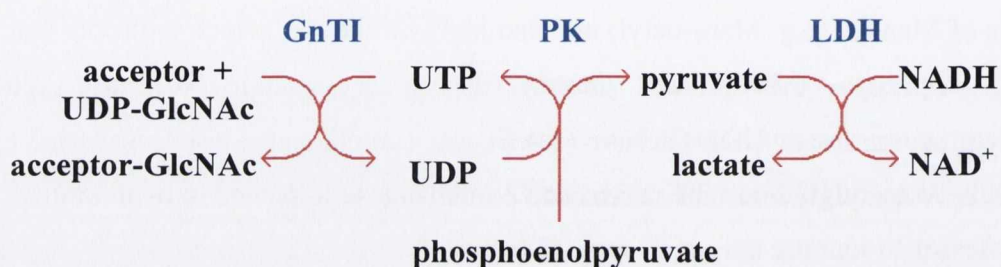


Figure 4.1 Spectrophotometric coupled assay.

In the spectrophotometric coupled assay, the UDP produced by this enzyme is coupled to NADH oxidation to NAD⁺ via pyruvate kinase (PK) and lactate dehydrogenase (LDH). The NAD⁺ is photometrically measured at 340 nm acceptor – high-mannose-type glycan (ovalbumin); donor, UDP-GlcNAc; GnTI, GlcNAc transferase I; PK, pyruvate kinase; LDH, lactate dehydrogenase.

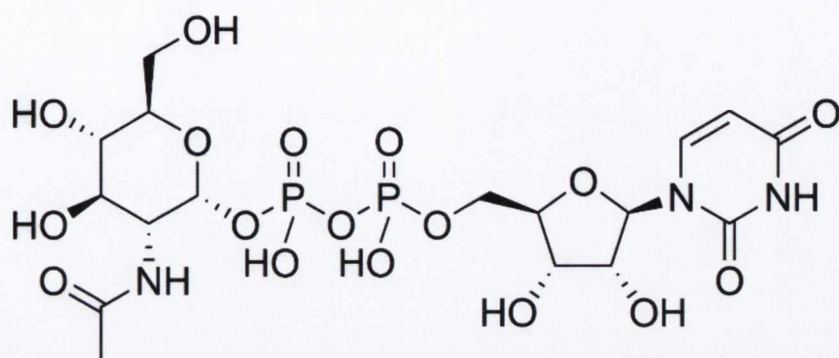


Figure 4.2 Chemical structure of UDP-GlcNAc.

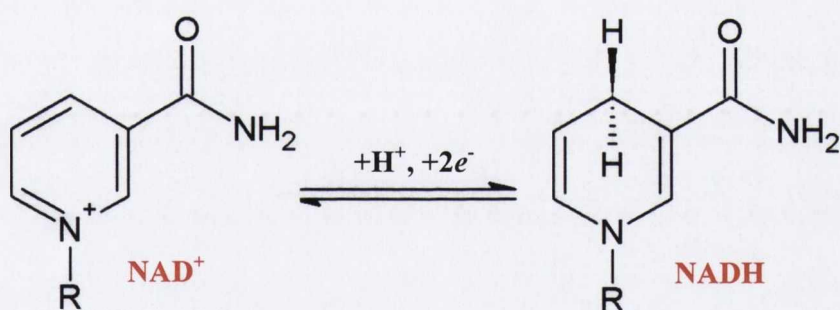


Figure 4.3 The redox reaction of nicotinamide adenine dinucleotide.

4.2 Aim of Chapter

The aim of this chapter was to obtain kinetic data for recombinant CHO GnTI that was previously cloned, expressed and purified from *E.coli* bacterial cells. GnTI was analyzed for its activity using a spectrophotometric coupled assay, in which UDP produced by GnTI was processed by pyruvate kinase (PK) and the product of PK, pyruvate was reduced by lactate dehydrogenase (LDH) to release NAD^+ from NADH, resulting in the decrease in absorbance at 340 nm. Kinetic constants, K_m and $V_{\max}$ of recombinant CHO GnTI were determined using the SoftMax Pro 4.8 (Version 3.13) software and Prism (Version 4.0), which employed the standard Michaelis-Menten equation represented below:

$$v_o = \frac{V_{\max} [S]}{K_m + [S]}$$

In the equation, v_o is the initial velocity of reaction, $V_{\max}$ is the maximal velocity at saturation, K_m is the Michaelis constant, and $[S]$ is the substrate concentration. The Michaelis constant (K_m) is the substrate concentration required to reach half-maximal velocity ($V_{\max}/2$).

Additionally, the effects of pH, temperature, MnCl_2 concentration and the concentration of other divalent cations (Co^{2+} , Mg^{2+} , Ca^{2+} and Zn^{2+}) on the activity of recombinant CHO GnTI were investigated under standard reaction conditions.

4.3 Methods

4.3.1 Expression and affinity purification of recombinant CHO GnTI

Recombinant CHO GnTI lacking 31 amino acids from the N-terminus was expressed and purified by a combination of ion-metal affinity chromatography as described in Sections 3.2.21 and 3.2.23, followed by ion exchange chromatography on the Äkta FPLC System from an equivalent of 3 L of bacterial culture.

4.3.2 Purification of recombinant CHO GnTI by ion-exchange chromatography

The enzyme-containing eluate from the affinity purification step was pooled to give a final volume of 10 ml and dialyzed at 4°C against three changes of low-salt ion-exchange buffer (20 mM MES, pH 6.0, 10 mM NaCl, 1 mM DTT) over a period of 18 h. The protein sample was centrifuged (10,000 g, 1 min, 4°C) in order to remove insoluble content that may appear after dialysis. Low-salt and high-salt ion exchange buffers were filtered under vacuum through 0.45 µm Nalgene filter disc, degassed and stored at 4°C until required. The dialysed protein was applied onto a Mono S™ 5/50 GL Tricorn™ high performance column that was pre-equilibrated with low-salt buffer until baseline, pH and conductivity were stable. The recommended flow rate was 1 ml/min. The elution begun using a gradient of an increasing ionic strength up to 0.5 M NaCl buffer and the protein was eluted with high-salt buffer (20 mM MES, pH 6.0, 1 M NaCl and 1 mM DTT). The protein-containing elution fractions were pooled, adjusted to 2.5 ml and applied onto a pre-washed PD10 column. GnTI was eluted with 3.5 ml of elution buffer (20 mM MES, pH 6.5, 150 mM NaCl) and concentrated using a 15 ml Amicon concentrating spin columns (15k MWCO, 2,000 g, 30 min, 4°C). The protein content was determined by the BCA protein assay as described in Section 2.7. Typically, 1.5 mg of purified recombinant CHO GnTI was obtained from 3 L of bacterial culture. Glycerol was added to the suspension to give a final concentration of 15% (v/v), in order to stabilise the protein. Recombinant CHO GnTI was stored at -70°C until required for characterisation of catalytic activity.

4.3.3 Spectrophotometric coupled assay for assaying GnTI

Kinetic studies were performed using a continuous coupled assay similar to that described by Gosselin *et al.* in 1994, in which UDP release is coupled to the oxidation of NADH

($\lambda=340\text{ nm}$, $\epsilon=6.22\text{ mM}^{-1}\text{ cm}^{-1}$). The reaction was carried out in a 96-well format and the results were taken in triplicates. Standard reaction conditions were as follows: 100 mM HEPES (pH 7.5), 0.1% (w/v) bovine serum albumin, 50 mM KCl, 10 mM MnCl_2 , 0.7 mM phosphoenolpyruvate (PEP), 0.25 mM NADH, 6 units of pyruvate kinase (PK), and 10 units of lactate dehydrogenase (LDH). The buffer components were added as a 10-fold concentrated buffer solution. The concentrations of UDP-GlcNAc and ovalbumin added depended upon the experiment and ranged from 0.03125 mM or 7.05 μM to 1 mM or 225.6 μM , respectively. Reactions were initiated by the addition of GnTI (0.025 $\mu\text{g}/\mu\text{l}$ final concentration), bringing the final volume to 150 μl . The change in the absorbance at 340 nm (37°C) was monitored for 30 min using a microplate reader. Kinetic parameters of recombinant CHO GnTI for UDP-GlcNAc and ovalbumin were determined from the initial reaction velocities obtained at five different substrate concentrations. The Prism Software (Version 4.0) was used to determine K_m and $V_{\max}$ from the above values and standard Michaelis-Menten and Lineweaver–Burk plots were generated.

4.3.4 Determination of the effect of pH on enzyme activity

Enzyme activity was determined at pH values varying between 5.5 and 10.5. The buffer solutions (100 mM) used were sodium acetate for pH of 5.5, MES for pH of 6.5, HEPES for 7.5, Tris for pH of 8.5 and glycine-NaOH buffer for pH of 10.5. Reactions were carried out in the standard reaction mixture for 30 min at 37°C and the relative activity (%) was determined.

4.3.5 Determination of the effect of temperature on enzyme activity

The effect of temperature on enzyme activity was examined under standard assay conditions at 10, 20, 30, 37 and 40°C and the decrease in the absorbance at 340 nm was recorded after 30 min.

4.3.6 Varying MnCl_2 concentration and its effect on enzyme activity

The effect of MnCl_2 concentration on enzyme activity was carried in a standard reaction mixture containing 5 mM up to 100 mM of MnCl_2 . Reactions were carried in the standard reaction mixture for 30 min at 37°C and the relative activity (%) was determined.

4.3.7 Effect of different metal ions on enzyme activity

Divalent metal ions, including MnCl_2 , CoCl_2 , MgCl_2 , CaCl_2 , and ZnCl_2 were used in a concentration of 10 mM. Reactions were carried in the standard reaction mixture for 30 min at 37°C and the relative activity (%) was determined.

4.3.8 Standard curve for NADH

The standard curve for β -NADH was performed in order to ensure reproducible data for all spectrophotometric coupled assays. Serial dilution of β -NADH (0 – 1 mM) was prepared in 100 mM HEPES, pH 7.5 in a total volume of 150 μl (see Figure 4.4). The absorbance (A) was measured at 340 nm using a standard microplate reader and the concentration of β -NADH was recalculated using the Lambert-Beer formula below:

$$A = \epsilon \times c \times l$$

where, **A** is absorbance, ϵ denotes a molar extinction coefficient for β -NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), **c** is the final concentration of β -NADH (M) and **l** is the path length (cm).

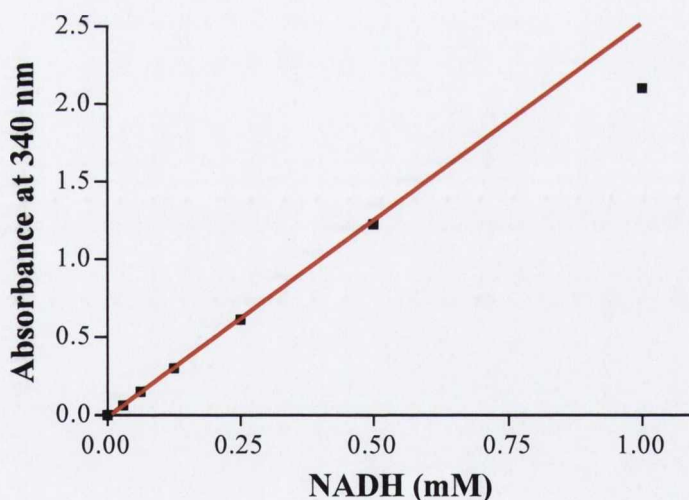


Figure 4.4 Standard curve of β -NADH at 340 nm.

The absorbance was recorded for the concentration ranging from 0 mM to 1 mM as described in Section 4.3.8. Absorbance values over 2.00 are considered inaccurate and were not taken into account when constructing a standard curve ($r^2 = 0.997$). Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol.

4.3.9 Statistical analysis and relative activity calculations

All statistical analyses were done using the Prism 4 (Version 4.0) package. The error bars represent a standard error of the mean (SEM). Statistical analysis was completed by the one-way ANOVA followed by post-hoc Bonferroni analysis. A value of at least $p < 0.05$ was considered statistically significant.

Relative activities were calculated as values obtained from the single experiment divided by the maximum value in the standard reaction mixture and are given as a percentage.

4.4 Results

4.4.1 Expression and purification of recombinant CHO GnTI

The soluble and functional recombinant CHO GnTI was constructed as a truncated, fusion protein lacking 31 amino acids from the N-terminus. The enzyme was initially purified by the affinity chromatography (immobilised-ion metal affinity chromatography; IMAC) on Ni Sepharose 6 Fast Flow resin and the partially purified GnTI of the apparent molecular weight of 50.5 kDa was pooled, dialyzed against the low salt buffer and subjected to the ion-exchange chromatography (IEX). CHO GnTI bound to the Mono S™ 5/50 GL Tricorn™ cation-exchanger was washed with the increasing concentration of NaCl up to 0.5 M as described in the Section 4.3.2. The protein yield from each purification step and the SDS-PAGE gel of purified CHO GnTI are shown in Table 4.1 and Figure 4.5, respectively.

Table 4.1 Yield of recombinant GnTI from the purification process.

Purification step	Volume (ml)	Protein concentration (mg/ml)*	Total protein (mg)*
1. Supernatant	50	14.1	705
2. IMAC	10	0.43	4.3
3. Dialysis	10	0.21	2.1
4. IEX	3	0.62	1.86
5. Final	1	1.58	1.58

*These values represent the average value taken from two separate purifications.

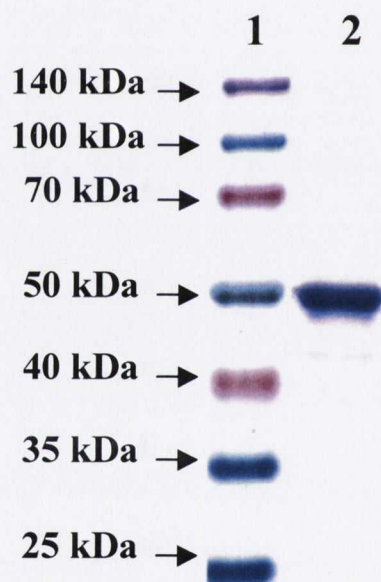


Figure 4.5 Resolution of recombinant CHO GnTI on SDS-PAGE gel.

GnTI was purified by a combination of affinity and ion-exchange chromatography. Experiment details are described in Section 3.2.22 and 3.2.23. The aliquot of protein sample was mixed with an equal volume of 2X SDS sample buffer and heated immediately for 5 min at 95°C. The SDS-PAGE gel was loaded with the following samples: **lane 1**, protein marker; **lane 2**, purified CHO GnTI.

4.4.2 A continuous spectrophotometric enzyme assay for GnTI activity

A continuous spectrophotometric assay for human and rabbit GlcNAc transferase I has been previously reported by Gosselin *et al.* in 1993 and Gordon *et al.* in 2006, respectively. Both publications report the enzyme activity towards the same acceptor molecule, Man₃-octyl, and both groups used similar reaction conditions. The same conditions were found to be optimal for recombinant CHO GnTI and the method was valuable to determine the kinetic constants.

Before carrying out the kinetic study using the coupled enzyme assay, it was necessary to determine the optimum amount of functional CHO GnTI. The relationship between the increasing amount of GnTI and the change in the absorbance at 340 nm per min, namely v ($\Delta\text{Abs}/\text{min}$) is shown in Figure 4.6. The linearity was retained in the concentration ranging from 1 μg to 5 μg per reaction. At higher concentrations, >10 μg , the reaction rate did not increase linearly, probably due to overloading of the enzyme. Additionally, in the assay performed under standard conditions described in Section 4.3.3, the absorbance at 340 nm decreased linearly over a period of approximately 30 min. After this time the reaction stabilized with no further change in the absorbance, therefore the 30 min time was applied in all assays.

It was important to verify the critical components of the reaction mixture, including MnCl_2 , phosphoenolpyruvate, pyruvate kinase (PK), lactate dehydrogenase (LDH), both added in excess, UDP-GlcNAc, ovalbumin and GnTI. MnCl_2 is essential for proper functioning of GnTI and pyruvate kinase. Phosphoenolpyruvate serves as a substrate for pyruvate kinase and UDP-GlcNAc with ovalbumin are substrates of GnTI. The reaction rates were measured under standard reaction conditions with all the components except for one from the “critical” group. Control assays showed no significant change in the absorbance at 340 nm indicating that GnTI is required to initiate the process (see Figure 4.7).

The kinetic parameters of GnTI for UDP-GlcNAc and ovalbumin were determined under varying concentrations of these in the standard reaction mixture. Substrate concentrations ranged from 31.2 μM to 1 mM for UDP-GlcNAc and from 7.05 μM to 225.6 μM for ovalbumin. Next, the rates were calculated by employing the SoftMax Pro 4.8 (Version

3.13) software from the initial reaction velocities obtained at five different substrate concentrations. Kinetic parameters of both substrates are represented as Michaelis-Menten, and Lineweaver-Burk plots (see Figures 4.8 and 4.9). From the double-reciprocal (Lineweaver-Burk) plot, the K_m values for UDP-GlcNAc and ovalbumin were calculated to be $81.06 \mu\text{M} \pm 5.04$ and $154.3 \mu\text{M} \pm 16.1$, respectively, indicating that GnTI has different affinity to them. Maximal velocities (V_{max}) for both UDP-GlcNAc and ovalbumin were calculated as $14.62 \mu\text{mole/min} \pm 0.24$ and $23.04 \text{ nmole/min} \pm 1.3$, respectively.

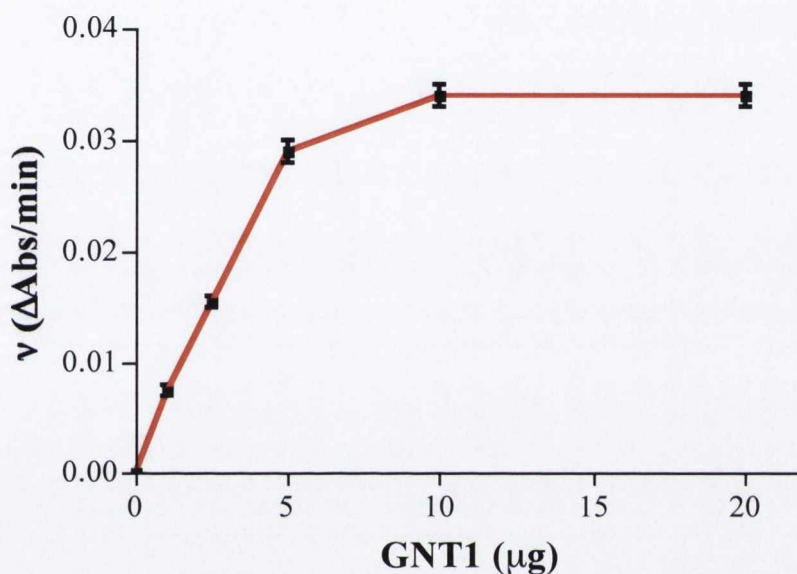


Figure 4.6 The relationship between the amount of GnTI and the reaction rate – v ($\Delta\text{Abs}/\text{min}$).

The assay was performed under varying concentrations of GnTI ranging from 0 μg to 20 μg in the standard reaction mixture. Experimental details are described in Section 4.3.3. Results are expressed as the mean $\pm$ SEM of the representative experiment performed in triplicate. At points where no error bar is shown, the SEM was within the size of the symbol.

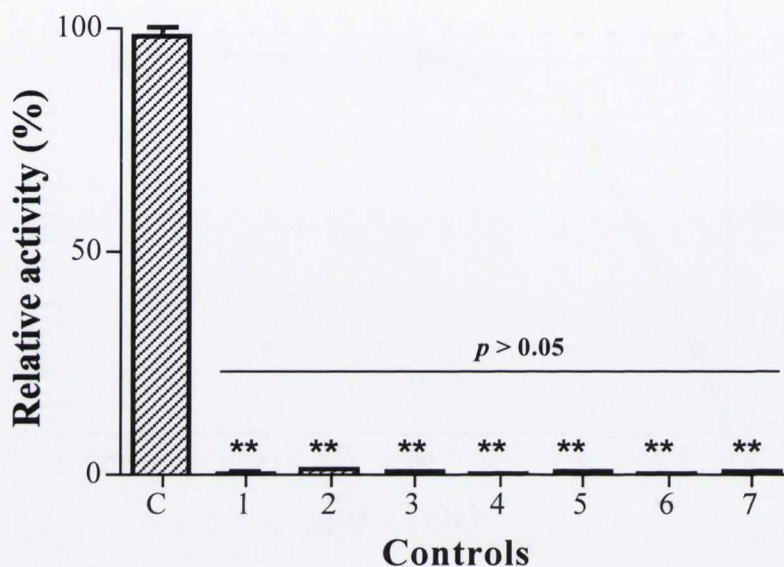


Figure 4.7 A set of controls in the assay for measuring activity of CHO GnTI.

The activity of GnTI in the control sample (C) is considered 100% whereas enzymatic activities of GnTI in samples (1) – MnCl_2 , (2) – PEP, (3) – PK, (4) – LDH, (5) – UDP-GlcNAc, (6) – ovalbumin are represented as relative activities in %. The sample denoted as (7), which lacks GnTI is treated as a negative control. Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was completed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism 4; ** = $p < 0.01$.

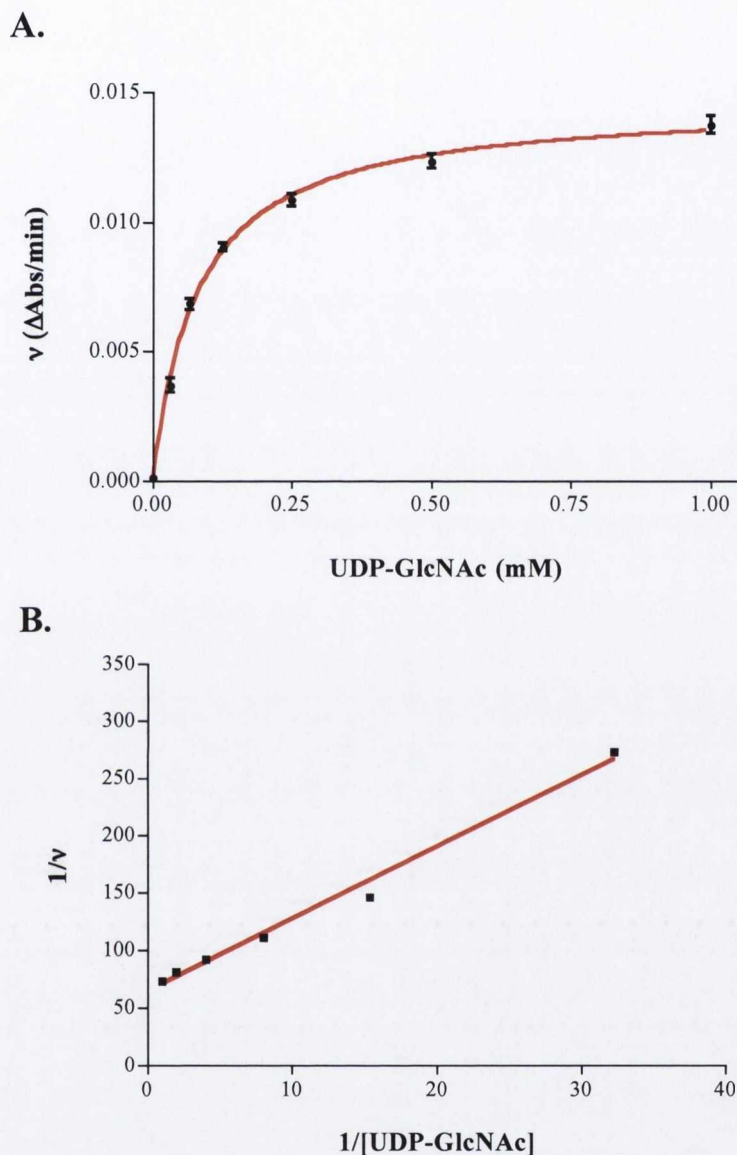
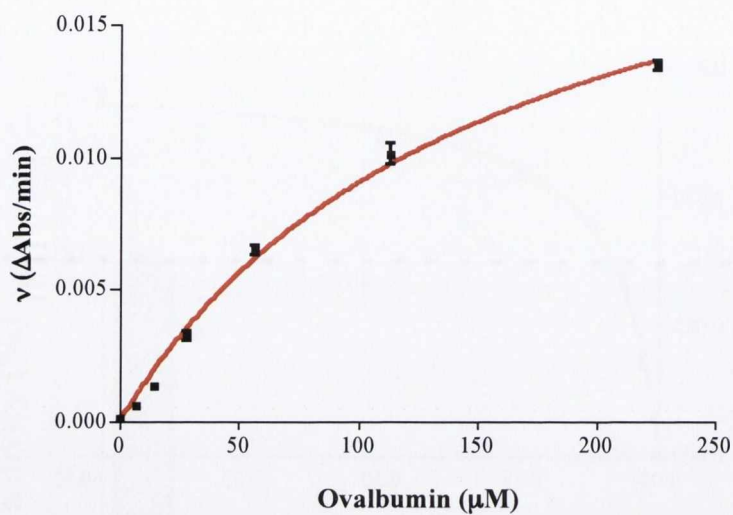


Figure 4.8 Effect of UDP-GlcNAc concentration on GnTI activity.

Assays were performed under varying UDP-GlcNAc concentration as described in Section 4.3.3, from 31.2 μ M to 1 mM while holding ovalbumin at 225.6 μ M. Kinetic data is represented as: **(A)** Standard Michaelis-Menten plot; **(B)** Double-reciprocal Lineweaver-Burk plot. Results are expressed as the mean $\pm$ SEM.

C.



D.

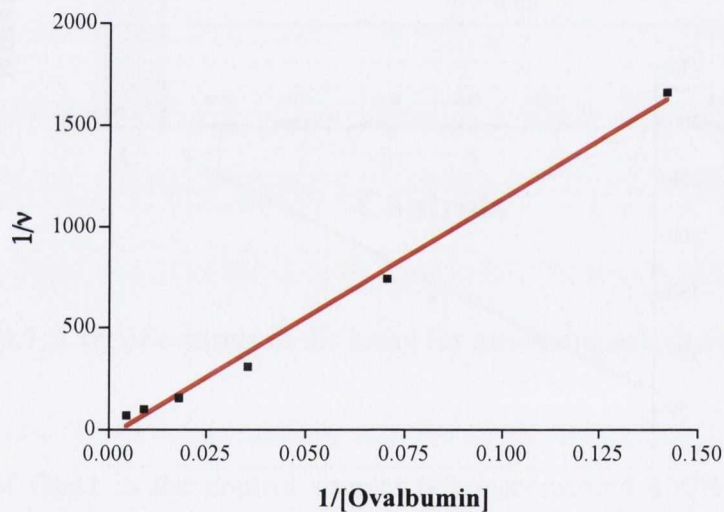


Figure 4.9 Effect of ovalbumin concentration on GnTI activity.

Assays were performed under varying ovalbumin concentration as described in Section 4.3.3, from 7.05 μM to 225.6 μM while holding UDP-GlcNAc at 0.5 mM. Kinetic data is represented as: **(A)** Standard Michaelis-Menten plot; **(B)** Double-reciprocal Lineweaver-Burk plot. Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol.

4.4.3 Determination of optimal conditions for assaying GnTI activity using the spectrophotometric coupled assay.

The optimal temperature for assaying GnTI using the spectrophotometric coupled assay was established at the temperature ranging from 10°C to 40°C. The decrease in the absorbance at 340 nm was recorded from the representative experiment performed in triplicate (see Figure 4.10). The highest decrease was observed at 37°C, which in this case represents 100% activity. The incubation at 10°C, 20°C, 30°C and 40°C showed 2.8%, 44.9%, 65.1% and 89.2% activity, respectively.

In addition to the temperature, GnTI activity was determined at pH varying between 5.5 and 10.5. The optimal pH values reported in the literature for GnTI, PK and LDH are 6.25, 7.4 and 7.5, respectively. Reactions were carried out in triplicate in the standard reaction mixture and the relative activity was determined (see Figure 4.11). The activity of GnTI was dependent on the optimal pH values for both PK and LDH and the enzyme reached its 100% activity at the pH of 6.5-7.5. At pH 8.5 the enzyme retained 25.4% activity and no activity was detected at pH below 6 and at 10.5.

The presence of divalent cations, e.g. MnCl_2 is essential for many enzyme-catalyzed reactions. In the spectrophotometric coupled assay, manganese chloride is required for activity of GnTI and pyruvate kinase. The activity of GnTI in the reaction mixture containing 5 mM MnCl_2 was 88% of the one with 10 mM, which was considered 100%. Increased concentrations of manganese ions up to 100 mM did not affect the final activity of the enzyme, which remained constant (see Figure 4.12).

In addition, the activity of GnTI was tested in the presence of other divalent cations including CoCl_2 , MgCl_2 , CaCl_2 and ZnCl_2 at the concentration of 10 mM, (see Figure 4.13). The effect of the Co^{2+} was similar to Mn^{2+} and the 93% activity was recorded. Magnesium ions gave only 46.5% activity and GnTI showed negligible activity of less than 5% in the presence of Ca^{2+} and Zn^{2+} .

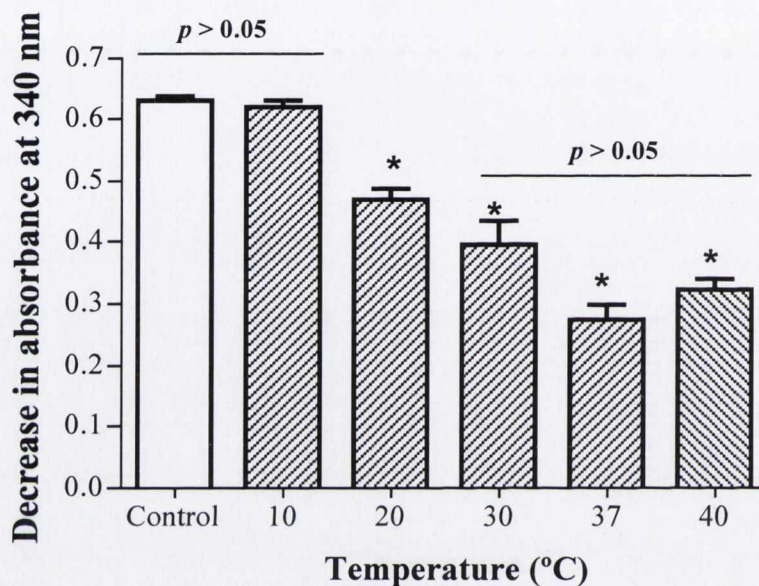


Figure 4.10 The effect of the temperature on GnTI activity.

The enzyme activity was examined under standard assay conditions as described in Section 4.3.5 and the decrease in the absorbance at 340 nm was recorded after 30 min of incubation. The control represents the absorbance value at the start of the experiment. Results are expressed as the mean $\pm$ SEM. Statistical analysis was completed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism 4; * = $p < 0.05$.

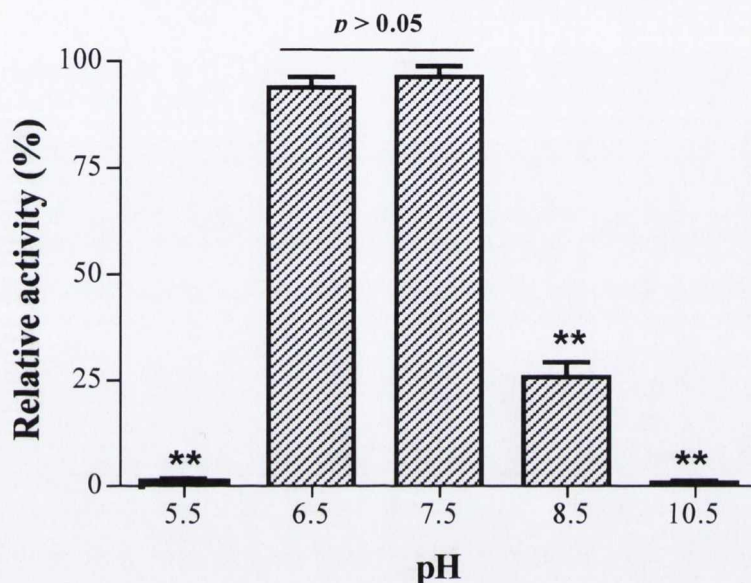


Figure 4.11 The effect of pH on GnTI activity.

The enzyme activity was determined at pH values varying between 5.5 and 10.5 as described in Section 4.3.4. The buffer solutions (100 mM) were sodium acetate for pH 5.5, MES for pH 6.5, HEPES for 7.5, Tris for pH 8.5 and glycine-NaOH buffer for pH 10.5. Reactions were carried in the standard reaction mixture for 30 min at 37°C and the relative activity was determined. Results are expressed as the mean $\pm$ SEM. Statistical analysis was completed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism 4; ** = $p < 0.01$.

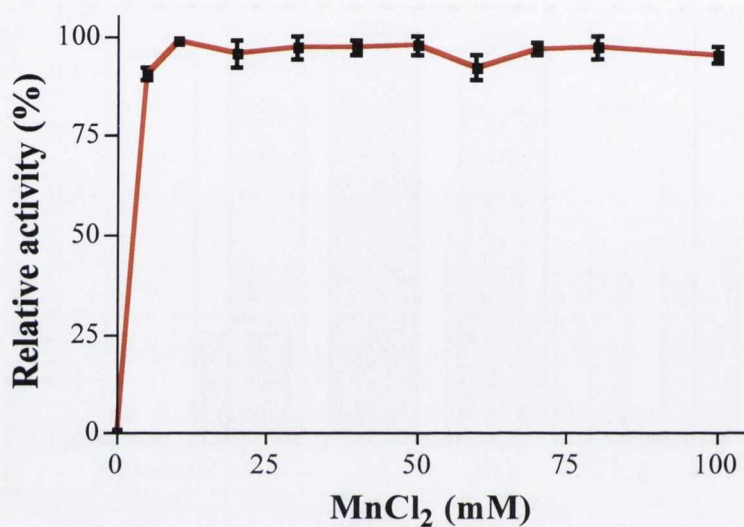


Figure 4.12 MnCl₂ concentration and its effect on GnTI activity.

The concentration of manganese ions in the standard reaction mixture was varied from 0 mM to 100 mM as described in Section 4.3.6 and the relative activity of GnTI was determined. Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol.

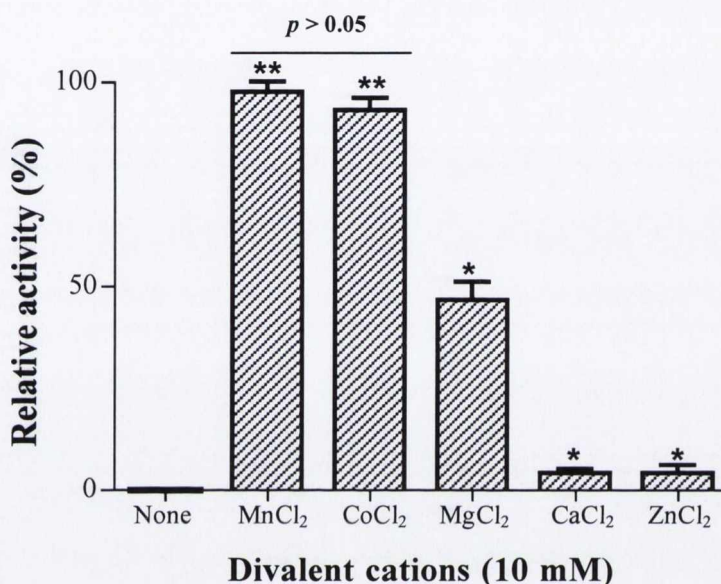


Figure 4.13 Effect of divalent cations on the activity of GnTI.

Relative activity is expressed as percentage of GnTI activity in the presence of 10 mM MnCl₂. Experimental details are described in Section 4.3.7. The reaction lacking divalent cations is represented as “None”. Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was completed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism 4; * = $p < 0.05$; ** = $p < 0.01$.

4.5 Discussion

4.5.1 Expression and purification of recombinant CHO GnTI

GnTI was expressed as a fusion protein with the N-terminal polyhistidine tag from pET15b expression vector in BL21(DE3) *E.coli* cells. The protein was a truncated version of full-length CHO GnTI (447 amino acids) lacking 31 amino acids from the N-terminus, which consists of the cytoplasmic tail and the transmembrane domain (TMD). The full-length protein was accumulated in the inclusion bodies when expressed from the same expression vector and the same *E.coli* cells. Since the protein was fused with the widely used His tag, it was possible to purify the enzyme on the affinity column equipped with Ni^{2+} ions that binds histidine from the fusion tag. GnTI was eluted from the column with 250 mM imidazole wash and the enzyme-containing eluate from the affinity purification step was pooled, dialyzed at 4°C against low-salt ion-exchange buffer containing 20 mM MES. The dialysis of GnTI against the low salt-buffer resulted in the substantial loss of approximately 50% of total protein collected from the first purification step. It was also necessary to remove all the precipitate that was formed during the dialysis and the enzyme containing sample was applied onto the preequilibrated FPLC Mono S™ 5/50 GL Tricorn™ high performance column. The reason for using a strong cation exchanger was the pI of 8.4 for recombinant CHO GnTI. The Mono Q anion exchange column has been design for pH values starting from 8, however, the pH of the buffer should be different from the calculated pI of the target protein of at least ± 1 unit and the cation exchange (Mono S; pH 6.0) column seemed the most appropriate. The elution of GnTI was initiated using a gradient of an increasing ionic strength up to 0.5 M NaCl, at which stage the protein was finally eluted in three separate 0.5 ml fractions. The high concentration of NaCl in the final buffer is not preferable by most proteins and the salt had to be removed from the sample using PD10 columns. The final protein content determined by BCA protein assay was 1.58 mg/ml and 15% (v/v) glycerol was added in order to stabilize the enzyme. Recombinant CHO GnTI enzyme was further assayed using the spectrophotometric coupled assay.

4.5.2 The spectrophotometric coupled assay

A final volume of 380 μl per well gives a path length of 1 cm (Galgani *et al.*, 1991), whereas if the assay is performed in 150 μl , the path length is decreased to 0.4 cm.

The final concentration of NADH in the reaction mixture was calculated using the Lambert-Beer formula and the absorbance value recorded for each assay separately.

HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) is a zwitterionic organic chemical buffering agent with a pKa of 7.55. HEPES is widely used in experiments where physiological pH has to be maintained. All reaction components were made up as 10X concentrated stocks on very concentrated buffer - 100 mM HEPES to prevent pH changes when adding PEP, which is very acidic. The pH of the mix was verified before adding PK, LDH and GnTI. Bovine serum albumin (BSA) in the final concentration of 0.1% was used as a carrier protein and as a stabilizing agent. Most enzyme-dependent reactions involve a divalent cation as a cofactor (typically Mg^{2+} or Mn^{2+}) therefore manganese chloride in the concentration of 10 mM was included in the reaction mixture. In addition, the commercial preparations of lactate dehydrogenase and pyruvate kinase are the ammonium sulphate based solutions and the addition of concentrated buffers directly to the assay can cause some precipitation. It was advisable to add diluted preparations of enzymes to the final reaction mixture (communication via email with Monica Palcic, Adjunct Professor, Department of Chemistry Carlsberg Laboratory, Denmark). Since several components of the coupled enzyme assay including BSA, PEP, β -NADH, PK, LDH, UDP-GlcNAc and ovalbumin tend to decompose rapidly when stored for longer periods, fresh stocks of those were prepared on the day of experiment. Extreme care was taken when preparing the β -NADH solution which is susceptible to oxidation and the presence of light and heavy metals can accelerate this process. It has been reported by Fawcett *et al.* in 1961 that potent inhibitors of lactate dehydrogenase form in the β -NADH solution stored for longer periods even at low temperatures.

4.5.3 Determination of kinetic constants

Many enzyme-catalyzed reactions are difficult to assay directly or direct assays require specialized equipment or time-consuming separation of products. In the coupled assays the product of the first reaction is made to react in the following reaction that is more amenable to assaying.

The scheme of the coupled enzyme assay is shown below:



where $A \rightarrow B$ is the reaction of interest (v_1) and $B \rightarrow C$ is the coupling reaction (v_2).

The main principal of coupled assays is the equality between v_1 and v_2 and it is important to validate this rule in the experimental conditions (Storer and Cornish-Bowden, 1974). The coupled enzyme assay for determining human and rat GnTI has been previously reported by several groups, including Gosselin, *et al.* in 1994 or Gordon *et al.* in 2006 and standard reaction conditions used in both protocols are identical therefore applied in the case of CHO GnTI.

There were two products in GnTI-mediated reaction. One was the glycosylated acceptor, ovalbumin, and the other was UDP. UDP was coupled to the conversion of phosphoenolpyruvate (PEP) to pyruvate using pyruvate kinase (PK). The pyruvate was catalyzed by lactate dehydrogenase (LDH) to its oxidative product, lactate, to generate the terminal product NAD^+ and the decrease in the absorbance was measured at 340 nm. The ovalbumin protein used in the assay has high-mannose type glycans ($Man_5GlcNAc_2-Asn$) attached to its surface, which serve as the actual acceptor molecules for subsequent addition of a GlcNAc residue from UDP-GlcNAc to mannose in a $\beta 1,2$ linkage.

In general, glycosyltransferases exhibit kinetic constants for nucleotide sugar substrates in the low micromolar range, when assayed *in vitro*. K_m values for acceptor substrates observed *in vitro* can vary dramatically for different species, ranging from low micromolar values to low millimolar values. The summary of kinetic constants of recombinant CHO GnTI is represented in Table 4.2.

Table 4.2 Kinetic parameters of recombinant CHO GnTI.

UDP-GlcNAc	Ovalbumin
$V_{max} = 14.62 \mu\text{mole/min} \pm 0.24$	$V_{max} = 23.04 \text{ nmole/min} \pm 1.3$
$K_m = 81.06 \mu\text{M} \pm 5.04$	$K_m = 154.3 \mu\text{M} \pm 16.1$

Table 4.3 represents the summary of kinetic properties of GnTI from other species, including human, rabbit, rat and mouse. Table 4.4 clarifies symbols used in the summary.

Table 4.3 Comparison of kinetic properties of GnTI from various sources

Source*	Assay*	Substrates	K _m (mM)	V _{max}	Other	References
Rabbit liver PE	R (¹⁴ C)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin	(1) 0.078 (2) 0.25	19.8 μmol/min/mg	pH optimum – 5.6; Mn ²⁺ - 20-100 mM	Schachter <i>et al.</i> , 1989
Human PEE	PA	(1) UDP-GlcNAc and synthetic (2) {M ₅ } (3) {M ₂ } (4) {M ₃ } (5) {M ₄ } (6) {M ₆ }	(1) - (2) 0.483 (3) 0% of {M ₅ } (4) 0% of {M ₅ } (5) 52% of {M ₅ } (6) 17% of {M ₅ }	101 nmol/min/mg	pH optimum 6.5-9.5; Mn ²⁺ - 10 mM,	Fujiyama <i>et al.</i> , 2001
Rat PEE	PA	(1) UDP-GlcNAc (2) synthetic {M ₅ }	(1) 0.046 (2) 0.46	5.30 μmol/min/mg	pH optimum – 7.0; optimum Mn ²⁺ of 20 mM	Nishiu <i>et al.</i> , 1995
Mouse PEE	R (³ H)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin		116.5 nmol/h/mg		Kumar <i>et al.</i> , 1992
Rabbit liver PE	R (¹⁴ C)	(1) UDP-GlcNAc and synthetic (2) {M ₅ } (3) {M ₃ -octyl} (4) {M ₃ -β-hexyl}	(1) - (2) 0.4 (3) 0.7 (4) 1.7			Schachter <i>et al.</i> , 2003
CHO WT CE	R (³ H)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin		3.70 nmol/h/mg		Kumar <i>et al.</i> , 1990
CHO WT CE	R (³ H)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin	(1) 0.17 (2) 0.26	10.4 nmol/h/mg	pH optimum - 6.2; optimum Mn ²⁺ of 25 mM	Chaney and Stanley, 1986
Rabbit liver PE	R (¹⁴ C)	(1) UDP-GlcNAc and synthetic (2) {M ₅ } (3) {M ₃ } (4) {M ₂ }	(1) - (2) 0.45 (3) 1.0 (4) 4.1	2.50 μmol/min/mg	pH optimum – 6.3	Oppenheimer and Hill, 1981
Rabbit liver PE	R (³ H)	(1) UDP-GlcNAc (2) synthetic {M ₃ -octyl}	(1) - (2) 0.585	50.0 pmol/min/mg		Kaur and Hindsgaul, 1991
Rabbit PEC	S	(1) UDP-GlcNAc (2) synthetic {M ₃ -octyl}	(1) 0.069 (2) 0.5			Gordon <i>et al.</i> , 2006
Human M	S	(1) UDP-GlcNAc (2) synthetic {M ₃ -octyl}	(1) - (2) 0.430			Gosselin <i>et al.</i> , 1994
Rabbit liver PE	R (¹⁴ C)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin	(1) 0.0384 (2) 2.03	16.3 μmol/min/mg	pH optimum – 5.7; Mn ²⁺ – 20 mM	Nishikawa <i>et al.</i> , 1988
Rat liver E	R (³ H)	(1) UDP-GlcNAc (2) {M ₅ } from hen ovalbumin	(1) - (2) 0.44		pH optimum – 7.2	Miyagi and Tsuiki, 1981

* See Table 4.4 for description of symbols

Table 4.4 Description of symbols used in Table 4.3.

Symbol	Description
PE	enzyme purified from extracts
PEE	truncated enzyme, expressed and purified from <i>E.coli</i> bacterial cells
CE	cell extracts
PEC	truncated enzyme, expressed and purified from CHO cells
M	enzyme purified from human milk
E	crude liver extracts
R	radiochemical assay
PA	Pyridylaminated (PA)-labeled substrates; HPLC separation of products
S	spectrophotometric coupled assay

The K_m values of recombinant CHO GnTI for UDP-GlcNAc and $\text{Man}_5\text{GlcNAc}_2$ $\{M_5\}$ from ovalbumin (81.06 μM and 154.3 μM , respectively) appeared to be comparable to the values obtained from the rabbit liver preparation (Schachter *et al.*, 1989). The K_m value for UDP-GlcNAc was also quite similar to rabbit GnTI overexpressed in CHO cells as described by Gordon *et al.*, 2006. In addition, GnTI from the wild-type CHO extract (Chaney and Stanley, 1986) gave approximately twice the value for UDP-GlcNAc. However, the K_m for $\{M_5\}$ reported in the same publication was similar to recombinant CHO GnTI. On average, the K_m values for $\{M_5\}$ from Table 4.3 were significantly higher, starting from 0.4 mM (Schachter *et al.*, 2003) up to 2.03 mM (Nishikawa *et al.*, 1988). The reason for that might be the fact that ovalbumin itself was not used as an acceptor molecule but $\{M_5\}$ oligosaccharide purified from ovalbumin. The preparation of $\{M_5\}$ from ovalbumin could not be performed due to equipment limitations.

4.5.4 Optimum conditions

Glycosylation enzymes tend to be most active in the pH range of 5.0 to 7.0, which reflects pH values found in various parts of the ER-Golgi-plasmalemma pathway and usually have their temperature optima at 37°C. In this case, the optimum pH range for recombinant CHO GnTI was approximately 6.5-7.5 and was slightly higher than pH values of GnTI isolated from rabbit liver (Schachter *et al.*, 1989) or assayed from CHO extracts (Chaney and Stanley, 1986). The rat liver preparation of GnTI gave comparable pH values of ~7.0. The additional polyhistidine tag on the N-terminus of recombinant CHO GnTI adds positive charges to the protein molecule and this may be the cause of a different pH optimum for recombinant GnTI. The maltose-binding protein (MBP) fusion tag used for

constructing human GnTI, provides the enzyme with additional positive charges therefore the optimum pH of up to ~9.5 was obtained (Fujiyama *et al.*, 2001). The enzyme was only 25.3% active when the pH of reaction mixture reached 8.5 and no activity was detected at pH over 10, which is far too basic for all three enzymes.

Previous experiments proved an absolute requirement for Mn^{2+} to maintain the activity of recombinant CHO GnTI, with 10 mM being shown to be optimal (Figure 4.16). The GnTI enzymes described in Table 4.3 had roughly the same properties at various concentration of Mn^{2+} of up 100 mM. The effectiveness of other divalent cations followed the pattern: $Mn^{2+} > Co^{2+} > Mg^{2+} > Ca^{2+} > Zn^{2+}$ (Schachter *et al.*, 1989; Fujiyama *et al.*, 2001; Chaney and Stanley, 1986), which is in good agreement with data obtained for GnTI from other sources.

4.5.5 Summary

For many reasons, kinetic data obtained from *in vitro* assays may not reflect the actual values from intracellular compartments. These assays often utilize acceptor analogs or part of the physiological substrate. Furthermore, the catalytic sites of most glycosyltransferases face the lumen of various compartments in the Golgi, and the sugar nucleotide-donors, which are made in the cytosol, have to be transferred across the Golgi membrane. Several other factors can regulate the activity of Golgi GlcNAc transferases, including intraluminal concentration of the sugar nucleotide and acceptors, competition by other enzymes for the same substrate or intracellular pH.

Also, one of the most important limitations would be the fact that differences between kinetic constants generated from the coupled assay and the data obtained from single-enzyme assays (e.g. radiolabelled assays) may be quite substantial. One reason could be differences between individual requirements (e.g. pH, temperature) for each enzyme, in this case GnTI, PK and LDH, that participates in the assay. Hence, the final outcome will always represent a balanced value for any given condition.

In summary, the spectrophotometric coupled enzyme assay was a valuable method for measuring the activity of recombinant CHO GnTI with several limitations that have been described previously. Theoretically, this method might be extended to evaluate other

glycosyltransferases that use nucleotide donors (e.g. CDP, GDP or ADP) and can be processed by pyruvate kinase. However, the assay is limited to pure enzyme preparations since the concentration of sugar nucleotides in cell extracts is too high and the results will not reflect the actual values.

CHAPTER 5

Localisation of CHO GlcNAc transferases in the Golgi apparatus

5.1 Introduction

The landmark studies completed by Kornfeld and Kornfeld in 1985 demonstrated that the enzymes responsible for *N*-glycan synthesis and remodelling were organized in the order in which the enzymes catalyzing earlier steps were located in *cis* and *medial* compartments and the late-acting enzymes were distributed mainly in the *trans* Golgi. This strict compartmentalization of enzymes was thought to ensure the efficient biosynthesis of oligosaccharide structures by providing optimal contact between enzyme, glycoprotein substrate and sugar nucleotide donor. With further investigation, it became clear that each enzyme forms a concentration gradient over a particular region of the Golgi. As a result of such gradients, the glycosylation enzymes represent an overlapping distribution and demonstrate cell-type-specific Golgi subcompartmentation (Rabouille *et al.*, 1995). Interestingly, gradients of enzyme distributions have been observed not only in the *cis*-to-*trans* direction of Golgi stacks. Sometimes the Golgi ribbon exhibits a gradient of enzyme concentrations along the same cisterna. Additionally, there are differences in the locations of glycosyltransferases between different cell types (cell lines vs. primary cells) even within the same tissue (Roth *et al.*, 1986) and there are other glycosyltransferase locations, such as the, plasma membrane, cytosol and nucleus (Berger, 2002).

The ultrastructure of the Golgi complex and its polarity was previously elucidated using the transmission electron microscopy (Balch *et al.*, 1986; Slusarewicz *et al.*, 1994; Polishchuk and Mironov, 2004; Jackson, 2009). The more recent and popular fluorescent and confocal imaging has been hampered by the resolution of the microscope. Electron microscopy (EM), unfortunately, is not able to provide analyses of living cells, however, the snap-shots representing specific distribution of glycosyltransferases in the Golgi apparatus can provide better insight into one of the most important post-translational modifications, which is glycosylation.

Initially the most intensely studied glycosyltransferases were the *trans*-Golgi/*trans*-Golgi network (TGN) enzyme, α 2,6 sialyltransferase, the *trans*-Golgi enzyme β 4GalT1, and the *medial*-Golgi enzymes, α -mannosidases and GnTI. The early view suggested that the α -mannosidases I and II (ManI and ManII) were found in the *cis* and *medial* Golgi, GnTI in the *medial* Golgi, GalT in the *trans* Golgi, and the ST in the *trans* Golgi and *trans* Golgi

network (TGN). However, ManI and ManII enzymes have been found to overlap within the *medial* and *trans* Golgi of most cell types (Goldberg and Kornfeld, 1983; Velasco *et al.*, 1993; Taguchi, 2003). In HeLa cells, the GnTI and ManII overlap in the *medial* and *trans* Golgi cisternae (Dunphy *et al.*, 1985; Balch *et al.*, 1986; Burke *et al.*, 1992), while the GalT and ST overlap in the *trans* cisternae and *trans* Golgi network (Nilsson *et al.*, 1993, Nilsson *et al.*, 1993a; Rabouille *et al.*, 1995).

In addition, detail biochemical analyses revealed that several Golgi-based enzymes form homo- or heterodimers as well as oligomers and these complexes were found to be the result of interactions between charged residues in the luminal domains (Nilsson *et al.*, 1996) or initiated through disulfides in the stem region (Sasai *et al.*, 2001). For instance, the physical association between α ManII and GnTI, two *medial* Golgi residents in HeLa cells, was reported by Nilsson *et al.* in 1994. Additionally, in transfected CHO cells the *medial* enzymes GnTI and GnTII were part of high-molecular-weight complexes (Opat *et al.*, 2000). Unlike other glycosyltransferases, GnTV forms disulfide-bonded homooligomers through cysteine residues localised in the stem region (Sasai *et al.*, 2001).

A number of studies of glycosyltransferase chimeras have shown the transmembrane domain of many different glycosyltransferases provides a dominant localisation signal. However, contributions from the luminal domain and cytoplasmic tail suggested that there may be multiple signals involved in the specific localisation of these Golgi enzymes (Colley, 1997 and Gleeson, 1998, Grabenhorst and Conradt, 1999). Additionally, due to the limited homology between glycosyltransferase, no common peptide motifs localising enzymes to specific compartments have been identified by sequence comparison. It is likely that the mechanisms that retain glycosyltransferases in the Golgi apparatus are highly conserved through evolution, as mammalian enzymes have been expressed in yeast and plant cells, and are correctly localised to the Golgi (Gomez and Chrispeels, 1994; Schwientek *et al.*, 1995).

5.2 Aim of Chapter

The aim of this study was to provide consistent and valuable localisation data for GlcNAc transferases (GnTs) in CHO cells. In order to do so, CHO GnT cDNAs were cloned and transiently expressed from the pcDNA3.1 expression vector. The C-terminal *myc*-epitope attached to each GnT sequence was used for detection of newly produced proteins by Western blotting, followed by immunocytochemistry (ICC), fluorescence and confocal microscopy. In addition, specific Golgi markers, giantin and TGN46, were used to co-localise GnTs to specific Golgi compartments. Giantin is the largest Golgi membrane protein (400-kDa), which is C-terminally anchored to *cis*- and *medial*-Golgi membranes (Linstedt and Hauri, 1993) whereas the *trans* Golgi network 46 protein (TGN46) cycles between the *trans*-Golgi network and the cell surface returning via endosomes and its rodent homologue is known as TGN38 (Prescott *et al.*, 1997).

5.3 Methods

5.3.1 Molecular cloning of CHO GnTs into pcDNA3.1/myc-His

The GnT cDNA clones in pBluescript SK+ were digested with *Xba*I restriction enzyme in a total volume of 40 µl as described in Section 3.3.14 and used directly as a template for polymerase chain reaction. PCR was performed in order to create restriction sites for sequential cloning into pBluescript SK+ and pcDNA3.1/*myc*-His. Sequences and corresponding restriction sites of the oligonucleotides used for amplification of the coding regions of GnT cDNAs are described in Table 5.1. The sense oligonucleotides were designed to introduce *Ap*aI, *Hind*III restriction sites and a Kozak translation initiation sequence (-GCCACC-) at the ATG initiation codon for proper translation (Kozak, 1987; Kozak 1990). The antisense oligonucleotides were designed to introduce *Ap*aI and *Xba*I sites and the stop codon was removed from the 3'- untranslated region in order to create a fusion protein with C-terminal peptides – *myc* and a His tag.

Table 5.1 Oligonucleotides used for amplification of GnT cDNAs;
(**F**-forward primer; **R**-reverse primer).

Name	Sequence	Restriction site
G1-F	5'-CAAGCTTCCGCCACCATGCTGAAGAAGCAG-3'	<i>Hind</i> III
G1-R	5'-GAAGGGCCCATAAGTCCAGCTAGGATC-3'	<i>Ap</i> aI
G2-F	5'-CAAGCTTCCGCCACCATGAGGTTCCGCAT-3'	<i>Hind</i> III
G2-R	5'-GAAGGGCCCACACTGCAGTCTTCTATAAC-3'	<i>Ap</i> aI
G4B-F	5'-CAAGCTTCCGCCACCATGAGGCTCCGC-3'	<i>Hind</i> III
G4B-R	5'-GGGCCCTCTAGAATAGTCGGCCTTTTTC-3'	<i>Xba</i> I
G5-F	5'-CAAGCTTCCGCCACCATGGCTTTCTTTAC-3'	<i>Hind</i> III
G5-R	5'-GGGCCCTCTAGAATATAGGCAGTCTTTGC-3'	<i>Xba</i> I

Amplification was completed in a thermal cycler, in a total volume of 25 µl as described in Section 3.3.18 and the PCR program used for amplifying GnT cDNAs is described in Table 5.2.

Table 5.2 PCR program.

Process		Temperature	Time
Initial denaturation		95°C	2 min
35 cycles	Denaturation	95°C	1 min
	Annealing	64°C	30 sec
	Elongation:	72°C	
	GnTI		3 min 30 sec
	GnTII		3 min 30 sec
	GnTIVB		3 min 30 sec
	GnTV		4 min 30 sec
Final elongation		72°C	7 min

The resulting amplification product was resolved by agarose gel electrophoresis and purified using a method described in Section 3.3.7 followed by cloning into pBluescript SK+ and pcDNA3.1/*myc*-His. Plasmid mini preparations of GnT-pcDNA3.1/*myc*-His were used for sequencing as described in Section 3.3.17 and endotoxin-free maxi preparations were purified from *E.coli* according to Section 3.5.2.

5.3.2 Endotoxin-free maxi-preparation of mammalian constructs

The PureLink™ HiPure Plasmid Filter Maxiprep kit allows purification of 500–850 µg of high-quality plasmid DNA from 100–200 mL overnight *E. coli* cultures when cloning high copy number plasmids. The purified DNA is ultrapure and suitable for downstream applications, including those requiring the highest purity, such as transfection of mammalian cells. Initially, RNase A was added to the Resuspension Buffer (R3) and the solution was kept at 4°C until required. TOP10 *E.coli* cells containing the plasmid of interest were grown overnight in a total volume of 100 ml of LB supplemented with 100 µg/ml (w/v) Amp. Bacterial cells were harvested by centrifugation at 4,000 g for 10 min and the supernatant was discarded. The cell pellet was resuspended in 10 ml of Resuspension Buffer (R3) until homogenous followed by addition of 10 ml Lysis Buffer (L7). The mixture was gently inverted until the lysate was homogenous and incubated at room temperature for 5 min. In the meantime, the purification column was equilibrated with 30 ml Equilibration Buffer (EQ1) and the solution was let to drain by gravity flow. Precipitation of proteins was carried out by adding 10 ml Precipitation Buffer (N3) and the suspension was mixed immediately by inverting the tube until the mixture was thoroughly homogeneous. The supernatant was separated from proteins by centrifugation at 16,000 g for 10 minutes at room temperature and then applied onto the equilibrated column. The

solution was let to drain by gravity flow followed by washing the column with 60 mL Wash Buffer (W8). DNA was eluted from the column by adding 15 mL Elution Buffer (E4). In order to precipitate DNA from the eluate, 10.5 mL isopropanol was added to the elution tube. The supernatant was separated from precipitated DNA by centrifugation at 16,000 g for 30 minutes at 4°C and the DNA pellet was washed in 5 mL 70% ethanol, followed by centrifugation at 4°C for 5 min. Finally, the DNA pellet was air-dried for 10 min, then resuspended in 50 µL TE Buffer (TE) and stored at -20°C until required. The DNA content was estimated as described in Section 2.2.9.

5.3.3 Mammalian transfections

Wild type CHO were transfected by nucleofection developed by Amaxa. In this technique, cells are exposed to a unique combination of cell-type specific solutions and electrical parameters in a Nucleofector device. During this procedure DNA enters directly into nucleus following and electrical pulse and as a result, transgene expression can occur rapidly. Cell Line Nucleofector Kit V was used for all transfection procedures. Only healthy, exponentially growing cultures were used for transfection procedures.

The U-024 program has been optimized for transfection of suspension CHO cells. Cells were cultured as described in Section 2.2.1. Prior to transfection, 18 µl of Supplement was added to 82 µl of Cell Line Nucleofector Solution, mixed gently and pre-warmed prior to use. One million cells were centrifuged at 100 g for 8 min at room temperature. Fresh culture medium was added to a 6 well plate and pre-incubated in a humidified 37°C/5% CO₂ incubator. The cell pellet was sequentially resuspended in 100 µl supplemented Cell Line Nucleofector Solution, mixed with 5 µg DNA (see Table 5.3) and transferred to Amaxa certified cuvette, avoiding air bubbles while pipetting. The cuvette was placed in the cuvette holder, the required program was selected and the cells were nucleofected. Pre-heated medium (500 µl) was subsequently added the cuvette, the cells pipetted using a Pasteur pipette provided with the kit and transferred to pre-incubated medium (1 ml). This procedure was also repeated for cells transfected with no DNA which were used as controls. Cells were allowed to recover for 48 hours in non-selective medium. After this time, proteins were extracted from cells as in Section 5.3.4, followed by Western blotting to confirm the expression as in Section 2.2.8.

Table 5.3 Mammalian constructs used in this project.

Name	Size	Form	Transfection	Cells
GnTI-pcDNA3.1/ <i>myc</i> -His	6837 bp	Circular	Transient	CHO WT
GnTII-pcDNA3.1/ <i>myc</i> -His	6822 bp	Circular	Transient	CHO WT
GnTIVB-pcDNA3.1/ <i>myc</i> -His	7140 bp	Circular	Transient	CHO WT
GnTV-pcDNA3.1/ <i>myc</i> -His	7716 bp	Circular	Transient	CHO WT

5.3.4 Protein extraction

CHO cells were collected by low-speed centrifugation at 100 x g for 5 min followed by three washes with cold PBS. Proteins were extracted with ice-cold RIPA buffer (50 mM Tris 150 mM NaCl, 1% Igepal (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v), pH 8.0) supplemented with protease inhibitors and Benzonase (1U/ μ l). Cell suspension was incubated on ice on a shaking platform for 30 min and the protein content was estimated by protein assay described in Section 2.2.7.

5.3.5 Immunocytochemistry

Freshly nucleofected CHO cells were collected from the plates and centrifuged at 100 g for 5 min. The pellet was rinsed twice with cold PBS, resuspended again in fresh PBS, and mixed with 6% paraformaldehyde solution, pH 7.4 (250 μ l: 250 μ l) to give a final concentration of 3%. After 30 min incubation on ice, the cells were centrifuged as before and resuspended in 50 μ l of PBS and 15 mM sodium azide (N₃Na) solution and transferred onto a poly-L-lysine-covered slide (0.05% of poly-L-lysine in water). Cells were allowed to attach for 20 min at room temperature prior to incubation with PBST-5% BSA (PBS, 15 mM N₃Na, 0.1% Triton X-100) for 30 min, in order to reduce non-specific binding. The blocking solution was removed and cells were washed three times in PBS for 5 min each. Primary antibodies (see Table 5.4) were added to PBST-5% BSA and the slides were incubated in petri dishes overnight at 4°C. Cells were washed as before prior adding secondary antibodies (see Table 5.4) in PBST-5%BSA followed by incubation for 1 h in the dark at room temperature. Slides were washed as before and cells were covered with a small amount of VECTASHIELD® fluorescence mounting medium containing DAPI. A coverslip was placed on each slide and the nail varnish was used to prevent from

drying and movement under the microscope. Cells were examined by fluorescence and confocal microscopy using Olympus FV1000 Point-Scanning Confocal Microscope.

Table 5.4 Primary and secondary antibodies used in the ICC-IF.

Primary antibodies			Secondary antibodies			Nucleic probe
Name	Source	Dilution	Name	Source	Dilution	DAPI (blue)
anti- <i>myc</i>	mouse	1:500	Alexa 488 (green)	chicken	1:1000	
anti-giantin	rabbit	1:500	Alexa 546 (red)	goat	1:1000	
anti-TGN46	rabbit	1:500	Alexa 546 (red)	goat	1:1000	

5.4 Results

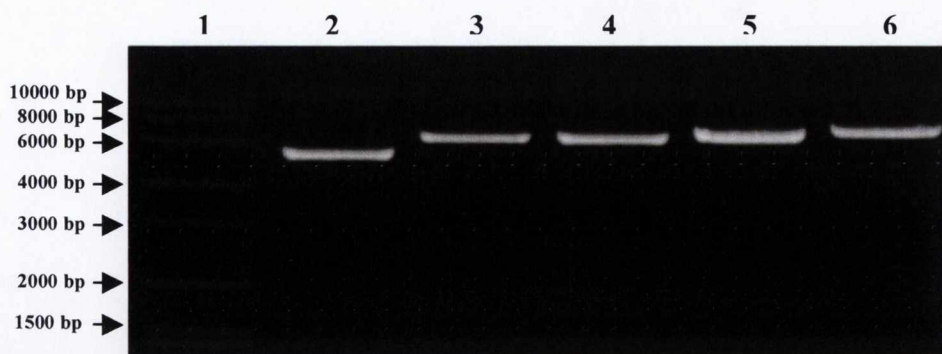
5.4.1 Intracellular localisation of GnTs in CHO cells

CHO GnT cDNAs were amplified from the linearized pBluescript SK+ clones followed by subcloning into pcDNA3/*myc*-His mammalian expression vector. Restriction analysis of the GnT-pcDNA3/*myc*-His constructs using *Hind*III confirmed the successful cloning procedure and showed DNA products of 6837 bp for GnTI construct, 6822 bp for GnTII construct, 7140 bp for GnTIVB construct and 7716 bp for GnTV construct (see Figure 5.1A). Transient overexpression of GnTs was achieved by nucleofection of wild type CHO cells with the circular GnT-pcDNA3/*myc*-His construct. The Western blotting, which was used to determine the overexpression level, showed distinctive protein products for GnTI, GnTII, GnTIVB and GnTV with apparent molecular weights of ~55 kDa, ~55 kDa, ~75 kDa and ~90 kDa, respectively (see Figure 5.1B).

To provide some localisation data for CHO cells using confocal microscopy, the paraformaldehyde-fixed cells were transferred onto a poly-L-lysine coated slide followed by specific staining with monoclonal anti-*myc*, polyclonal anti-giantin or anti-TGN46 antibodies. Giantin and TGN46 protein are markers specific to *medial* and *trans* Golgi network, respectively. The fluorescent stain, DAPI, was routinely used to stain cell nuclei. Figures 5.2A and 5.2B show a population of CHO cells grown in suspension using a phase contrast microscopy at 20X and 60X magnification. The Golgi apparatus is typically localised around the large nucleus as shown in Figure 5.2C, in which this organelle was immunostained with the anti-giantin antibody. Not surprisingly, all CHO GnTs, including newly characterized GnTII and GnTIVB, were perfectly co-localised with specific Golgi markers as shown in Figures 5.3-5.6 confirming their natural embedment in Golgi membranes. The *myc* tag, which is only 11 amino acids in length, had no apparent effect on the localisation of GnTs and in fact no significant changes in Golgi morphology, volume, or surface area were detected for wild type CHO cells.

Unfortunately, the resolution of the confocal microscope does not allow for distinction specific *cis*, *medial* and *trans* compartments and further investigation using transmission electron microscopy is required.

A.



B.

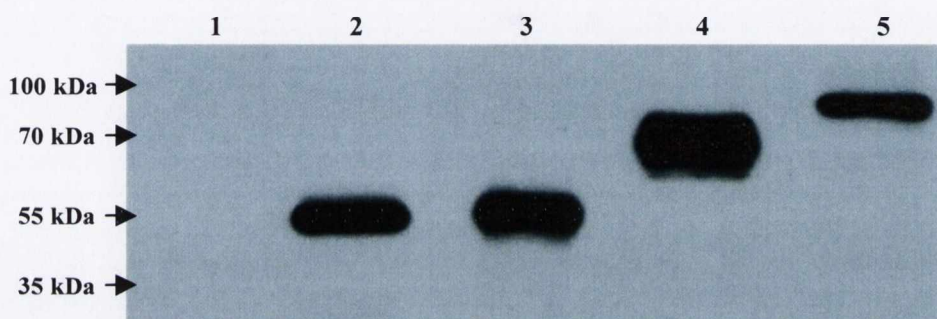


Figure 5.1 Molecular cloning and expression of GnTs in CHO cells.

The restriction analysis with *Hind*III (**A**) of CHO GnTI- (6837 bp), GnTII- (6822 bp), GnTIVB- (7140 bp) and GnTV-pcDNA3.1/*myc*-His (7716 bp) constructs was carried as described in Section 3.3.14. The agarose gel was loaded as follows: **lane 1**, DNA marker; **lane 2**, pcDNA3.1/*myc*-His (5493 bp); **lane 3**, GnT1-pcDNA3.1/*myc*-His; **lane 4**, GnTII-pcDNA3.1/*myc*-His; **lane 5**, GnTIVB-pcDNA3.1/*myc*-His; **lane 6**, GnTV-pcDNA3.1/*myc*-His; (**B**) shows the expression profile of *myc*-tagged GnTs in CHO cells. Equal protein aliquots (10 μ g) were mixed with 2X SDS sample buffer and boiled at 95°C for 5 min. CHO lysates were resolved on a 12% SDS-PAGE gel prior to transfer onto PVDF membranes as in Section 2.2.7 and 2.2.8. Blots were probed with mouse anti-*myc*, 1:5000 and rabbit anti-mouse HRP, 1:10000. The SDS-PAGE gel was loaded with the following samples: **lane 1**, no DNA control; **lane 2**, *myc*GnTI; **lane 3**, *myc*GnTII; **lane 4**, *myc*GnTIVB; **lane 5**, *myc*GnTV.

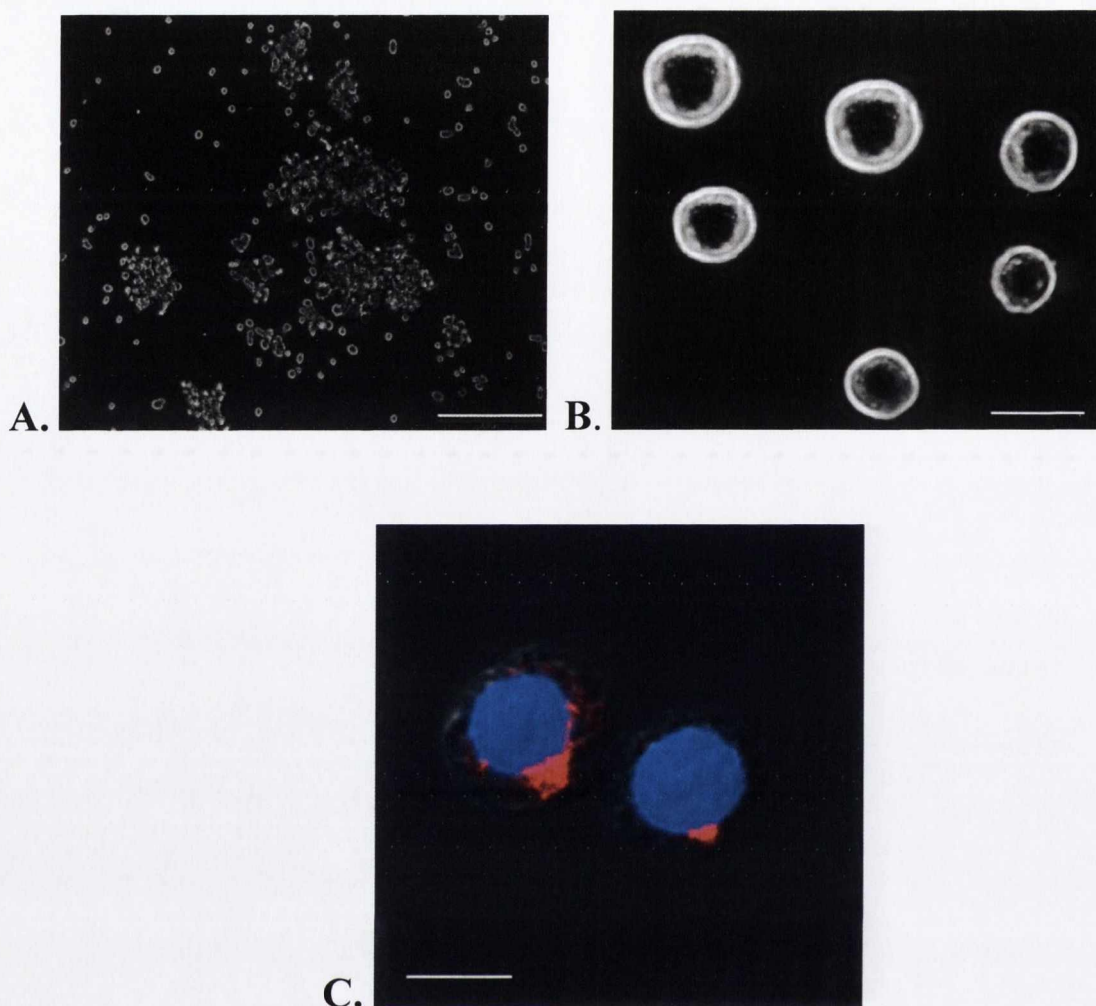


Figure 5.2 Visualisation of Golgi structures in Chinese hamster ovary (CHO) cells.

Chinese hamster ovary (CHO) cells are derived from a subclone of the parental CHO line originated by Theodore T. Puck in 1957. Wild type cells used in this project (ECACC, No. 00102307) have been adapted for growth in suspension. Cells were visualised using Olympus IX81 long focal length fluorescent microscope; **(A)** 20X magnification and scale bar represents 200 μm ; **(B)** 60X magnification scale bar represents 15 μm ; **(C)** Cells visualised using Olympus FV1000 Point-Scanning Confocal Microscope; paraformaldehyde-fixed wild type CHO cells were transferred onto a poly-L-lysine coated slide and stained with the rabbit polyclonal anti-giantin at 1:500 dilution and goat anti-rabbit Alexa Fluor 547 (red) at 1:1000 dilution. DAPI stains the nucleus (blue). Scale bar, 10 μm .

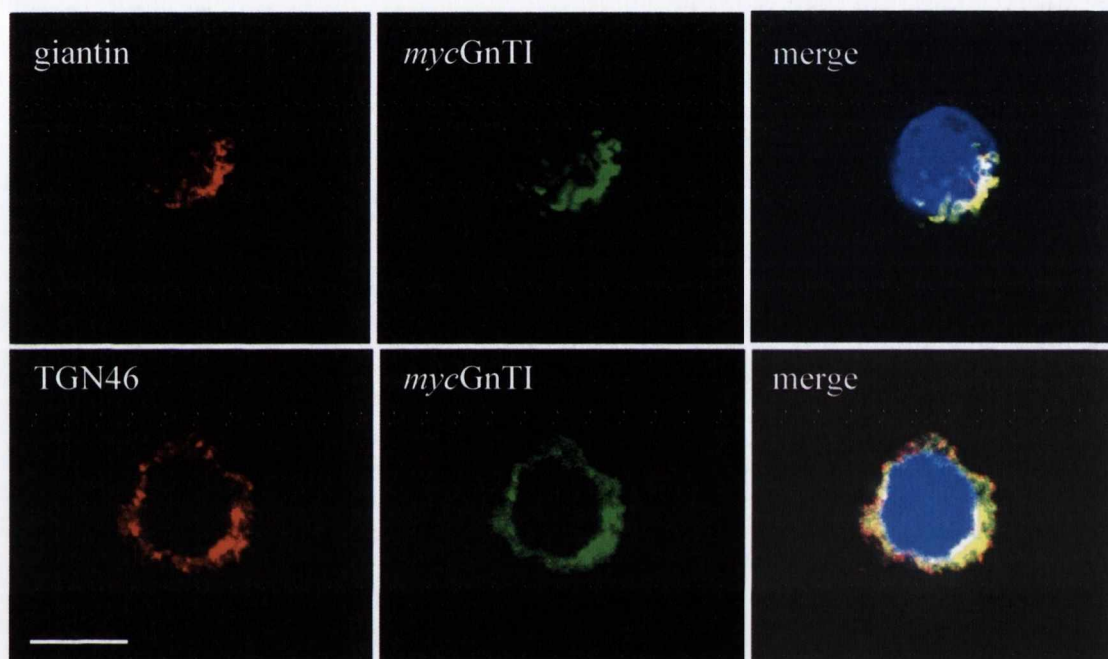


Figure 5.3 Localisation of *mycGnTI* in the Golgi apparatus of CHO cells.

Wild type CHO cells transfected with GnTI-pcDNA3.1/*myc*-His, paraformaldehyde-fixed were transferred onto a poly-L-lysine coated slide and stained with the mouse monoclonal anti-*myc*, rabbit polyclonal anti-giantin or rabbit polyclonal anti-TGN46 antibodies. Bound antibodies were detected with the chicken anti-mouse Alexa Fluor 488 (green) and goat anti-rabbit Alexa Fluor 547 (red). DAPI stains the nucleus (blue). Bar, 10 μ m.

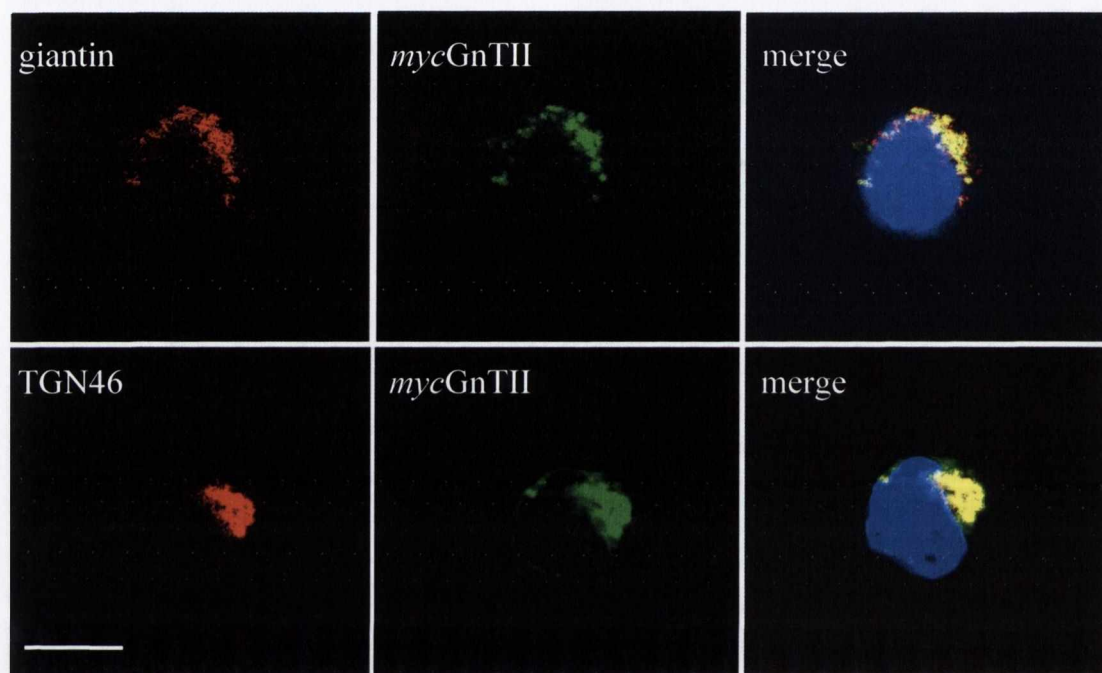


Figure 5.4 Localisation of *mycGnTII* in the Golgi apparatus of CHO cells.

Wild type CHO cells transfected with GnTII-pcDNA3.1/*myc*-His, paraformaldehyde-fixed were transferred onto a poly-L-lysine coated slide and stained with the mouse monoclonal anti-*myc*, rabbit polyclonal anti-giantin or rabbit polyclonal anti-TGN46 antibodies. Bound antibodies were detected with the chicken anti-mouse Alexa Fluor 488 (green) and goat anti-rabbit Alexa Fluor 547 (red). DAPI stains the nucleus (blue). Bar, 10 μ m.

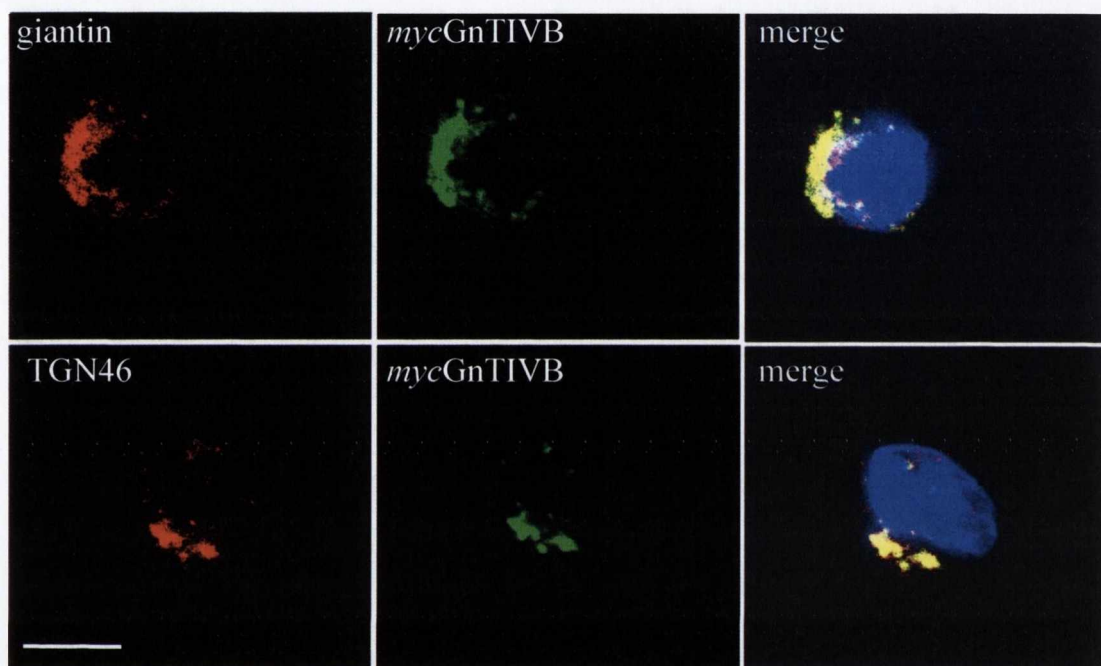


Figure 5.5 Localisation of *mycGnTIVB* in the Golgi apparatus of CHO cells.

Wild type CHO cells transfected with *GnTIVB-pcDNA3.1/myc-His*, paraformaldehyde-fixed were transferred onto a poly-L-lysine coated slide and stained with the mouse monoclonal anti-*myc*, rabbit polyclonal anti-giantin or rabbit polyclonal anti-TGN46 antibodies. Bound antibodies were detected with the chicken anti-mouse Alexa Fluor 488 (green) and goat anti-rabbit Alexa Fluor 547 (red). DAPI stains the nucleus (blue). Bar, 10 μm .

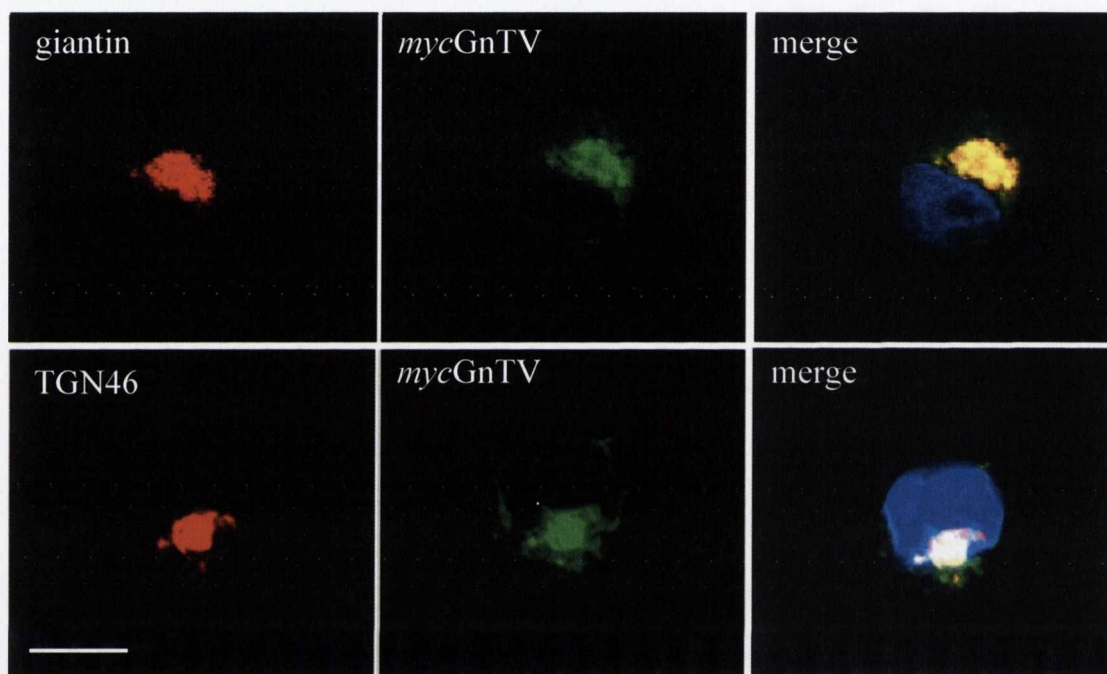


Figure 5.6 Localisation of *mycGnTV* in the Golgi apparatus of CHO cells.

Wild type CHO cells transfected with GnTV-pcDNA3.1/*myc*-His, paraformaldehyde-fixed were transferred onto a poly-L-lysine coated slide and stained with the mouse monoclonal anti-*myc*, rabbit polyclonal anti-giantin or rabbit polyclonal anti-TGN46 antibodies. Bound antibodies were detected with the chicken anti-mouse Alexa Fluor 488 (green) and goat anti-rabbit Alexa Fluor 547 (red). DAPI stains the nucleus (blue). Bar, 10 μ m.

5.5 Discussion

Ever since the discovery of the remarkable morphology of the stacked Golgi cisternae and then the later realization that the Golgi was the site of major steps of glycosylation, there has been much interest in defining the sub-Golgi locations of glycosyltransferases. These enzymes are the integral membrane protein constituents of Golgi membranes and the majority of glycosyltransferases are type II integral membrane proteins comprised of a cytoplasmic tail (CT), a transmembrane (TM) spanning region, a stem region, and an enzymatic domain (Tu and Banfield, 2009). Glycosyltransferases reach the *cis* cisternae of Golgi apparatus via COPII-coated transport vesicles from the endoplasmic reticulum (ER). The distribution of enzymes throughout the Golgi (i.e., whether its steady-state localisation is predominantly in the *cis*, *medial*, or *trans* cisternae) is not homogeneous, and often reflects the sequential order in which these enzymes act controlling the protein and lipid traffic in both forward (anterograde) and reverse (retrograde) directions (Opat, 2001). Examination of the mechanisms and signals that dictate the characteristic non-uniform distribution of glycosyltransferases across cisternae of the Golgi have revealed that, for many glycosyltransferases, their steady-state localisations are often mediated by a number of features within the cytoplasmic tail, transmembrane domain and stem region (CTS) of the protein, which may vary from one enzyme to another (Wildt and Gerngross, 2005; Hamilton and Gerngross, 2007). In addition, several studies indicate that glycosyltransferases from different Golgi compartments form high molecular mass complexes and that the inclusion into such complexes increases the efficiency and stability of Golgi localisation (Nilsson *et al.*, 1993). Mislocalisation of glycosyltransferases within the Golgi can have profound effects on glycan synthesis and in turn on the normal physiology of cells (Dennis *et al.*, 1999)

The large diversity of carbohydrate structures is the product of over 200 different glycosyltransferases, however, the precise localisation of only a small number of the glycosyltransferase proteins within the Golgi apparatus has been determined (Opat, 2001). Localisation studies have been further confounded by the observation that different cell types from the same organism can show distinct localisation patterns for the same enzyme (Berger, 2001). GlcNAc transferases (GnTs), which were investigated in this Chapter, are minor proteins constituting <0.2% of Golgi membrane protein (Nishikawa *et al.*, 1988). Four different GnTs (I, II, IV and V) have been characterised in wild type CHO cells,

however their specific localisation in the Golgi has not been experimentally verified. Transient overexpression of *myc*-tagged GnTs in CHO cells resulted in their targeting to the Golgi apparatus. Co-localisation with specific Golgi markers (giantin and TGN46) was confirmed by immunocytochemistry (ICC) followed by fluorescence and confocal imaging. Unfortunately, no further distinction between specific Golgi compartments can be made unless higher resolution, for instance electron microscopy, is used.

In summary, a significant difference in the distribution of GnTs could be possibly detected between the *cis*-most and *trans*-most cisternae of the central Golgi domain, and not between adjacent cisternae of the same stack. The main problem is still the limitation of the spatial resolution of methods used for the analysis of Golgi structure. Biochemical isolation leads to loss of integrity and Golgi morphology. Popular fluorescent protein (FP) video imaging has been hampered by the resolution of light microscopy. Immunolocalisation by electron microscopy (EM) provides a snap-shot of the distribution of a glycosyltransferases at any moment in time and thus represents a steady-state distribution, however, it gives no information on whether the proteins are actively retained or constantly moving.

CHAPTER 6

Glycoengineering human chorionic gonadotropin (HCG)

6.1 Introduction

Glycosylation is an important post-translational modification for many recombinant therapeutic proteins. The presence or absence and the specific content of carbohydrates can have a major impact on physical stability, resistance to protease attack, antigenicity, pharmacokinetics, tissue distribution, and specific biological activity. The heterogeneous populations of glycoproteins are called glycoforms, which share the same polypeptide backbone but oligosaccharides at glycosylation sites are different (Cumming, 1991; Jenkins *et al.*, 1996). Bioengineering of recombinant glycoproteins by creating novel glycoforms or glycoform distributions can potentially lead to improved therapeutic features (Bailey, 1991; Stanley, 1992). As a result, various techniques have been used to engineer the sequence of therapeutic glycoproteins to increase their activity and half life and consequently reduce the frequency of administration (Lee *et al.*, 2006; Fauser *et al.*, 2009). In the majority of the studies carried out to date, genetic manipulations of enzymes involved in the *N*-glycan processing have been reported to yield novel oligosaccharide structures attached to endogenous or recombinant glycoproteins (Lee *et al.*, 1989; Minch *et al.*, 1995). Surprisingly, very little attention has been paid to genetic manipulation of enzymes already present in the cells with a few unsuccessful attempts as reported by Youakim and Shur in 1993. Considering the complexity of the glycosylation pathway, effective engineering of therapeutic glycoproteins can be aided by a mathematical model, whose main function is to calculate the expected qualitative trends in the *N*-linked oligosaccharide distribution resulting from changes in the levels of one or more enzymes involved in the network of enzyme catalyzed reactions. These simulations allow for a comparison of different strategies, such as simultaneous overexpression or silencing of several enzymes acting at different points in the pathway. Additionally, the mathematical modeling approach may also maximize the fractions of beneficial glycoforms of a target glycoprotein and its distribution which can be preferable in the biotechnology industry in large scale production of therapeutic glycoproteins such as antibodies (Bibila and Robinson, 1995).

This work involves the use of a CHO cell line that overexpresses human chorionic gonadotrophin (HCG). HCG is a glycoprotein hormone of heterodimeric structure consisting of an α subunit that is covalently associated with the homologous

β subunit. The α subunit has two *N*-linked oligosaccharides whereas the β subunits are *N*- and *O*-glycosylated at six positions in the peptide chain.

First investigations into the specific *N*-linked glycan structures attached to urinary forms of HCG were carried out in 1979 (Endo *et al.*, 1979; Kessler *et al.*, 1979). Results showed that a biantennary, galactosylated and potentially disialylated structure predominated, with the added possibility of core fucosylation as well as hybrid structures where the α 1-3 mannose arm was substituted with a GlcNAc, Gal and NeuAc residue. These structures were confirmed by more sophisticated techniques developed in the past 20 years (Weisshaar *et al.*, 1991; Liu and Bowers, 1997; Kobata and Takeuchi, 1999; Gervais *et al.*, 2003; Toll *et al.*, 2006). A small proportion of tri-antennary structures with variable sialylation and one tetra-antennary glycan were identified in recombinantly expressed HCG in CHO cells (Gervais *et al.*, 2003). Toll *et al.* in 2006 report a much smaller number of triantennary species that were identified by HPLC combined with mass spectroscopy analysis. The discrepancies between the glycosylation pattern of urinary HCG and the recombinant version of this hormone indicate that *in vitro* production and sourcing of HCG may alter the *N*-glycan antennarity.

The importance of glycosylation of HCG in the folding and proper activity was investigated via recombinant expression in *E.coli*, which lacks post-translational modifications (Strickland *et al.*, 1985; Huth *et al.*, 1994) and site directed mutagenesis of glycosylation sites (Matzuk and Boime, 1988). Additionally, the complete desialylation of HCG reduces the half-life as reported in rat to less than 1 min (Van Hall *et al.*, 1971). Removal of *O*-glycans attached to the β subunit has no impact on the activity, but the half life decreases dramatically as reported by Kalyan and Bahl in 1983. It has been previously described that removal of sialic acid residues increases the affinity of the protein for its receptor while reducing the stimulation of the intracellular cAMP cascade by 50% (Amano *et al.*, 1990). Removal of all sugars attached to HCG increases the affinity even more, while reducing the activity to almost zero. To summarize, interaction of HCG with its receptor and subsequent transduction of a signal, molecular stability and proper folding are greatly influenced by the glycans.

6.2 Aim of Chapter

According to Dr. McDonald's (Trinity College Dublin) computer model predictions, the simultaneous 25-fold overexpression of CHO GlcNAc transferase V (GnTV) with the knockdown of CHO β 4galactosyltransferases (β 4GalT) to 10% of their initial amount, is expected to increase the level of triantennary and tetra-antennary structures attached to human chorionic gonadotropin (HCG) whereas the amount of mono- and biantennary forms will likely be reduced. GnTV catalyzes the attachment of a GlcNAc residue of an *N*-glycan to form a tetra-antennary structure (Pierce *et al.*, 1987). Galactosyltransferases are important enzymes for the extension of the glycan chains of glycoproteins by catalyzing the transfer of galactose to GlcNAc, forming Gal(β 1,4)GlcNAc (lactosamine) units in glycoconjugates (Brockhausen *et al.*, 2006).

The aim of this experiment was to establish a genetically modified CHO7J stable cell line, which overexpresses GnTV and has an RNAi-induced knockdown of β 4GalTs, and further investigate the effect of such genetic manipulations on the glycosylation pattern of HCG. For this purpose, human chorionic gonadotropin was purified from the genetically modified CHO7J cells and the *N*-glycans that were formed on its surface were analyzed using the platform developed by Prof. Pauline Rudd's team based in the Dublin-Oxford Glycobiology Laboratory at NIBRT. The analysis was a combination of the normal-phase HPLC (NP-HPLC), exoglycosidase digestions and weak-anion exchange HPLC (WAX-HPLC) whereas specific glycan structures were assigned using the GlycoBase (Version 2.0) software.

6.3 Methods

6.3.1 Growth and maintenance of CHO7J-HCG cell line

The CHOK1 cell line overexpressing HCG was supplied by MSD, Oss, The Netherlands. Cells were cultivated in CD-CHO medium supplemented with 8 mM L-glutamine and HT supplement (100 μ M sodium hypoxanthine and 16 μ M thymidine) to aid cell growth. The initial inoculation of a culture and routine maintenance including cryopreservation and manual cell counting were completed as described in Section 2.2-2.5 for the wild-type CHO cells.

6.3.2 Determination of the optimal concentration of blasticidin

Blasticidin is a nucleoside antibiotic produced by the bacterium *Streptomyces griseochromogenes* (see Figure 6.1). It is a potent translational inhibitor in both prokaryotic and eukaryotic cells. Blasticidin has a fast, potent mode of action, causing rapid cell death at low antibiotic concentrations. The pcDNA6/V5-*myc* vector was constructed such that it contains a blasticidin resistance gene. Resistance to blasticidin is conferred by the product of the *bsd* gene that confers resistance to the antibiotic blasticidin S.

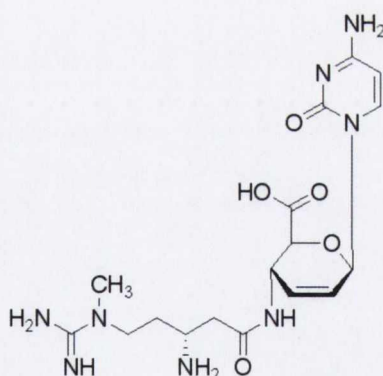


Figure 6.1 Chemical structure of blasticidin.

The *bsd* gene is driven by the SV40 mammalian promoter (Simian Virus 40) in tandem with the bacterial EM7 promoter. In order to determine the optimal concentration of blasticidin S required to kill non-transfected cells, CHO7J cells were seeded according to Section 6.3.1. The next day, the medium was substituted with fresh medium containing blasticidin S at following concentrations: 0 μ g/ml, 2 μ g/ml, 4 μ g/ml, 5 μ g/ml, 6 μ g/ml,

8 µg/ml and 10 µg/ml and the cells were allowed to grow for further 10 days. The cell viability was determined by manual counting of viable cells using Trypan Blue as described in Section 2.2.5 and cell death was observed microscopically every day. The lowest concentration which resulted in cell death within 10 days was 5 µg/ml and was selected for further experiments.

Blasticidin stock (10 mg/ml) was prepared by dissolving 1 mg in 100 µl of sterile MilliQ water. The solution was sterile filtered and stored in small aliquots (10 µl) at -20°C for the maximum of 6 weeks.

6.3.3 Determination of the optimum concentration of puromycin

Puromycin is an aminonucleoside antibiotic produced by the bacterium *Streptomyces alboniger* (see Figure 6.2). Puromycin inhibits protein synthesis by disrupting peptide transfer on ribosomes causing premature chain termination during translation. It is a potent translational inhibitor in both prokaryotic and eukaryotic cells.

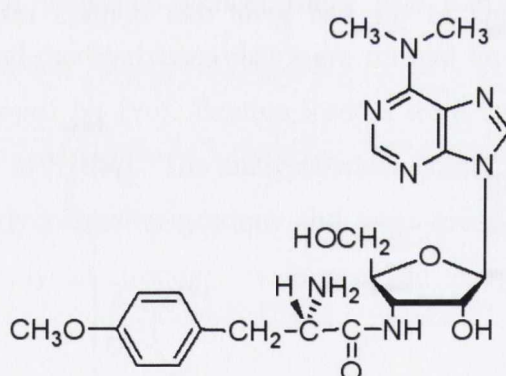


Figure 6.2 Chemical structure of puromycin.

Resistance to puromycin in pRNAT-U6.2/Lenti siRNA expression vectors is conferred by the puromycin N-acetyl-transferase gene (*pac*) from *Streptomyces*. The *pac* gene is driven by the SV40 mammalian promoter (Simian Virus 40). Determination of the optimal concentration of puromycin required to kill non-transfected cells was carried out as for blasticidin in Section 6.3.2 with the exception of the final concentrations, which were 0 µg/ml, 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, 4 µg/ml, 4.5 µg/ml and 5 µg/ml. The

lowest concentration resulting in cell death within 10 days was 4 µg/ml and was selected for further experiments.

6.3.4 Molecular cloning of GnTV cDNA into the pcDNA6/V5-His vector

The GnTV cDNA clone (in pBluescript SK+) was digested with *Xba*I in a total volume of 40 µl as described in Section 3.2.14, and was used directly as a template for polymerase chain reaction. PCR was performed in order to introduce restriction sites for sequential cloning into pBluescript SK+ and pcDNA6/V5-His (see Figure 6.3). pcDNA6/V5-His is a 5.1 kb vector derived from pcDNA3.1(+) and designed for high-level expression, selection on blasticidin, purification, and detection of recombinant proteins in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The vector contains the following elements: human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells, multiple cloning site (MCS) facilitating in-frame cloning with C-terminal tags - V5 epitope and a polyhistidine metal-binding peptide, blasticidin resistance gene (*BSD*) for selection in prokaryotic and eukaryotic cells, and bovine growth hormone (BGH) polyadenylation signal for efficient transcription termination and polyadenylation of mRNA.

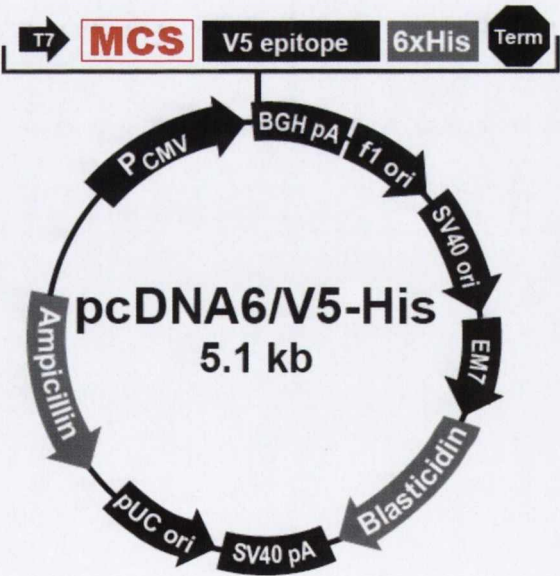


Figure 6.3 pcDNA6/V5-His mammalian expression vectors.

The sense oligonucleotide was designed to introduce a *Hind*III site and a Kozak translation initiation sequence (-GCCACC-) at the ATG initiation codon for proper translation (Kozak, 1987; Kozak, 1990) whereas the antisense oligonucleotide was designed to introduce a *Sac*II restriction site. The stop codon was removed from the 3' untranslated region in order to create a fusion protein with C-terminal peptides – V5 and polyhistidine tags. The sequences and corresponding restriction sites of the oligonucleotides used for amplification of the coding regions of the GnTV are described in Table 6.1.

Table 6.1 Oligonucleotides used for amplification of GnTV cDNA;
(F-forward primer; R-reverse primer).

Name	Sequence	Restriction site
G5-F	5'-CTAAGCTTGCCACCATGGCTTTCTTTACTC-3'	<i>Hind</i> III
G5-R	5'-GTCCGCGGATATAGGCAGTCTTTGCATAG-3'	<i>Sac</i> II

Amplification was completed in a thermal cycler, in a total volume of 25 µl of 1X *Pfu* DNA Polymerase buffer (200 mM Tris-HCl, pH 8.2, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1 % Triton X-100 and 1 mg/ml nuclease-free BSA) containing 1 µl of linearised GnTV-pBluescript SK+, 2 pmol/µl of each primer, 0.2 mM dNTPs, and 0.625 units of *Pfu* DNA polymerase. The PCR program used for amplifying GnTV cDNA is described in Table 6.2.

Table 6.2 GnTV PCR program.

Process		Temperature	Time
Initial denaturation		95°C	2 min
35 cycles	Denaturation	95°C	1 min
	Annealing	64°C	30 sec
	Elongation	72°C	4 min 30 sec
Final elongation		72°C	7 min

The resulting amplification product was resolved by agarose gel electrophoresis as described in Section 3.2.6, and purified using a method described in Section 3.3.7. The GnTV product and pBluescript SK+ were digested with *Hind*III and *Sac*II and the products were purified using a QIAquick PCR purification kit as described in Section 3.3.8. Ligation was completed overnight at 16°C and ligated products were transformed into

competent TOP10 *E. coli* cells as described in Sections 3.3.15. The GnTV sequence was excised from pBluescript SK+ with the restriction enzymes (*Hind*III and *Sac*II), ligated with restriction digested pcDNA6/V5-His and transformed into competent TOP10 *E. coli*. Successful ligations containing the GnTV-encoding insert were analyzed on agarose gel and sent to MWG for full sequence analysis. Maxi preparations were then prepared from *E. coli* according to Section 5.3.2 and the plasmid was linearized as described in Section 6.3.5.

6.3.5 Linearisation and phenol:chloroform:isoamyl alcohol DNA extraction

Phenol:chloroform:isoamyl alcohol extraction is an easy way to remove proteins from nucleic acid samples in a way that nucleic acids remain in the aqueous phase, proteins lie at the interface and RNA or lipids, if present in a mixture, separate into the organic phase. Restriction digest of GnTV-pcDNA6/V5-His was performed in a total volume of 200 µl of 1X reaction buffer containing 10 µg of DNA (0.2 µg/µl final) and 2.5 µl of *Sca*I. Restriction digest was carried out at 37°C for 2 h. Removal of proteins (BSA from the reaction buffer and *Sca*I enzyme) from the digestion mixture was achieved by adding 600 µl of cold phenol:chloroform:isoamyl alcohol solution (25:24:1). The sample was placed on a shaking device for 10 min followed by centrifugation at 16,000 g for 30 min at 4°C. The aqueous layer, which was formed during the previous step, was removed and placed in a fresh tube. In order to precipitate DNA from the suspension, 100 µl of 5M sodium acetate were used followed by adding 800 µl of ice-cold isopropanol, which had been stored at -20°C for at least an hour. The mixture was placed in a -20°C freezer for 30 min and centrifuged as before. The supernatant was removed and washed with cold 70% ethanol followed by 10 min centrifugation at 16,000 g. Ethanol was removed and the pellet was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

6.3.6 Establishing a stable line overexpressing GnTV in CHO7J cells

CHO7J cells overexpressing HCG were transfected by nucleofection (see Section 5.3.3) with the linearized GnTV-pcDNA6/V5-His construct and were allowed to recover for 48 hours in non-selective medium. After this time, cells were treated with selective medium containing 5 µg/ml blasticidin, as determined previously and aliquoted into small volumes that contained approximately 500 cells/well on a 96 well plate. Selective medium was changed every 3 days. The stably transfected cells were screened for the overexpression of

GnTV by Western blotting after 14 days of antibiotic selection (see Sections 2.2.7 and 5.3.4). The cultures with the highest expression of GnTV were expanded for further week and then frozen in the liquid nitrogen as described in Section 2.2.3.

6.3.7 Designing and production of *GalT* siRNA constructs

All siRNA constructs used in this project were designed and prepared by Dr. Tania O'Connor (Trinity College Dublin). A small DNA insert (about 70 bp) encoding a short hairpin RNAs (shRNA) targeting six galactosyltransferases (see Figure 6.4) were cloned into pRNAT-U6.2/Lenti between *Bam*HI and *Xho*I sites (see Figure 6.5). The key features of this vector are the strong U6.2 human promoter, the puromycin resistance cassette and expression of the coral GFP (cGFP) marker under CMV promoter control to track the transfection efficiency. The pRNAT lenti-constructs were transformed into chemically competent Stbl3 *E.coli* cells, which were designed for cloning direct repeats found in lentiviral expression vectors. In order to produce lentiviral particles, 293T HEK packaging cells were transfected with the endotoxin-free maxi preparations of pRNAT lentivectors using ViraPower Packaging Mix according to manufacturer's instructions. Cell supernatants from 293T HEK cells were collected and the viral titer was determined.

BamHI Antisense Loop Sense XhoI

GGATCCCGACCGAAGTCCAGTTGCTCTTGATATCCGAGCAACTGGACTTCGGTCTTTTTTCCAAC TCGAG

Termination Signal

BamHI Antisense Loop Sense XhoI

GGATCCGTCATAGGTAGCATCCTCCTTCTTGATATCCGGAAGGAGGATGCTACCTATGATTTTTTCCAAC TCGAG

Termination Signal

BamHI Antisense Loop Sense XhoI

GGATCCCGTATTGGTCAGGAGTGAGCGCTTTTGATATCCGAGCGCTCACTCCTGACCAATATTTTTCCAAC TCGAG

Termination Signal

BamHI Antisense Loop Sense XhoI

GGATCCCGTATTGGTCAGGAGTGAGCGCTTTGATATCCGAGGTGAACGTGGACCGGATGATTTTTTCCAAC TCGAG

Termination Signal

BamHI Antisense Loop Sense XhoI

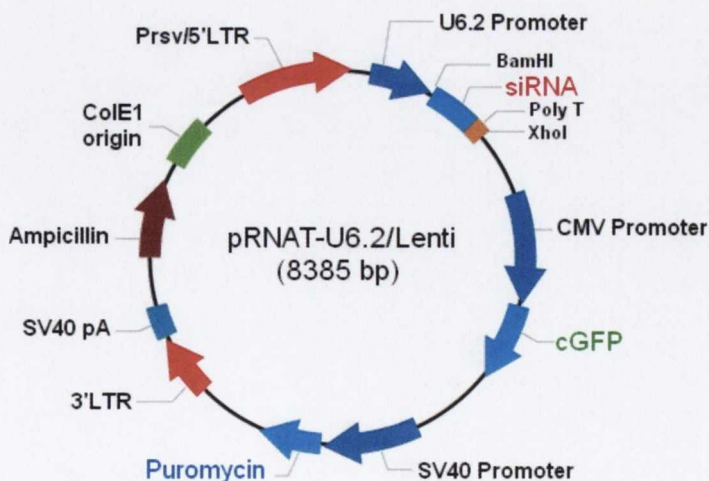
GGATCCGTTGTTTGCAAAGTAGTTCAGGTTTGATATCCGACCTGA⁺ACTACTTTGCAAACA⁺TTTTTTCCA⁺ACTCGAG

Termination Signal

BamHI Antisense Loop Sense XhoI

GGATCCGTTGTTCAATCCATCGATGTACTTGATATCCG**G**TACATCGATGGATTGAACA**A**TTTTTCCA**A**CTCGAG

Termination Signal



6.3.8 Establishing a stable cell line expressing cGFP and GalT siRNAs

The overview of the RNAi strategy used for silencing CHO galactosyltransferases is shown in Figure 6.6. Lentiviral constructs containing six GalT siRNAs were prepared as described in Section 6.3.7. Three days before transduction, the GnTV-CHO7J cells were seeded in normal growing medium at 2×10^5 cells/ml. Prior transduction the medium was substituted with fresh conditioned medium so that the final cell density was 5×10^5 cell/ml. All procedures were carried out in the viral hood and the cells were kept in a designated viral incubator. GalT siRNA lentiviral constructs and the control siRNA (0.5 ml each) were introduced into GnTV-CHO7J cells (10 ml) followed by addition of 6 μ l polybrene (10 mg/ml). Cells were left to grow for two more days in the viral incubator. After this time, cells were spun down at 100 g for 5 min followed by the medium replacement and further growth for two more days. In the meantime, the effectiveness of transduction was monitored by fluorescence microscopy. Medium was replaced again and the cells were grown for further 5 days in the culture medium containing 4 μ g/ml puromycin. In order to select puromycin resistant but also GFP positive clones, cell cultures were selected by FACS as described in Section 6.3.9. After FACS, each GalT-GnTV-CHO7J cell line (10 ml) was placed in a T75 flask and expanded to a volume of 50 ml (1×10^6 cell/ml). An aliquot of cells was taken from each flask in order to extract the total RNA as described in Section 6.3.10 followed by the first-strand cDNA synthesis and the quantitative real-time PCR as described in Section 6.3.12. Frozen cell stocks were prepared as described in Section 2.2.3.

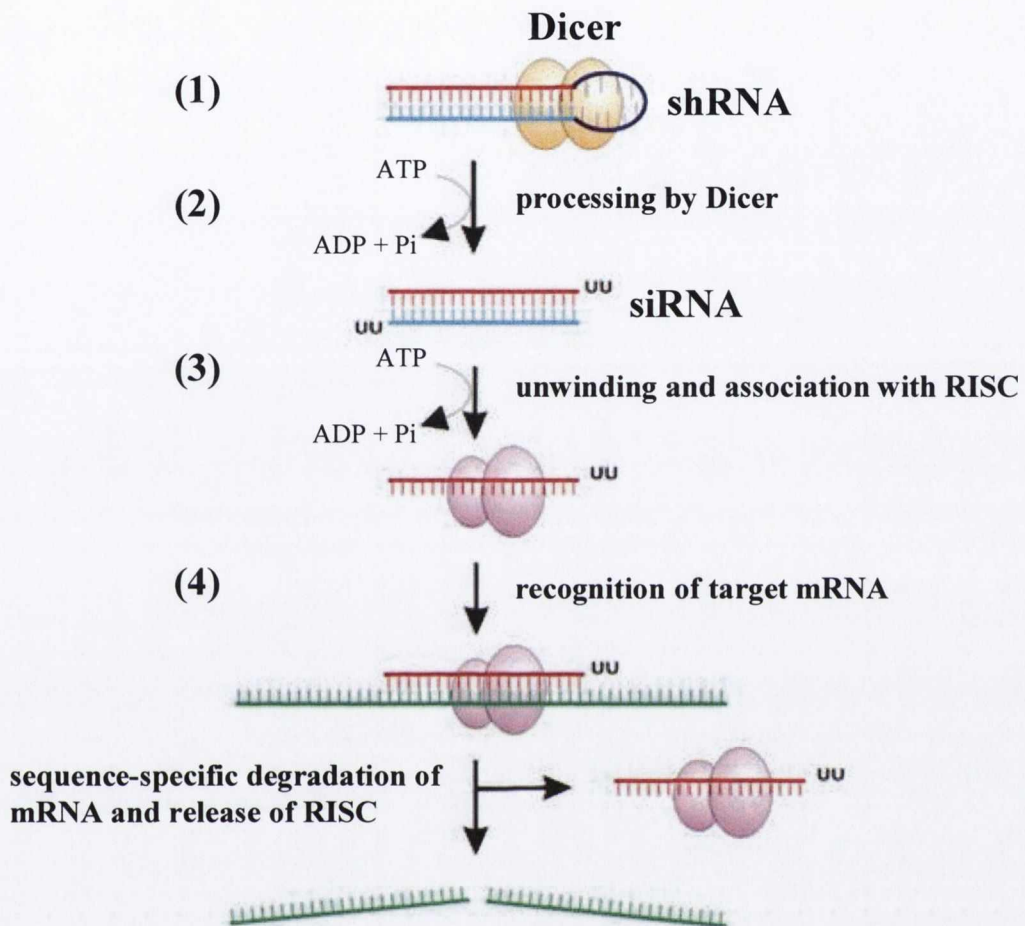


Figure 6.6 RNA silencing overview.

The siRNA construct is introduced into target cells and expresses short hairpin RNA (shRNA) molecules for long term gene silencing (1). The short hairpin RNA is rapidly processed by the cellular machinery (Dicer) into 19-22 bp double stranded RNA (siRNA) (2). The siRNA unwinds and assembles into RNA-induced silencing complexes (RISCs) (3). The antisense siRNA strand guides RISC to the complementary target mRNA to cleave it, leading to specific gene silencing (4).

6.3.9 Fluorescence-activated cell sorting (FACS)

GalT-GnTV-CHO7J cells from each T75 flask (15 ml) were centrifuged for 5 min, at 100 g and room temperature. The supernatant was carefully removed, and the cells were resuspended in 400 µl of fresh medium. A cell strainer was used to obtain a single cell suspension. Cells were further processed by FACS (see Figure 6.7). GFP positive cells were expanded and frozen in liquid nitrogen.

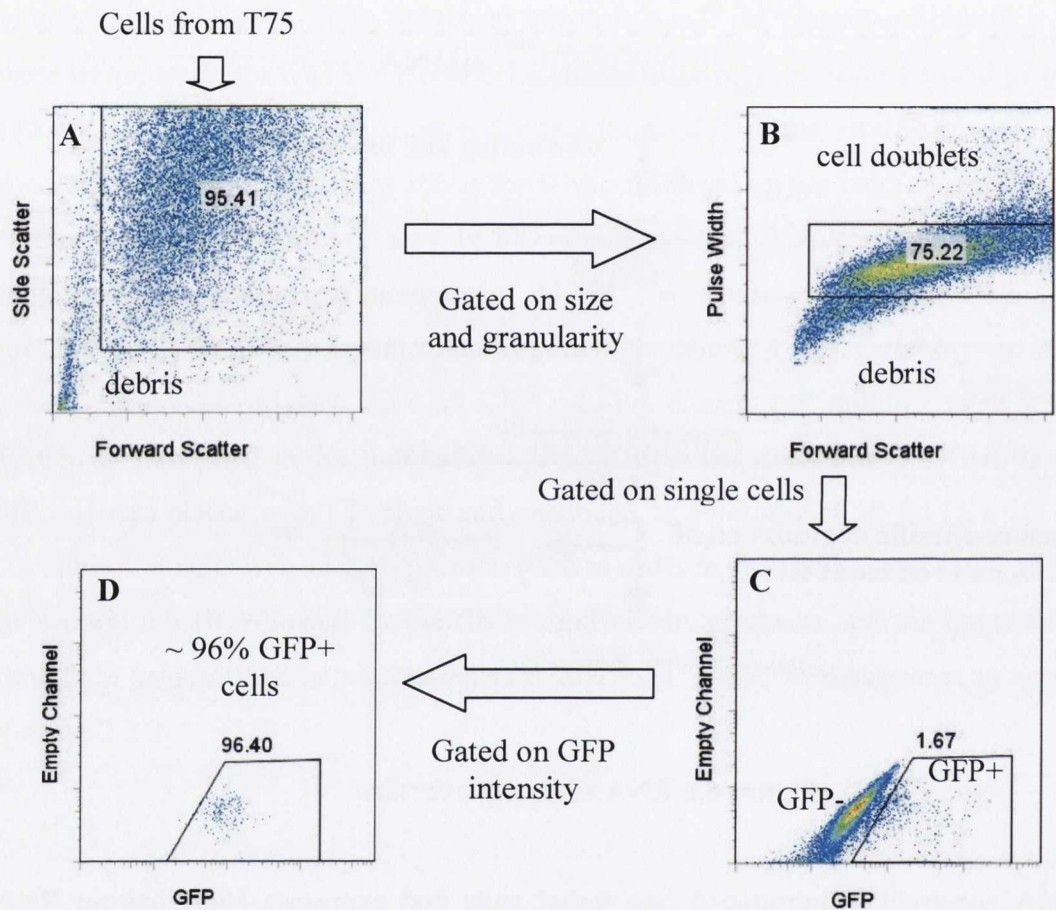


Figure 6.7 Selection of GFP+ cells using FACS.

Figure A illustrates the scatter of size (forward scatter) and granularity (side scatter) in the cell population. Figure B shows the accurate gating of single cells based on propidium iodide incorporation, with the ungated areas containing cell debris and aggregates. Diagrams C and D illustrate the gating of the single cells based on fluorescence and the percentage of GFP+ cells in the final cell population, respectively.

6.3.10 Extraction of RNA using RNeasy Mini Kit

RNeasy Mini Kit is a complete set of reagents for the isolation of total RNA from diverse biological material. The isolated RNA can be used directly for any downstream procedures including quantitative real-time PCR. The cell pellet (1×10^7 cells) was resuspended in 600 μ l of Buffer RLT and vortexed for 3 sec to obtain a homogenous suspension. The homogenate was then applied onto a QIAshredder spin column to improve the lysis. Then, 600 μ l of 70% ethanol were added, mixed well and the suspension was transferred onto an RNeasy spin column. The supernatant was collected by centrifugation at 10,000 g for 15 min at room temperature and discarded. Buffer RWI (350 μ l) was applied onto the column and the sample was centrifuged as before. In order to eliminate genomic DNA, the sample was treated with 10 μ l DNase I that was prepared in Buffer RDD according to manufacturer's instructions. The reaction was incubated for 5 min at room temperature followed by applying Buffer RW1 (350 μ l and then 700 μ l). The column was washed twice with 500 μ l Buffer RPE and the sample was centrifuged as before. RNA was collected to a fresh RNase-free tube by applying 50 μ l of RNase-free water and by centrifugation at 10,000 g at room temperature. The RNA concentration was estimated as described in Section 2.2.9.

6.3.11 AffinityScript QPCR cDNA Synthesis

The AffinityScript QPCR cDNA Synthesis Kit was designed for the highest efficiency conversion of RNA to cDNA and is fully optimized for two-step quantitative reverse transcription-PCR (QRT-PCR) applications. The kit includes the genetically engineered version of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), which is highly thermostable. The system can be used with as little as 1 ng or as much as 5 μ g of total RNA. The first-strand cDNA synthesis reaction mixture was prepared, with each component being added in the following order: RNase-free water, 10 μ l of 2 x first strand master mix, 3 μ l of 100 ng/ μ l random primers, 1 μ l of AffinityScript RT/RNase Block enzyme mixture and 2 μ g of total RNA extracted from CHO cells in a total of 20 μ l. All steps were carried out in the PCR machine that was set to the following program: 25 °C for 5 min, 42°C for 15 min and 95°C for 5 min. The cDNA synthesis reaction was used directly for QPCR or stored at -20°C until required.

6.3.12 Quantitative Real-Time PCR

The quantitative real-time PCR (QPCR) was used to verify the percentage of GnTV overexpression and GalT knockdown in the genetically modified CHO7J cells. Primers were designed using Beacon Designer 7 software (see Table 6.3) and were based on the sequence data shown in Table 6.4. The optimal final concentrations and binding efficiencies of primers are shown in Table 6.5 and 6.6, respectively.

Table 6.3 Properties of QPCR primers.

Name	Sequence	% GC	Tm (°C)	Ta (°C)	qRT product
GalT1-F	5'-TCAACATAGGCTTCCAAGAGG-3'	48	57.9	51.2	168 bp
GalT1-R	5'-GAACATAAGGCAGGCTAAACC-3'	48	57	53.0	
GalT2-F	5'-CTCAGAGGTTTAGTAAGATTC-3'	38	54.0	49.0	171 bp
GalT2-R	5'-TTGTCCATTAGTGTCAGC-3'	44	51.4	46.4	
GalT3-F	5'-TATAGTAACCTCAGTCACC-3'	42	52.4	47.4	123 bp
GalT3-R	5'-GCTAAATGATACCGACAC-3'	44	51.4	46.4	
GalT4-F	5'-AATCCTGATGTGGGTAAATAC-3'	38	54.0	49.0	200 bp
GalT4-R	5'-ACACCAGTCCAGAAATCC-3'	50	53.7	48.7	
GalT5-F	5'-GTCGTCTTCGCTGCTCTAC-3'	58	58.8	53.8	150 bp
GalT5-R	5'-GGCTGCTGTTCTCTTGG-3'	61	58.2	53.2	
GalT6-F	5'-GCATCGCCAACACATACCTC-3'	55	59.4	54.4	194 bp
GalT6-R	5'-TGTGAAGTTCTGTGGAAGATACG-3'	43	58.9	53.9	
GnTV-F	5'-AATGTGTATTGCCTCCTATG-3'	40	53.2	48.2	179 bp
GnTV-R	5'-TGCTCTCCAAGGTAAACG-3'	50	53.7	48.7	
β-actin-F	5'-GTGCGTGACATCAAAGAG-3'	50	53.7	48.7	195 bp
β-actin-R	5'-GCCACAGGATTCCATACC-3'	56	56.0	51.0	

Table 6.4 Sequence data for each template used in QPCR experiments.

Gene	Length	Accession Number
CHO GalT1	1262 bp	AF318896
CHO GalT2	1176 bp	AY117536
CHO GalT3	1338 bp	AY117537
CHO GalT4	1188 bp	AY117538
CHO GalT5	1255 bp	AY117539
CHO GalT6	1149 bp	AF506839
CHO GnTV	2223 bp	U62587
CHO β-actin	1128 bp	U20114

Table 6.5 Final concentrations of QPCR primers.

Name	Optimum Sense primer (nM)	Optimum Antisense primer (nM)
GalT1	600	400
GalT2	400	200
GalT3	600	400
GalT4	600	200
GalT5	400	600
GalT6	400	600
GnTV	600	200
β -actin	600	200

Table 6.6 The binding efficiencies of QPCR primers.

Primer set	% efficiency
GalT6	99.2%
GalT1	113%
GalT2	113.4%
GalT3	112.2%
GalT4	128.5%
GalT5	112.4%
GnTV	108.8%
β -actin	123.2%

Working primer stocks were made so that only 1 μ l of forward and 1 μ l reverse primer were added to each QPCR reaction to give the optimum primer concentration as described in Table 6.7.

Table 6.7 QPCR primer stocks.

Final concentration (nM)	Stock (μ M)	Stock 100 μ M (μ l)	RNase-free dH ₂ O (μ l)
200	5	2	38
400	10	4	36
600	15	6	34

The equivalent of 90 ng (4.5 μ l of first strand cDNA - 20 ng/ μ l) of input RNA was used for all reaction, as this was on the linear part of the standard curves and gave an appropriate Ct value. The components of each reaction mixture are described in Table 6.8.

Table 6.8 QPCR reaction mixtures.

Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
A1, A2, A3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
A4, A5, A6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT1	1 µl GalT1	12.5 µl
B1, B2, B3	6 µl	4.5 µl of 20 ng/µl GalT1	1 µl β-actin	1 µl β-actin	12.5 µl
B4, B5, B6	6 µl	4.5 µl of 20 ng/µl GalT1	1 µl GalT1	1 µl GalT1	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
C1, C2, C3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
C4, C5, C6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT2	1 µl GalT2	12.5 µl
D1, D2, D3	6 µl	4.5 µl of 20 ng/µl GalT2	1 µl β-actin	1 µl β-actin	12.5 µl
D4, D5, D6	6 µl	4.5 µl of 20 ng/µl GalT2	1 µl GalT2	1 µl GalT2	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
E1, E2, E3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
E4, E5, E6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT3	1 µl GalT3	12.5 µl
F1, F2, F3	6 µl	4.5 µl of 20 ng/µl GalT3	1 µl β-actin	1 µl β-actin	12.5 µl
F4, F5, F6	6 µl	4.5 µl of 20 ng/µl GalT3	1 µl GalT3	1 µl GalT3	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
G1, G2, G3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
G4, G5, G6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT4	1 µl GalT4	12.5 µl
H1, H2, H3	6 µl	4.5 µl of 20 ng/µl GalT4	1 µl β-actin	1 µl β-actin	12.5 µl
H4, H5, H6	6 µl	4.5 µl of 20 ng/µl GalT4	1 µl GalT4	1 µl GalT4	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
I1, I2, I3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
I4, I5, I6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT5	1 µl GalT5	12.5 µl
J1, J2, J3	6 µl	4.5 µl of 20 ng/µl GalT5	1 µl β-actin	1 µl β-actin	12.5 µl
J4, J5, J6	6 µl	4.5 µl of 20 ng/µl GalT5	1 µl GalT5	1 µl GalT5	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
K1, K2, K3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
K4, K5, K6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GalT6	1 µl GalT6	12.5 µl
L1, L2, L3	6 µl	4.5 µl of 20 ng/µl GalT6	1 µl β-actin	1 µl β-actin	12.5 µl
L4, L5, L6	6 µl	4.5 µl of 20 ng/µl GalT6	1 µl GalT6	1 µl GalT6	12.5 µl
Position in PCR racks	dH ₂ O	1 st strand cDNA	For Primer	Rev Primer	SYBR mix
M1,M2,M3	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl β-actin	1 µl β-actin	12.5 µl
M4,M5,M6	6 µl	4.5 µl of 20 ng/µl CHO7J	1 µl GnTV	1 µl GnTV	12.5 µl
N1, N2, N3	6 µl	4.5 µl of 20 ng/µl GnTV	1 µl β-actin	1 µl β-actin	12.5 µl
N4, N5, N6	6 µl	4.5 µl of 20 ng/µl GnTV	1 µl GnTV	1 µl GnTV	12.5 µl

The MxPro software on Stratagene Mx3000P machine was used to comparatively quantitate the DNA levels according to user-defined parameters (see Table 6.9).

Table 6.9 QPCR plate set-up.

calibrator 1 A NORM (Wild Type)	calibrator 1 A NORM (Wild Type)	calibrator 1 A NORM (Wild Type)	calibrator 2 A GalT1 primers (Wild Type)	calibrator 2 A GalT1 primers (Wild Type)	calibrator 2 A GalT1 primers (Wild Type)
unknown 3 B NORM (GalT1 KD)	unknown 3 B NORM (GalT1 KD)	unknown 3 B NORM (GalT1 KD)	unknown 4 B GalT1 primers (GalT1 KD)	unknown 4 B GalT1 primers (GalT1 KD)	unknown 4 B GalT1 primers (GalT1 KD)
calibrator 5 C NORM (Wild Type)	calibrator 5 C NORM (Wild Type)	calibrator 5 C NORM (Wild Type)	calibrator 6 C GalT2 primers (Wild Type)	calibrator 6 C GalT2 primers (Wild Type)	calibrator 6 C GalT2 primers (Wild Type)
unknown 7 D NORM (GalT2 KD)	unknown 7 D NORM (GalT2 KD)	unknown 7 D NORM (GalT2 KD)	unknown 8 D GalT2 primers (GalT2 KD)	unknown 8 D GalT2 primers (GalT2 KD)	unknown 8 D GalT2 primers (GalT2 KD)
calibrator 9 E NORM (Wild Type)	calibrator 9 E NORM (Wild Type)	calibrator 9 E NORM (Wild Type)	calibrator 10 E GalT3 primers (Wild Type)	calibrator 10 E GalT3 primers (Wild Type)	calibrator 10 E GalT3 primers (Wild Type)
unknown 11 F NORM (GalT3 KD)	unknown 11 F NORM (GalT3 KD)	unknown 11 F NORM (GalT3 KD)	unknown 12 F GalT3 primers (GalT3 KD)	unknown 12 F GalT3 primers (GalT3 KD)	unknown 12 F GalT3 primers (GalT3 KD)
calibrator 13 G NORM (Wild Type)	calibrator 13 G NORM (Wild Type)	calibrator 13 G NORM (Wild Type)	calibrator 14 G GalT4 primers (Wild Type)	calibrator 14 G GalT4 primers (Wild Type)	calibrator 14 G GalT4 primers (Wild Type)
unknown 15 H NORM (GalT4 KD)	unknown 15 H NORM (GalT4 KD)	unknown 15 H NORM (GalT4 KD)	unknown 16 H GalT4 primers (GalT4 KD)	unknown 16 H GalT4 primers (GalT4 KD)	unknown 16 H GalT4 primers (GalT4 KD)
calibrator 13 I NORM (Wild Type)	calibrator 13 I NORM (Wild Type)	calibrator 13 I NORM (Wild Type)	calibrator 14 I GalT5 primers (Wild Type)	calibrator 14 I GalT5 primers (Wild Type)	calibrator 14 I GalT5 primers (Wild Type)
unknown 15 J NORM (GalT5 KD)	unknown 15 J NORM (GalT5 KD)	unknown 15 J NORM (GalT5 KD)	unknown 16 J GalT5 primers (GalT5 KD)	unknown 16 J GalT5 primers (GalT5 KD)	unknown 16 J GalT5 primers (GalT5 KD)
calibrator 13 K NORM (Wild Type)	calibrator 13 K NORM (Wild Type)	calibrator 13 K NORM (Wild Type)	calibrator 14 K GalT6 primers (Wild Type)	calibrator 14 K GalT6 primers (Wild Type)	calibrator 14 K GalT6 primers (Wild Type)
unknown 15 L NORM (GalT6 KD)	unknown 15 K NORM (GalT6 KD)	unknown 15 L NORM (GalT6 KD)	unknown 16 L GalT6 primers (GalT6 KD)	unknown 16 L GalT6 primers (GalT6 KD)	unknown 16 L GalT6 primers (GalT6 KD)
calibrator 13 M NORM (Wild Type)	calibrator 13 M NORM (Wild Type)	calibrator 13 M NORM (Wild Type)	calibrator 14 M GnTV primers (Wild Type)	calibrator 14 M GnTV primers (Wild Type)	calibrator 14 M GnTV primers (Wild Type)
unknown 15 N NORM (GnTV KD)	unknown 15 N NORM (GnTV KD)	unknown 15 N NORM (GnTV KD)	unknown 16 N GnTV primers (GnTV KD)	unknown 16 N GalT6 primers (GnTV KD)	unknown 16 N GnTV primers (GnTV KD)

The “calibrator” is the wild type sample with the β -actin and GnTV or GalT primers. “NORM” stands for the normalizer housekeeping primer set (ie. β -actin). “Unknown” is the overexpressed or knock-down sample with the β -actin and GnTV or GalT primers. The QPCR is described in Table 6.10.

Table 6.10 QPCR program.

Process		Temperature	Time
Initial denaturation		95°C	10 min
40 cycles (association curve)	Denaturation	95°C	30 sec
	Annealing	50°C	1 min
	Elongation	72°C	30 sec
1 cycle (dissociation curve)	Denaturation	95°C	1 min
	Annealing	55°C	30 sec
	Elongation	72°C	30 sec

6.3.13 Immunoaffinity purification of HCG from CHO7J culture medium

A culture supernatant was filtered through a 1.2-micron filter and stored at -20°C until required. A column of 1 cm diameter and 10 cm length was carefully filled with NHS-activated Sepharose 4 FF resin conjugated to the monoclonal anti- β -HCG 119A antibody, which was a kind gift from MSD (Oss, The Netherlands). Buffers were filtered through a 0.22 μ m filter and stored at 4°C. All purification steps were carried out at 4°C. A simplified flow diagram of the purification process is shown in Figure 6.8. The cell free culture medium was thawed overnight at 4°C, mixed thoroughly to ensure homogeneity and then diluted 1:1 with PBS-methionine buffer (4.1 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.76 mM KH_2PO_4 , 0.13 M NaCl, 0.1 mg/ml methionine, pH 7.2). The column was equilibrated using at least 50 ml of PBS-methionine at a flow rate of 2 ml/min. After loading, two washing steps were performed: using 50ml of wash 1 buffer (2M NaCl, 4.1 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.76 mM KH_2PO_4 , 0.1 mg/ml methionine, pH 7.2), and then with 50 ml of PBS-methionine. Elution of HCG from the column was achieved using 45 ml of 10 mM ammonium acetate buffer at pH 2.7. This elution fraction was neutralized immediately to a pH of 7-8, using 25% ammonia. The column was neutralised using 50 ml of 1M Tris, pH 8.0 and was stored in the cold room after flowing 50ml of PBS (6.5 mM phosphate, 0.13 M NaCl, pH 7.2) in 20% ethanol through the system. The neutralised elution fraction was concentrated using Amicon Ultra-15 units with a molecular weight limit of 10 kDa. The HCG containing eluate (2.5 ml) was applied onto a PD-10 column that had been pre-equilibrated with 25 ml of MilliQ water. Samples were eluted in 3.5 ml MilliQ water followed by BCA protein assay. HCG was aliquoted into minitubes (45 μ g each), snap-frozen in liquid nitrogen and freeze dried overnight.

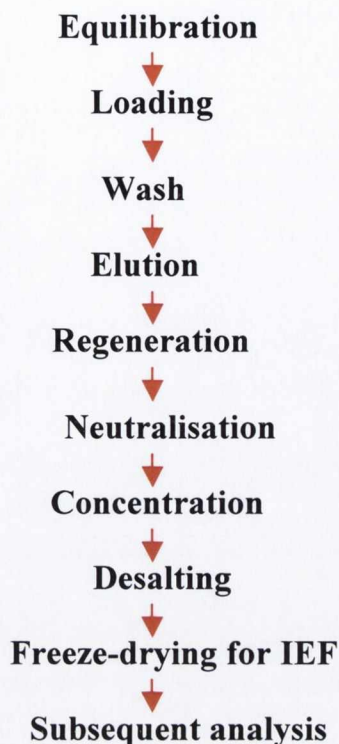


Figure 6.8 Immunoaffinity purification scheme.

6.3.14 Desalting Ovitrelle for use as a HCG standard in IEF

Recombinantly produced HCG (Ovitrelle) was used as an IEF standard. A sample of Ovitrelle (2.5 ml) was carefully transferred onto a pre-equilibrated PD-10 desalting column and the flow-through was discarded. The sample was eluted with 3.5 ml of water and the protein content was determined by BCA assay as described in Section 2.2.7. The resulting eluate was aliquoted into minitubes (45 µg each), freeze-dried overnight and the remaining pellet was processed as described in Section 6.3.15 and 6.3.16.

6.3.15 Isoelectric Focusing of HCG

Isoelectric focusing (IEF) was performed on a Multiphor II Electrophoresis System with an EPS3501XL power pack and the temperature was controlled using a waterbath. Kerosene (3 ml) was applied to the electrophoresis plate which had been precooled to 4°C. A precast pI 3-10 gel was carefully laid down (red side at the anode) to ensure no air bubbles were trapped underneath it. One of the electrode wicks was wetted in anode fluid 3 and placed on the red side of the gel. Another was soaked with cathode fluid 10 and carefully placed to the black side of the gel. The sample application strip was placed 1 cm away from the

cathode wick. The system was connected to the power supply and prefocused according to the conditions detailed in Table 6.11.

Table 6.11 Prefocusing and Focusing Conditions of IEF.

	Prefocusing	Focusing
Temp (°C)	4	4
Time	30 min	~ 3 hours
Potential over time	-	7000
Potential (V)	2000	2000
Current (mA)	Adjust till potential reaches about 200 V (approx 7 mA)	Adjust till potential reaches about 200 V (approx 7 mA)
Power (W)	12	12

After this time, freeze-dried samples were reconstituted in 15 µl of 3M urea (freshly made). The electrophoresis system was disconnected and the samples were then applied onto the application strip together with the pI markers (3 – 10). The gel was subject to the focusing conditions detailed in the above table until 7000 Vh was reached. After focusing, excess kerosene was removed from the back of the gel, and the gel was placed in fixation buffer (20% trichloroacetic acid) for 40 minutes, at room temperature with agitation. The gel was washed twice for 1 minute in destain (3% orthophosphoric acid) and then stained for 15 minutes in the freshly made stain (equal volumes of 20% orthophosphoric acid solution and Serva Violet 17 (2g/L) solution). The gel was destained with three changes of buffer every 8 minutes, and washed with MilliQ water twice for 5 minutes.

6.3.16 Analysis of N-Glycan structures of HCG

The analysis of glycan structures attached to HCG was performed as previously described by Royle *et al.*, 2006 and Campbell *et al.*, 2008 (see Figure 6.9).

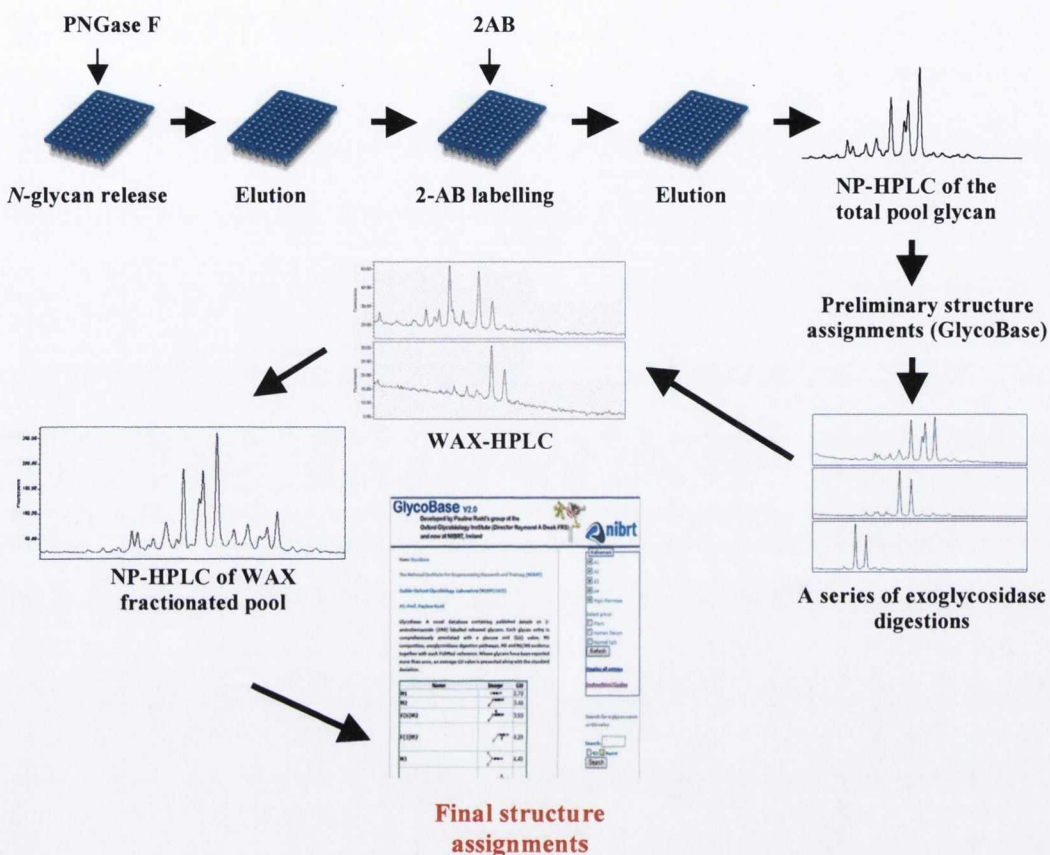


Figure 6.9 Overview of the process to determine the *N* – glycans attached to HCG.

The three bands of HCG from SDS-PAGE were excised and incubated with PNGase F (**P**eptide **N**-**G**lycosidase **F**) overnight in order to release the glycans from the protein. The released glycans were eluted and dried down before labelling with fluorescent 2-aminobenzamide (2-AB). The labelled glycans were eluted, purified and dried followed by NP-HPLC profiling of the entire glycan pool for each sample. Preliminary assignments for glycan structures were made with the use of GlycoBase (Version 2.0). A series of exoglycosidase digestions were carried out alongside WAX fractionation and collection of the glycan pool into differentially charged species, followed by NP-HPLC profiling in order to complete the process of final structural assignments.

6.3.16.1 Denaturing SDS-PAGE

Purified HCG samples were resolved by 12% SDS-PAGE gel as described in Section 2.2.6.

6.3.16.2 N-Glycan release

The excised gel pieces were transferred to a 96-well filter plate, washed with acetonitrile (1ml) and placed on a shaker for 10 min. After this time, the liquid was removed with the use of a vacuum manifold for 96-well plates. This procedure was repeated with 1 ml of PNGase F buffer (20mM NaHCO₃, pH 7.0), 1 ml of acetonitrile, 1 ml of PNGase F buffer and 1 ml of acetonitrile. The filter plate was placed over a new collection block (2 ml polypropylene 96 well blocks). The glycans were released by overnight incubation with a 100 mU/ml solution of PNGase F at 37°C. A sufficient volume of enzyme solution was added to ensure all gel pieces had re-swollen (approximately 50 µl). After five minutes the gel pieces were covered with an additional 50 µl of PNGase F buffer and sealed with adhesive film.

6.3.16.3 N-glycan extraction

MilliQ water (200 µl) was added to the gel pieces, and the plate was placed on the shaker for 10 minutes. After this time, the released glycans were vacuumed to the collection block and retained. The procedure was repeated with 200 µl of MilliQ water, 200 µl of MilliQ water, 200 µl of acetonitrile, 200 µl of MilliQ water and 200 µl of acetonitrile. The sample was then dried down in a vacuum centrifuge. The released glycans were converted to aldoses by reaction with 20 µl of 1% formic acid at room temperature for 40 minutes. The glycans were dried down to remove the formic acid and resuspended in MilliQ water. At this point a portion of the unlabelled glycans could also be retained for further analysis. Samples to be labelled with 2-AB were dried down once more.

6.3.16.4 2-AB labelling and glycan purification

The 2-aminobenzamide labelling solution (2-AB) was added to the tube containing the dried glycans. The tube was vortexed and centrifuged before incubation at 65°C for 30 minutes. After this time, the samples were vortexed again, centrifuged as before and incubated for another 2.5 hours. During this time 1 cm² pieces of 3MM Whatman chromatography paper were prepared by washing in MilliQ water followed by drying at

65°C for an hour. The paper was then folded in four and placed into a Whatman protein precipitation plate (pre-washed with 200 µl acetonitrile vacuumed to waste and repeated with 200 µl water). The 2-AB sample was applied to the paper and allowed to dry for 15 min at room temperature. The free 2-AB was removed with acetonitrile washing steps as follows - addition of 1.5 ml acetonitrile to each well, agitation on a shaker for 15 min followed by removal. The glycans were eluted by placing the Whatman plate on top of a new collection block followed by addition of 900 µl MilliQ water and shaking for 30 minutes. The liquid was removed by vacuum and the procedure was repeated once more. The sample was dried overnight in a vacuum centrifuge and resuspended in 100 µl MilliQ water.

6.3.16.5 NP-HPLC profiling of 2-AB labelled N-glycans

The 2-AB labelled glycans were separated using a Waters 2695 separations module with a 2475 fluorescence detector. The detector was set to maximum sensitivity with excitation and emission wavelengths set to 330 and 420 nm respectively. A TSKgel Amide-80 column was used (3 µm x 250 mm x 4.6 mm). Solvent A was composed of 50 mM ammonium formate, pH 4.4 while solvent B was acetonitrile. Samples were prepared in 80% acetonitrile. The elution gradient was as follows: a linear gradient of 35 to 47% solvent A over 48 minutes, followed by 1 minute at 47% to 100% A, returning to 35% A over 1 minute and then finishing with 35% A for 6 minutes, at a flow rate of 0.48 ml/min, and 30°C. All samples were prepared in 80% acetonitrile. In order to pre-equilibrate the column before use a blank was used to initiate a run. A dextran ladder was run every 24 hours in order to calibrate and assign GU values for the resulting peaks.

6.3.16.6 Exoglycosidase digestions

In order to identify the sequence, monosaccharide type and linkage of the sugar residues exoglycosidase digestion were carried out. The enzymes used were sialidase A (ABS), sialidase S (NANI), bovine kidney fucosidase (BKF), bovine testis galactosidase (BTG), *N*-acetylglucosaminidase (GUR) and jack bean mannosidase (JBM) and the detailed specificities are described in Table 6.12. A total of 6 digests were set up, with the ABS, BKF, BTG, GUR digests performed with double the amount of glycans. This was done in order to carry out a JBM digest on appropriate structures and under the correct conditions. A pool of representative samples was made and aliquots were taken for digestion analysis

(and WAX HPLC, Section 6.3.14.7). Aliquots (20 μ l) were dried down in a 96 well plate followed by addition of the required enzyme array to each well as described in Table 6.13. The buffer used was 500 mM sodium acetate, pH 5.5. Samples were vortexed before incubation at 37°C overnight. The next day, half of the ABS, BKF, BTG, and GUH digest was further treated with JBM to digest to the 2-AB core (this digest was set up initially with double the amount of glycans to accommodate for this). This involved addition of 4 μ l of enzyme, 4 μ l of MilliQ water and 21 μ l of JBM buffer (100 mM sodium acetate, 2 mM Zn²⁺, pH 5.0). This digest was incubated at 37°C for a further 24 hours. The enzymes were removed from the mixture with use of a Nanosep centrifugal device (10k MWCO). The membrane was pre-washed with 200 μ l MilliQ water by centrifuging at 14,000 g for 10 minutes. The flow through was discarded and the digested sample was applied to the membrane and centrifuged for 2 minutes at 14,000 g. The digestion tube was rinsed with 20 μ l of MilliQ water, which was also applied to the membrane and the centrifugation process was repeated. Finally, the membrane was rinsed with a final 100 μ l of water and centrifuged at 14,000 g for 2 minutes. Samples were dried down and resuspended in 20 μ l water.

Table 6.12 Exoglycosidases used in *N*-glycan release. All enzymes were used according to manufacturer's instructions.

Exoglycosidase	Specificity	Source
Sialidase A (ABS)	α 2,6, α 2,3 and α 2,8 non-reducing terminal sialic acids	<i>Arthrobacter ureafaciens</i>
Sialidase S (NANI)	α 2-3 and α 2,8 non-reducing terminal sialic acids	<i>Streptococcus pneumoniae</i>
α-Fucosidase (BKF)	α 1,2, α 1,3, α 1,4, α 1,6 fucose	Bovine kidney
β-Galactosidase (BTG)	β 1,3, β 1,4 galactose	Bovine testes
Hexosaminidase (GUH)	β G1cNAc (not bisecting GlcNAc linked to Man)	<i>Streptococcus pneumoniae</i>

Table 6.13 The composition of digests reaction used for HCG *N*-glycan structure assignment.

	Digest pool	Buffer (μl)	Water (μl)	ABS (μl)	BKF (μl)	BTG (μl)	GUH (μl)	NAN1 (μl)
1	ABS	1	8	1	-	-	-	-
2	ABS, BKF	1	7	1	1	-	-	-
3	ABS, BTG	1	6	1	1	2	-	-
4	ABS,BKF, BTG	1	5	1	1	2	-	-
5	ABS, BKF, BTG, GUH	1	3	1	1	2	2	-
6	NAN1	1	7	-	-	-	-	1

6.3.16.7 WAX – HPLC fractionation

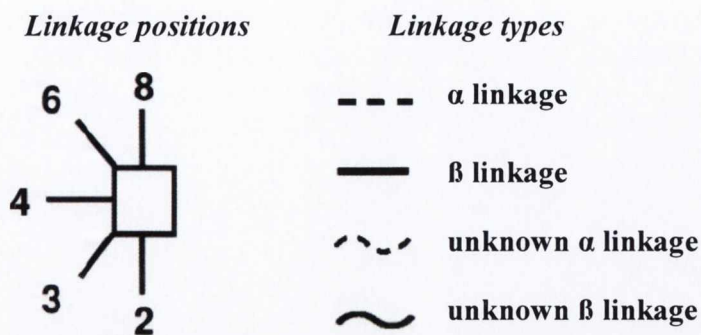
In order to confirm the structures present in the undigested *N*-glycan pools additional weak-anion exchange HPLC analysis was carried out. Separation was performed on a GlycoSep C polymeric DEAE anion exchange column (10 μm x 75 mm x 7.5 mm). Solvent A was 20% acetonitrile and solvent B was 0.1 M ammonium acetate buffer pH 7.0 in 20% (v/v) acetonitrile. The elution gradient was as follows: 100% A for 5 minutes, a linear gradient of 100% to 0% A over 15 minutes, a hold at 0% A for 2.5 minutes, 0 to 100% A over 0.5 minutes and a final 7 minutes at 100% A. Throughout the separation a flow rate of 0.75 ml/min and a temperature of 30°C was maintained. All samples were prepared in MilliQ water, and a fetuin *N*-glycan standard was used for calibration. The remainder of the glycan pool was separated and collected in fractions corresponding to neutral, mono-, di-, tri-, and tetrasialylated species. These fractions were then run on NP-HPLC again as described previously.

6.3.16.8 Assigning *N*-glycans structures

HPLC traces were integrated with the use of Empower software and the GU value of each peak was determined with reference to the dextran ladder. Assigning glycan structures was carried out by use of all digest and WAX fractionation results, and GlycoBase (Version 2.0), an online database for glycan structure determination and GU values.

6.3.16.9 Abbreviations used to identify 2-AB labelled N-glycans

All complex N-linked glycans have 2 core GlcNAc and 3 mannose residues, which are assumed in all structures. The number of GlcNAc antennae is represented by A1, A2 (biantennary with both GlcNAcs as β 1,2-linked), A3 (triantennary with a β 1,2-linked GlcNAc to both mannose and a third β 1,4-linked GlcNAc to the α 1,3-linked mannose) and A4 (GlcNAc linked as A3 with additional β 1,6-linked GlcNAc to the α 1,6-linked mannose). Galactose residues β -linked to the antennae are represented by G whereas S indicates sialic acids linked to galactose. The following numbers indicate the number of galactose residues or sialic acids linked to galactose residues. F at the start of the abbreviation indicates an α 1,4-linked fucose. For mannose structures the number of mannose residues on core GlcNAc is indicated after an M. Legends for sugar symbols, linkage type and position are shown in Figure 6.12.



Monosaccharide symbols

- N-acetylglucosamine (GlcNAc)
- Mannose (Man)
- Galactose (Gal)
- Fucose (Fuc)
- N-acetylneuraminic acid (NeuNAc)

Sugar	GU increment
GlcNAc	0.4-0.5
Man	0.7-0.9
Gal (G)	0.8-0.9
Fuc (F)	0.45-0.5
NeuNAc (S)	0.2-0.4

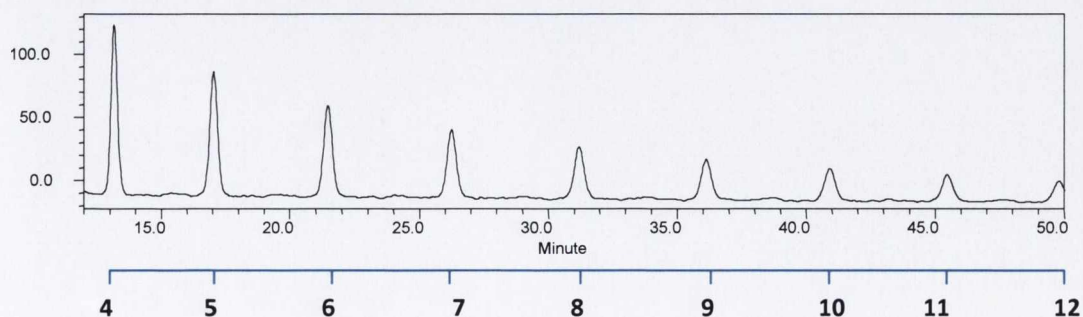


Figure 6.12 Legends for sugar symbols, linkage position and dextran ladder with assigned GU values.

6.3.17 Computational modelling of the biosynthesis of N-linked glycoforms in CHO cells (This project was the responsibility of Dr. Andrew McDonald, TCD)

The major objective of this study was to construct a mathematical model of *N*-glycosylation in Chinese hamster ovary (CHO) cells and investigate the factors affecting antennarity of *N*-glycans.

6.3.17.1 Model

A mathematical model of the *N*-glycosylation reactions of CHO cells was developed using ordinary differential equations. The model is thermodynamically open and allows for compartmentalisation of the Golgi into *cis*-, *medial*-, *trans*- and *trans*-Golgi network (Umaña and Bailey, 1997; Krambeck and Betenbaugh, 2005). In the in-silico experiments described in this work, a single-compartment model of the Golgi was used.

6.3.17.2 Commercial software

The model equations were integrated numerically by means of Berkeley Madonna (www.berkeleymadonna.com). Steady-state analysis of models was done with Mathematica® (Wolfram Research). The steady-state concentrations were initially set to 1 μ M whereas in the actual simulations, the concentrations of the intermediates were usually set to zero.

6.3.17.3 Custom software

A Perl script was written to generate the network of reactions of the eleven Golgi based enzymes shown in Table 6.14 as described previously (Krambeck and Betenbaugh, 2005). The script was used as the basis for a series of in-silico overexpression and knockout experiments, in which each of the eleven enzymes of *N*-glycosylation were either overexpressed or eliminated.

Table 6.14 Golgi-based enzymes present in the network.

EC number	Abbreviation	Accepted name
2.4.1.38	GalT	β -N-acetylglucosaminylglycopeptide β -1,4-galactosyltransferase
2.4.1.68	FucT	glycoprotein 6- α -L-fucosyltransferase
2.4.1.101	GnTI	α -1,3-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase
2.4.1.143	GnTII	α -1,6-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase
2.4.1.144	GnTIII	β -1,4-mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase
2.4.1.145	GnTIV	α -1,3-mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase
2.4.1.149	GnTE	N-acetyllactosaminide β -1,3-N-acetylglucosaminyltransferase
2.4.1.155	GnTV	α -1,6-mannosyl-glycoprotein 6- β -N-acetylglucosaminyltransferase
2.4.99.6	SialT	N-acetyllactosaminide α -2,3-sialyltransferase
3.2.1.113	ManI	mannosyl-oligosaccharide 1,2- α -mannosidase
3.2.1.114	ManII	mannosyl-oligosaccharide 1,3-1,6- α -mannosidase

6.3.17.4 Parameters

ExplorEnz (McDonald *et al.*, 2009) and the online resource BRENDA (Schomburg *et al.*, 2004) were the primary source of information on the enzymes of *N*-glycosylation. Kinetic parameters of the models were obtained from the literature. Table 6.15 shows the default set of kinetic parameters used in the model and these values were used as a baseline and varied in order to look for trends in the data.

Table 6.15 Maximal velocities ($k_f \times [E]_t$) and Michaelis constants used in the models (Krambeck and Betenbaugh, 2005).

Enzyme	$[E]_t$ (μM)	k_f (min^{-1})	Acceptor K_m (μM)	Donor K_m (μM)
ManI	1.04	1924	100	N/A
ManII	1.04	1924	200	N/A
FucT	1.00	253	25	6
GnTI	3.03	990	260	170
GnTII	0.71	1320	190	960
GnTIII	1.10	607	4000	420
GnTIV	0.36	187	3400	8300
GnTV	0.05	1410	130	129
GnTE	1.00	25	3850	5800
GalT	0.44	8712	1091	12.5
SialT	1.00	484	260	100

6.4 Results

6.4.1 Computer model predictions

The mathematical model developed by Dr. McDonald calculates the expected qualitative trends in the *N*-linked oligosaccharide distribution resulting from changes in the levels of one or more enzymes involved. The aim was to maximize the fractions that contained tri- and tetra-antennary structures attached to HCG. Computer simulations facilitated the selection of enzymes acting at different stages in the *N*-glycosylation pathway, such as GlcNAc transferase V (GnTV) and six galactosyltransferases (β 4GalTs). The genetic manipulations of these enzymes and the corresponding steady-state values for different forms of glycans as well as graphical representation of the table are shown in Figure 6.13. According to computer model predictions the mono- and biantennary structures represent the majority in the total pool of *N*-glycans in wild-type CHO cells. The 25-fold overexpression of GnTV is predicted to increase the amount of triantennary structures from 3% to 18.4% and decrease the biantennary forms from 21.1% to 5.4%. The RNAi-induced knockdown of CHO GalT enzymes to 10% of their wild-type level is predicted to decrease monoantennary forms to 26.7% with the opposite effect for bi- and tetra-antennary structures which are expected to increase to 50.1% and 2.5%, respectively. The combination of two genetic modifications is predicted to reduce the amount of biantennary forms further, to 2.9%, resulting in a large increase in triantennary forms to 62.9% and tetra-antennary forms to 5%.

A.

Experiment	Steady-state value (mole %)					
	zero	mono	bi	tri	tetra	Total
WT	2.6	73.3	21.1	3.0	0.0	100.0
+GnTV*	2.6	73.3	5.4	18.4	0.2	100.0
- GalT**	2.6	26.7	50.1	18.2	2.5	100.0
+ GnTV*- GalT**	2.6	26.7	2.9	62.9	5.0	100.0

B.

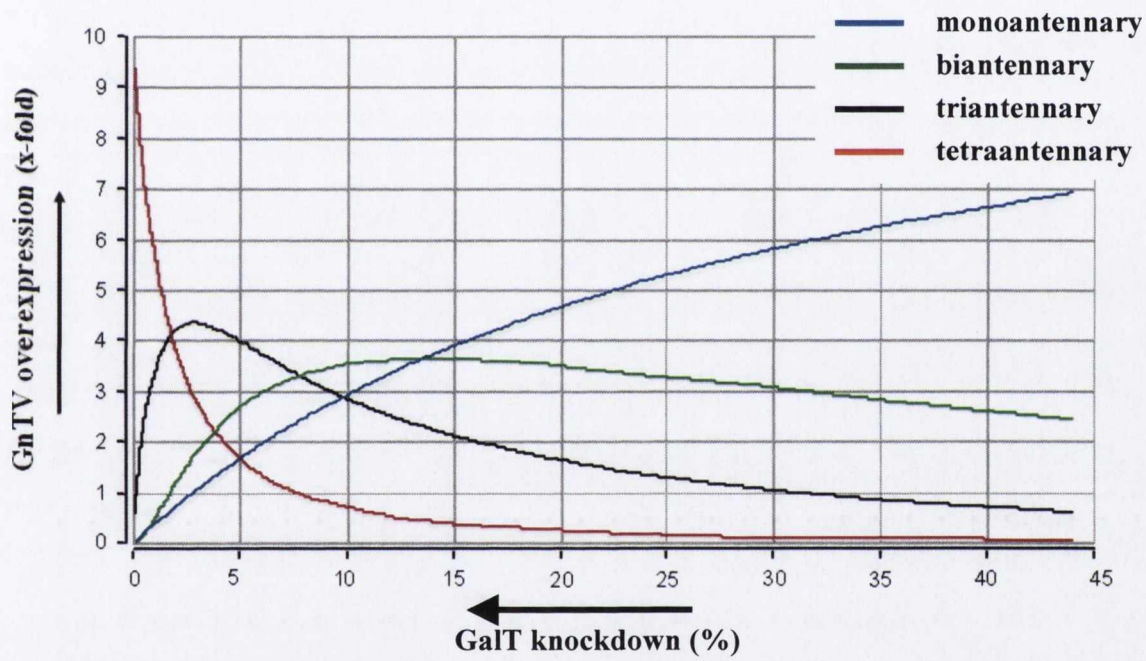


Figure 6.13 Computer model predictions for *N*-glycans based on Dr. McDonald’s *in silico* experiments.

(A) shows steady-state values for glycans and (*) denotes a 25-fold overexpression whereas (**) denotes a 10% knockdown of the wild-type concentration. (B) is a representation of computer model predictions, where the *x* and *y* axis are the percentages of GalT knockdown and the x-fold of GnTV overexpression, respectively.

6.4.2 Establishing a stable CHO7J cell line overexpressing GnTV with the RNAi-induced knockdown of GalTs

CHO GnTV clone (in pBluescript SK+) was linearized and used as a template for PCR. A specific primer set was used to create new restriction sites (*HindIII* and *SacII*) for sequential cloning into pBluescript SK+ followed by subcloning into pcDNA6/V5-His. Figure 6.14A and B show the GnTV cDNA flanked by *HindIII* and *SacII* restriction sites and the restriction analysis on pcDNA6/V5-His construct with the GnTV cDNA (GnTV-pcDNA6/V5-His) cloned in frame with the C-terminal V5 peptide and a His tag. Stable expression of GnTV was achieved by integration of the GnTV gene into the target CHO cell chromosome by nucleofection. A fresh culture of CHO7J cells was transfected with the linearized GnTV-pcDNA6/V5-His construct and the cells were selected for GnTV overexpression using blasticidin followed by Western blotting. Three representative cultures (Clone 7JG5-B2, 7JG5-D4 and 7JG5-F6) with V5-tagged GnTV of the correct molecular weight of 86.5 kDa are shown in Figure 6.14C. The highest expression was determined by the visual comparison of bands from the Western blotting together with the GAPDH control for equal loading (data not shown).

The stable GnTV-CHO7J cell line was used for the RNAi-induced knockdown of GalT1, GalT2, GalT3, GalT4, GalT5 and GalT6. DNA inserts (about 70 bp) encoding a shRNA targeting individual GalT sequence was cloned into pRNAT-U6.2/Lenti and was confirmed by restriction analysis using *BamHI*, *BglII* and *EcoRI* as shown in Figure 6.15.

Viral particles were obtained from the medium of 293T HEK packaging cells and were used to transduce GnTV-CHO7J cells. Co-expression of coral GFP in the positively transduced cells allowed their visualisation by fluorescence microscopy (see Figure 6.16) and selection by fluorescence activated cell sorting (FACS) to obtain a homogenous population of cells (see Table 6.14). The percentage of GFP positive cells from the entire population was not greater than 2.4%. The purity of the population was generally very high, starting from 92.3% (GalT6) up to 97.92% for the GalT3 knockdown.

The expression of GnTV in the genetically modified CHO7J cell lines was investigated again by Western blotting (see Figure 6.17) and was considered equal with the exception for the GalT1 knockdown, where the expression level was reduced by approximately 50%

according to the visual determination. The GAPDH housekeeping gene was used as a control for equal loading.

Quantitative real-time PCR was used to determine the relative amounts of mRNA for GnTV and galactosyltransferases following the genetic modifications (see Figure 6.18) in respect to the wild-type CHO mRNA, which was used as a control. The ~11-fold increase in the mRNA level for GnTV was obtained from the GnTV-CHOJ7 stable cell line. The efficiency of the RNAi-induced knockdown of galactosyltransferases appeared to be more diverse starting from the value of 3% for the GalT1 siRNA construct, which was considered statistically insignificant ($p > 0.05$) based on the one-way ANOVA followed by post-hoc Bonferroni analysis. The remaining levels of mRNA for other glycosyltransferases after the RNAi-induced knockdown were: GalT2 - 53.9%, GalT3 - 79.5%, GalT4 - 37.4%, GalT5 - 87.8% and GalT6 - 61.3%. The knockdown of GalT4 resulted in large decrease in the overall cell viability with further effects in cell morphology. Similarly, the knockdown of GalT5 resulted in the significant growth inhibition accompanied by morphological changes. All other cell lines grew stably without visible alterations (data not shown).

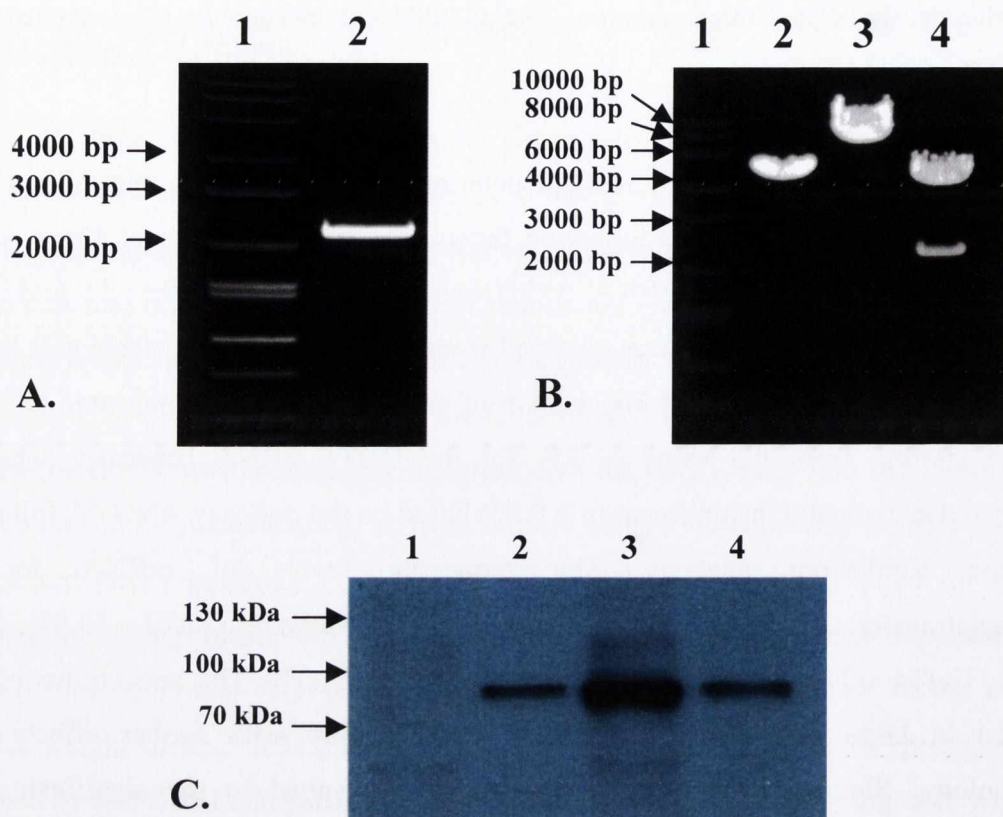


Figure 6.14 Establishing a stable cell line overexpressing GnTV in CHO7J cells.

CHO GnTV cDNA was amplified by PCR and new restriction sites, *Hind*III and *Sac*II, were introduced. Amplification of the coding region was performed as described in Section 6.3.4 and the representative gel is shown in (A). Agarose gel was loaded with the following samples: **lane 1**, DNA marker; **lane 2**, PCR product of GnTV cDNA (2223 bp). (B) shows the restriction digest of GnTV-pcDNA6/V5-His (7323 bp). Agarose gel was loaded with the following samples: **lane 1**, DNA marker; **lane 2**, (control) pcDNA6/V5-His digested with *Hind*III (5144 bp); **lane 3**, *Hind*III digest (7323 bp); **lane 4**, *Hind*III and *Sac*II digest (5144 bp and 2223 bp). (C) shows the representative blot of the GnTV protein (86.5 kDa) detected by the anti-V5 antibody from the stably transfected CHO7J cell lysate. The cell lysate was prepared as described in Section 2.2.6 and the Western blotting procedure is described in Section 2.2.8. The primary anti-V5 antibody, 1:10000, secondary goat anti-mouse, dilution 1:20000, **lane 1**, no DNA transfection control; **lane 2**, Clone 7JG5-B2; **lane 3**, Clone 7JG5-D4; **lane 4**, Clone 7JG5-F6.

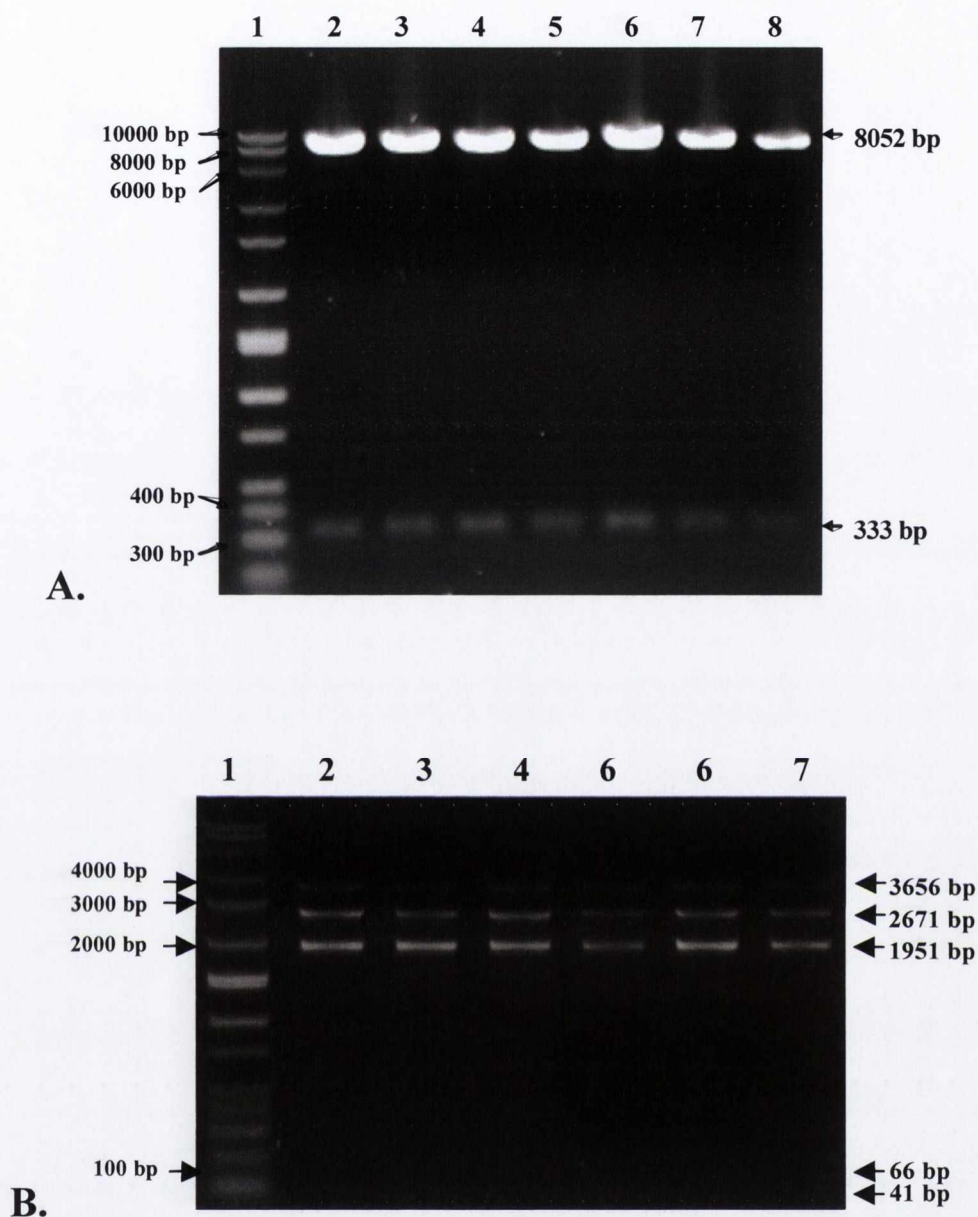


Figure 6.15 Restriction digest of endotoxin-free GalT siRNA constructs.

GalT siRNA constructs were digested as described in Section 3.3.14 with *Bam*HI and *Bgl*II enzymes (**A**) or *Bam*HI and *Eco*RI (**B**). The agarose gel from (**A**) was loaded as follows: **lane 1**, DNA marker; **lane 2-7**, GalT siRNA from 1 to 6 (3656 bp, 2671 bp, 1951 bp, 66 bp and 41 bp). The agarose gel from (**B**) was loaded as follows: **lane 1**, DNA marker; **lane 2-7**, GalT siRNA from 1 to 6 (8052 bp and 333 bp); **lane 8**, negative control (no GalT insert).

Table 6.14 Fluorescence-activated cell sorting (FACS) of GalT knockdown cell lines.

Name	% of GFP positive cells	Cell density (cells/ml)	Purity (%)
Control	-	0	-
GalT1	1.99	2.4×10^4	96.4
GalT2	2.15	3.1×10^4	97.06
GalT3	2.21	2.1×10^4	97.92
GalT4	1.55	1.5×10^4	96.99
GalT5	0.53	1.9×10^4	95.24
GalT6	2.39	2.9×10^4	92.31

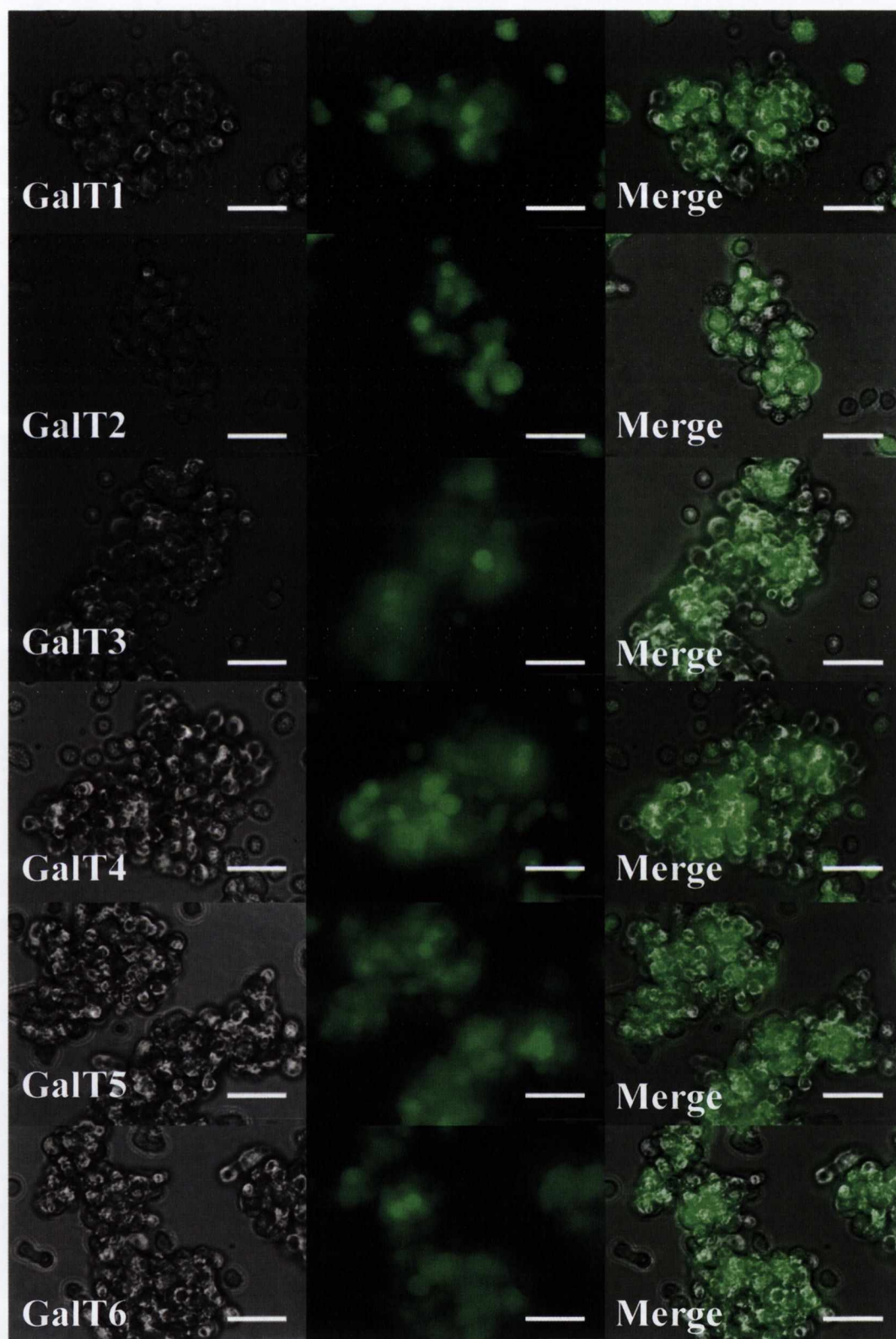


Figure 6.16 Representative populations of GFP positive cells sorted by FACS.

Cells were visualised at 40X magnification using Olympus IX81 long focal length fluorescent microscope; scale bar represents 50 μm.

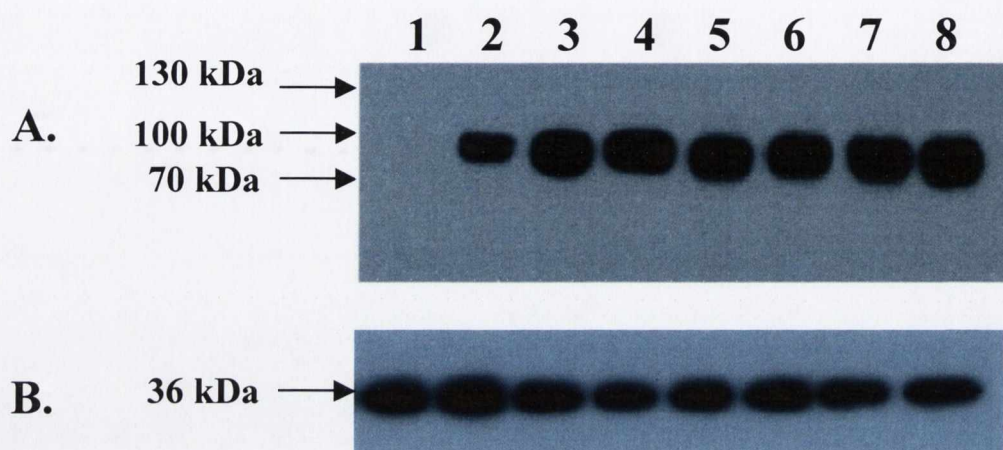


Figure 6.17 Expression profile of GnTV in CHO7J cells with the RNAi-induced GalT knockdown (KD).

Equal protein aliquots (10 μ g) were prepared as described in Section 5.3.4 and separated on a 8% SDS-PAGE gel prior to transfer onto PVDF membranes. Blots were probed with the anti-V5 antibody at 1:5000 dilution (A). The blot was reprobed with the anti-GAPDH antibody at a dilution of 1:5000 to ensure equal loading (B). The secondary goat anti-mouse HRP was used at a 1:10000 dilution for both incubations. The SDS-PAGE gel was loaded with the following samples: **lane 1**, CHO7J lysate; **lane 2**, GnTV-CHO7J lysate; **lane 3**, GalT1 KD; **lane 4**, GalT2 KD; **lane 5**, GalT3 KD; **lane 6**, GalT4 KD; **lane 7**, GalT5 KD; **lane 8**, GalT6 KD.

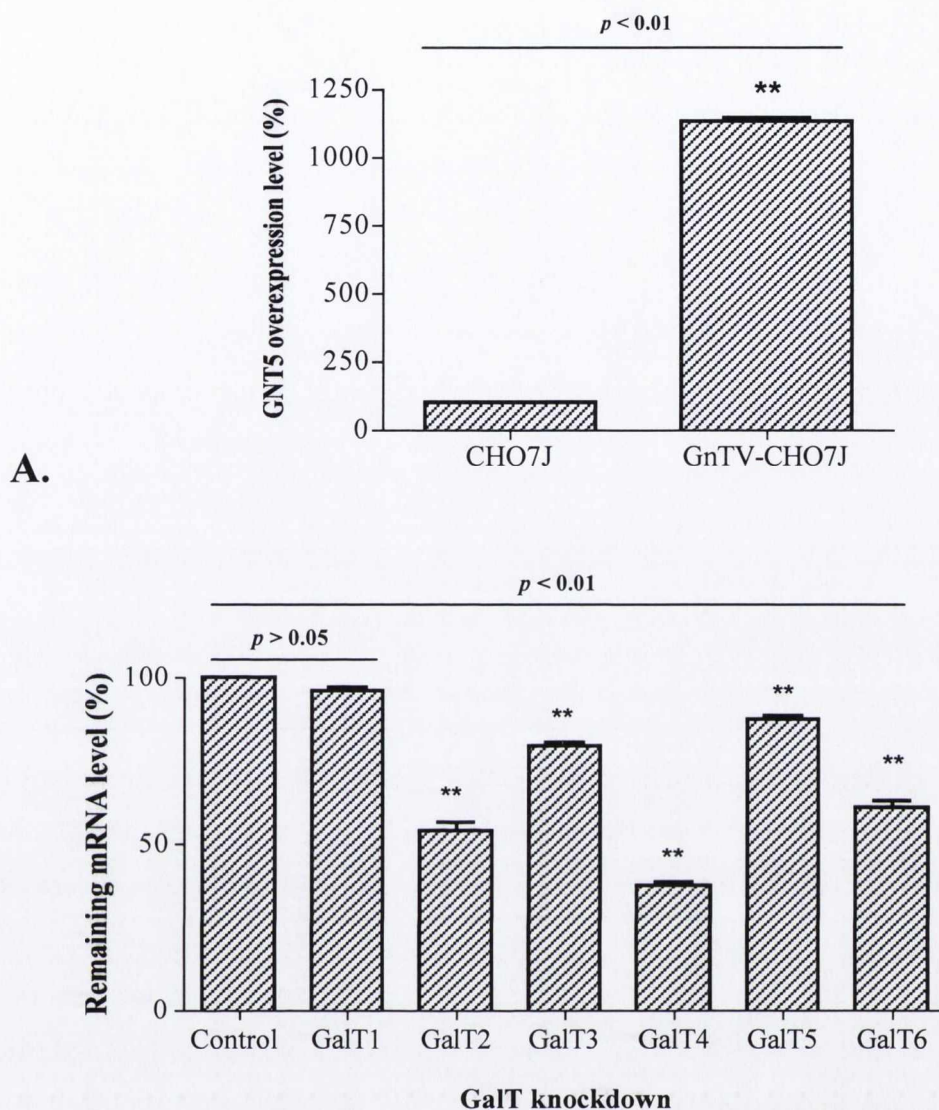


Figure 6.18 Quantitation of mRNA levels of GnTV and GalTs in the stable CHO7J cell lines by the real-time quantitative PCR (QPCR).

Total RNA was extracted from CHO7J cells that were transduced with GalT siRNA constructs. RNA was subsequently reverse transcribed into cDNA and used as a template in QPCR. **(A)** shows the relative increase of GnTV mRNA in the stable GnTV-CHO7J cell line. **(B)** shows the mRNA level that remained after RNAi-induced knockdown of GalTs. Values are represented as % in comparison to adequate wild-type control treated as 100%. Results are expressed as the mean $\pm$ SEM. At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was completed using unpaired t test or one-way ANOVA followed by post-hoc Bonferroni analysis using Prism 4; ** = $p < 0.01$.

6.4.4 Analysis of *N*-glycans attached to HCG

HCG was purified from CHO7J cell culture medium by immunoaffinity chromatography using a monoclonal antibody specific to the β -subunit of HCG. Purified HCG was analyzed using IEF (see Figure 6.19A) and SDS-PAGE gel (see Figure 6.19B). The molecular weight of the α and β subunits resolved by the SDS-PAGE gel are quoted in the literature as 14.9 kDa and 23 kDa, respectively (de Medeiros, 2009). However, glycosylated proteins appear to have larger molecular weight due to water molecules that associate with the carbohydrate moieties of the proteins. As a result, the α subunit appears at a molecular weight of 18 kDa and the β subunit is seen to separate into two bands at 28 kDa and 33 kDa, most likely due to changes in glycosylation site occupancy.

Following release from the SDS-PAGE gel slices, *N*-glycans were labelled with 2-aminobenzamide (2-AB) and subjected to normal phase HPLC (NP-HPLC) as shown in Figure 6.20. The main feature of the 2-AB label used in HPLC is that the order of elution on the column is related to the number of sugar residues in the glycan. Literally, the larger the molecule the longer it takes to elute it. The masses of the eluted glycans are expressed in glucose units (GU) by reference to a dextran ladder. Individual glycan structures have corresponding GU values and these can be used to predict structures and specific linkage types. There was no visible change in the glycosylation pattern of HCG purified from CHO7J cells that were stably overexpressing GnTV. A similar conclusion could be made for CHO7J cell that had the stable knockdown of GalT1. In contrast to previous results, the proportion of tri- and tetra-antennary structures changed significantly in CHO cells overexpressing GnTV combined with the RNAi-induced knockdown of GalT2, GalT3, GalT4, GalT5 and GalT6 corresponding to GU values ranging from 8.5 to 10.5.

Weak anion exchange HPLC (WAX-HPLC) was used to separate glycans based on charge of sialic acid content. The WAX-HPLC trace in Figure 6.21 shows an increased ratio of trisialylated structures in the pool of *N*-glycans from the bioengineered HCG in contrast to the control, wild type HCG.

NP-HPLC of the WAX fractions separated the pool of glycans into neutral, mono-, bi-, tri- and tetrasialylated forms (see Figure 6.22 and 6.23). In addition, the profile confirmed the

increased level of triantennary structures present in the pool of *N*-glycans from the glycoengineered HCG.

The combination of exoglycosidase digestions with NP-HPLC profiling and WAX collection allowed for the identification of individual peaks. The assignments of glycan structures were made with the use of GlycoBase (Version 2.0). Confirmation of any initial assignments by exoglycosidase sequencing was essential as a number of glycan structures co-eluted in the initial NP-HPLC profile giving a single peak. The assignments of the structures commenced with the sample digested with sialidase S (ABS), β -galactosidase (BTG), α -fucosidase (BKF) and hexosaminidase (GUH) (see Figure 6.24). This resulted in the elution of three peaks that correspond to the structures M3 (Man₃; 4.4), M4 (Man₄; 5.3) and M5 (Man₅; 6.2). In the trace in Figure 6.25, which lacks the GUH enzyme (adds GlcNAc residues and changes the antennarity), the M3 structure appeared as three distinctive peaks indicating A1 (GlcNAcMan₃) at 4.8, A2 (GlcNAc₂Man₃) at 5.3 and A3 (GlcNAc₃Man₃) at 5.8. These peaks are 0.45 GU, 0.95 GU and 1.42 GU higher than the 4.4 GU value which was assigned to M3 and corresponds to the GU increment value of the GlcNAc residue (see Figure 6.12). Additionally, the percentage area of the A3 (GlcNAc₃Man₃) structure increased significantly confirming the computer model predictions. The omission of fucosidase (Fuc; adds Fuc residues to the core GlcNAc) and hexosaminidase in the reaction mixture (see Figure 6.26) results in the new digestion pattern containing fucosylated structures, e.g. FA2 - 5.9, FA3 - 6.3, and the changes in their GU values correspond to the GU increments of 0.45 - 0.5 for the core fucose. Two additional peaks appeared in Figure 6.26B (bioengineered HCG) at 6.2 (FA3 and M5) and 6.6 (A4) indicating the increased level of tri- and tetra-antennary structures. The *N*-glycan pool digested with sialidase A (ABS) contains additional galactoses but lacks sialic acids as shown in Figure 6.27. Additional peaks were identified by their GU values combined with the GU increments for the galactose residues (0.8 - 0.9). The increased percentage areas for the following glycans, A3G3 (Gal₃GlcNAc₃Man₃) at 8.5 and FA3G3 (Gal₃GlcNAc₃Man₃ + Fuc) at 8.8 indicate the increased level of triantennary forms in the total pool of *N*-glycans attached to the glycoengineered HCG. No differences were observed between the sialidase S (NAN1) and ABS digests, indicating that all sialic acid linkages were α 2,3 and α 2,8 (data not shown). Figure 6.28 represents the total undigested pool of *N*-glycans with accompanying structure symbols and differences between the levels of tri- and tetra-antennary structures.

The 2-AB label is attached to a single glycan structure therefore the fluorescence intensity is related to the number of moles of a particular labelled glycan present in a sample. The % areas of the HPLC peaks can be quantified and the relative proportions of different glycans can be compared. The GU values with corresponding percentage areas (% areas) are represented in Table 6.15 for the control *N*-glycan pool and 6.16 for the pool of *N*-glycans attached to the bioengineered HCG.

Finally, the distribution of *N*-glycans based on their GU values and percentage areas is illustrated in Figure 6.29. As previously predicted by the computer simulations, the final level of biantennary structures (peaks 6 to 15) has decreased resulting in increased levels of tri- and tetra-antennary *N*-glycan structures (peaks 15-22).

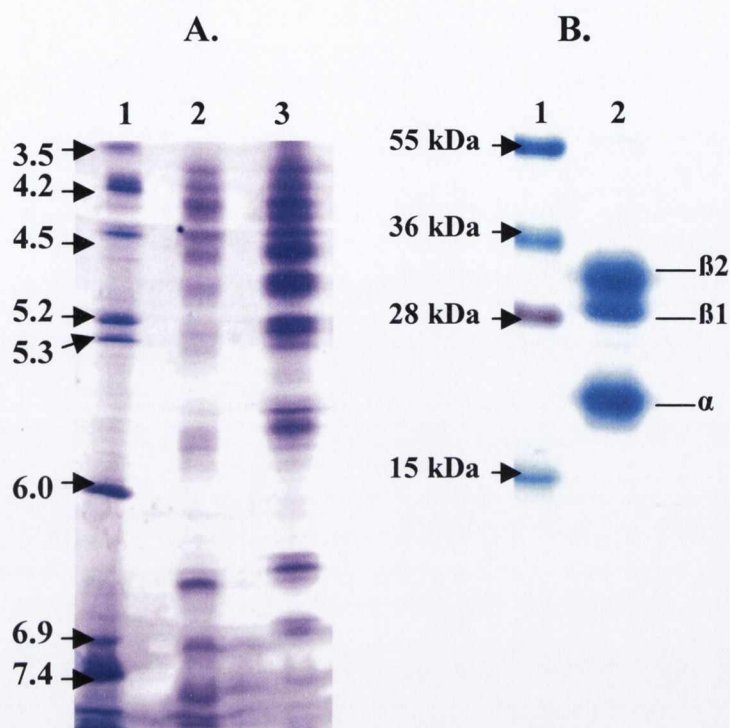


Figure 6.19 The representative IEF profile and denaturing SDS-PAGE gel of purified HCG.

HCG was purified from CHO7J supernatants followed by isoelectrofocusing and SDS-PAGE analysis. The numbers from the IEF profile (A) of HCG denote the migration pattern of the pI marker for 1, Ovitrelle (HCG standard) and recombinant HCG are 2 and 3, respectively. The SDS-PAGE gel (B) shows purified HCG (5 μ g), stained with Coomassie Brilliant Blue, which results in a complete separation of three subunits of HCG of apparent size $\beta 2$ -33kDa, $\beta 1$ -28kDa and α -18kDa. All three bands were cut out and merged for analysis of *N*-linked glycans structures.

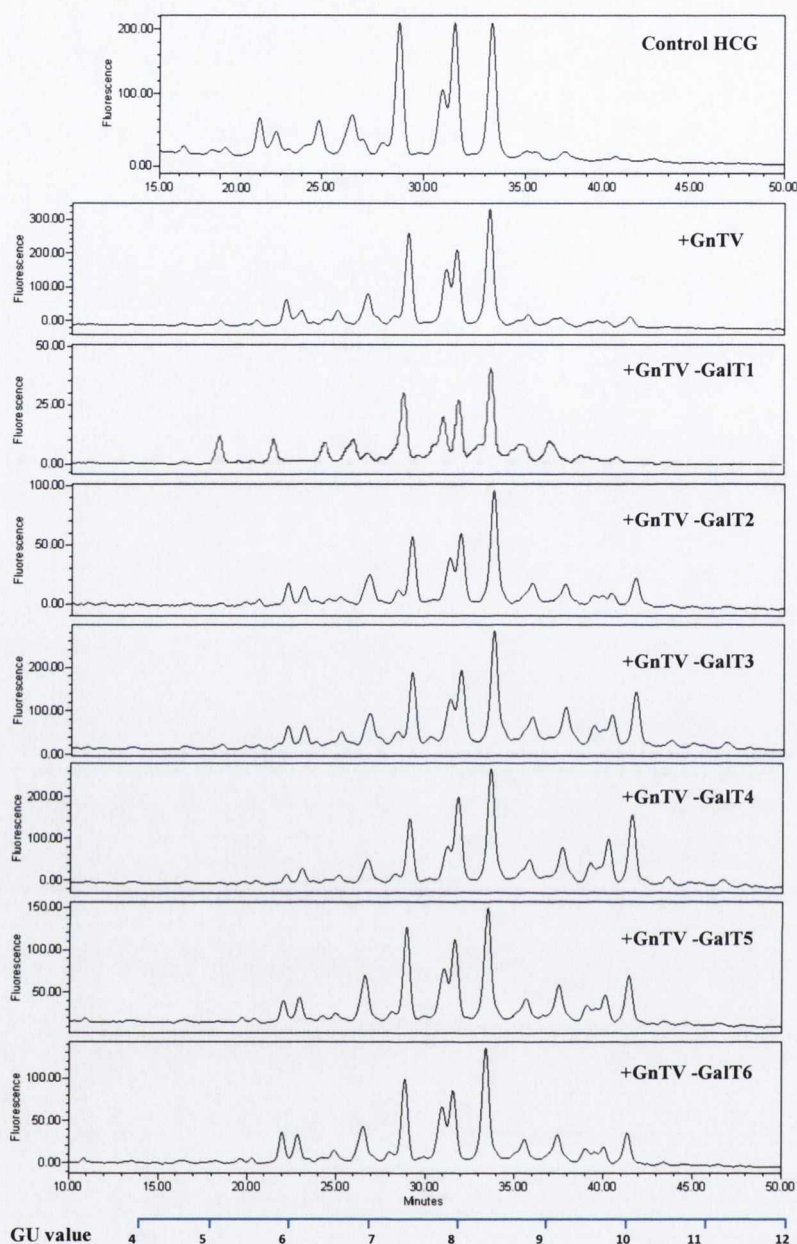


Figure 6.20 The increased level of tri- and tetra-antennary *N*-glycan structures attached to bioengineered HCG separated by the NP-HPLC.

Experimental details are described in Section 6.3.16.5. The mass of the eluted glycans are expressed in glucose units (GU) by reference to a dextran ladder. The proportion of tri- and tetra-antennary structures (GU values ranging from 8.5 to 10.5) changed significantly in GnTV-CHO7J cells with the RNAi-induced knockdown of GalT2, GalT3, GalT4, GalT5 and GalT6. HCG was purified from the following cell lines: control CHO7J, +GnTV: stable overexpression of GnTV, +GnTV -GalT: stable overexpression of GnTV with the RNAi-induced knockdown of GalTs.

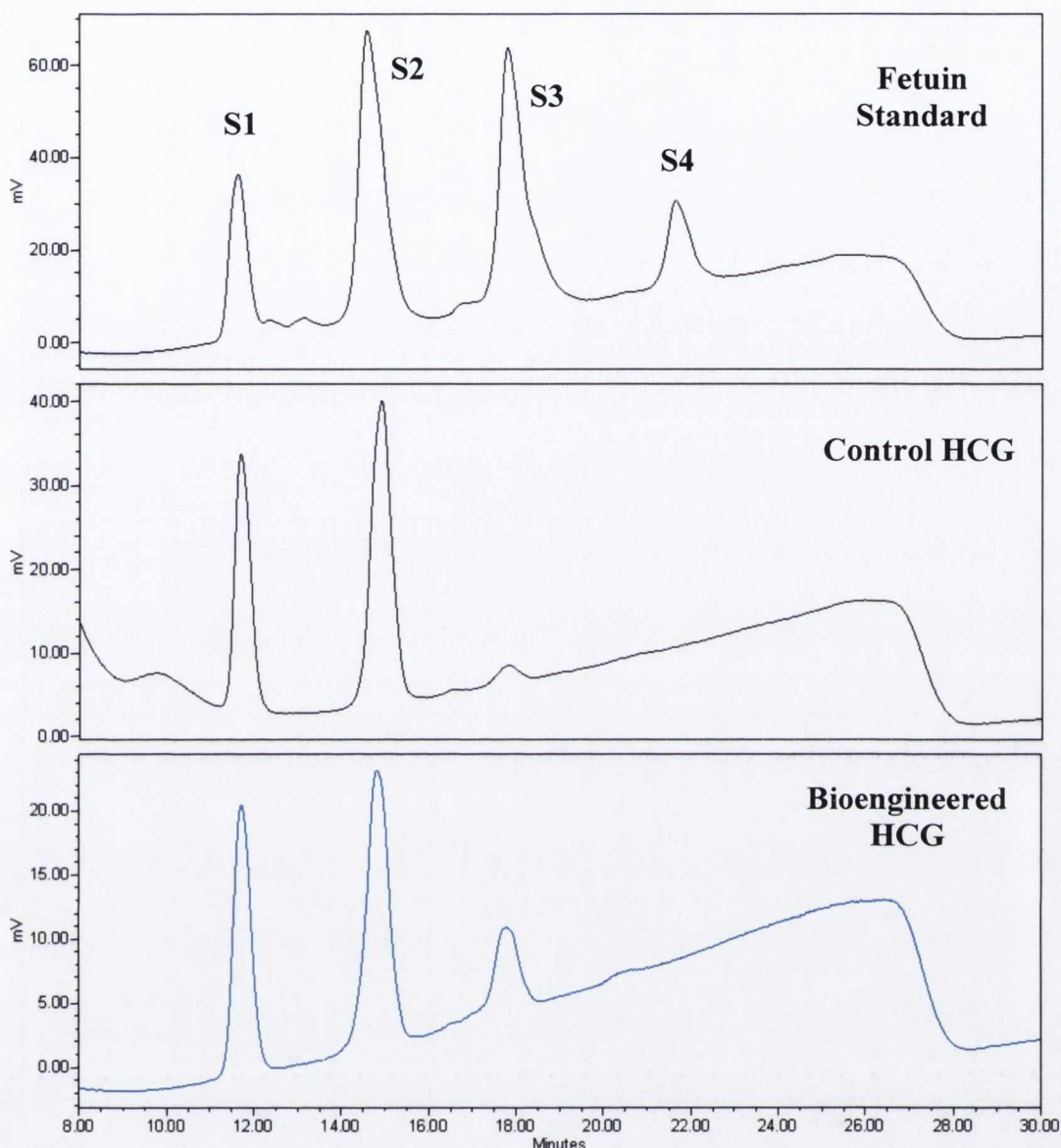


Figure 6.21 The increased level of trisialylated *N*-glycan structures attached to bioengineered HCG separated by the WAX HPLC.

Fetuin standard profile, pool of control and bioengineered *N*-glycans were run on weak-anion exchange HPLC (WAX-HPLC) as described in Section 6.3.16.6. The fetuin standard separates into neutral, mono-, bi-, tri-, and tetrasialylated structures (N, S1, S2, S3, and S4, respectively). The trace of bioengineered HCG shows an increased ratio of trisialylated structures in the pool of *N*-glycans in contrast to the control, wild type HCG. All undigested and digested fractions were run on NP-HPLC to aid the process of assigning structures in the total glycan pool.

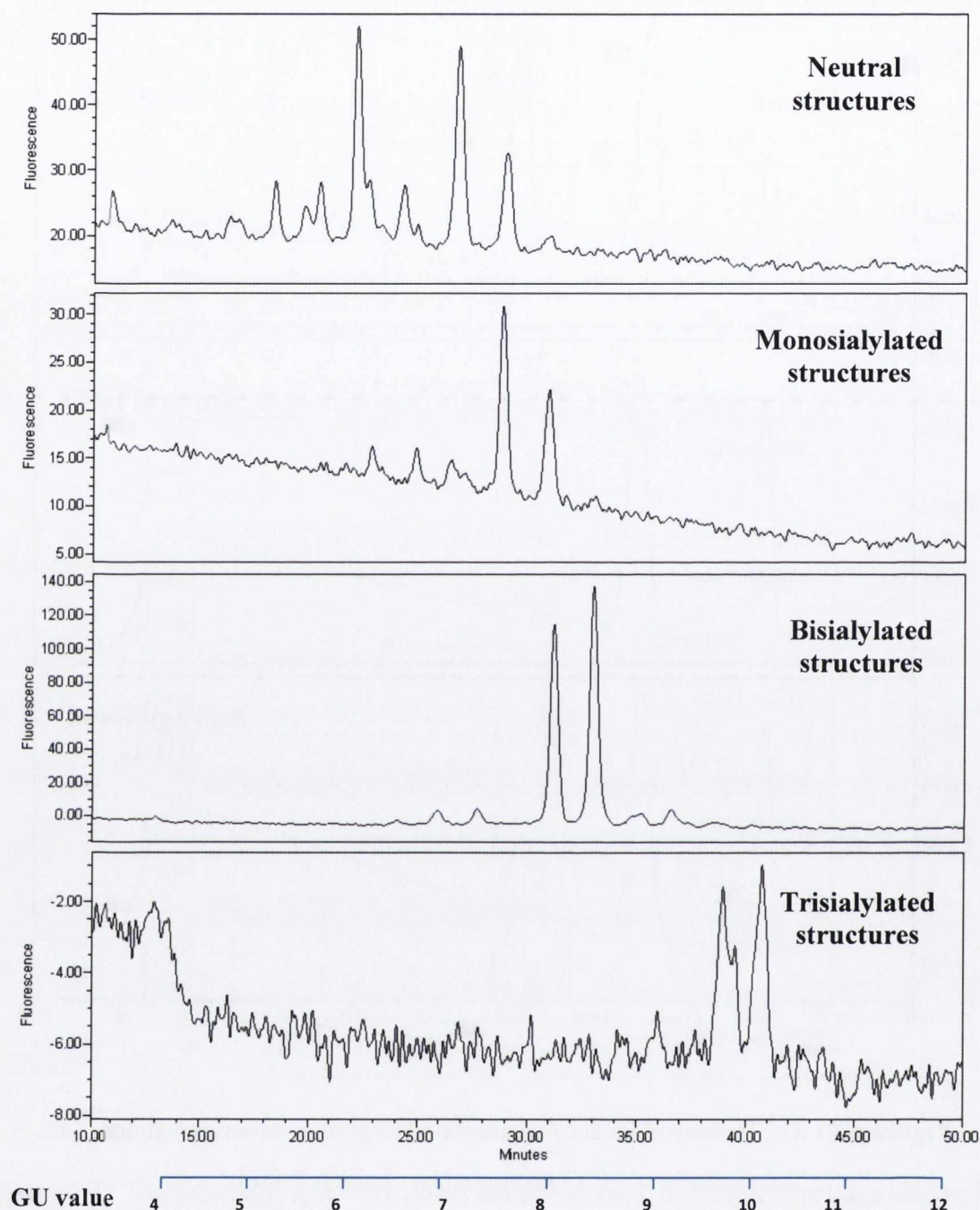


Figure 6.22 NP-HPLC of WAX separated fractions from the control HCG.

The neutral, mono-, bi-, tri- and tetra-sialylated fractions collected from the WAX-HPLC were dried down and resuspended in 80% acetonitrile for NP-HPLC. In the control HCG, the amount of bisialylated structures is relatively high comparing to the neutral and monosialylated *N*-glycans and no trisialylated structures were detected.

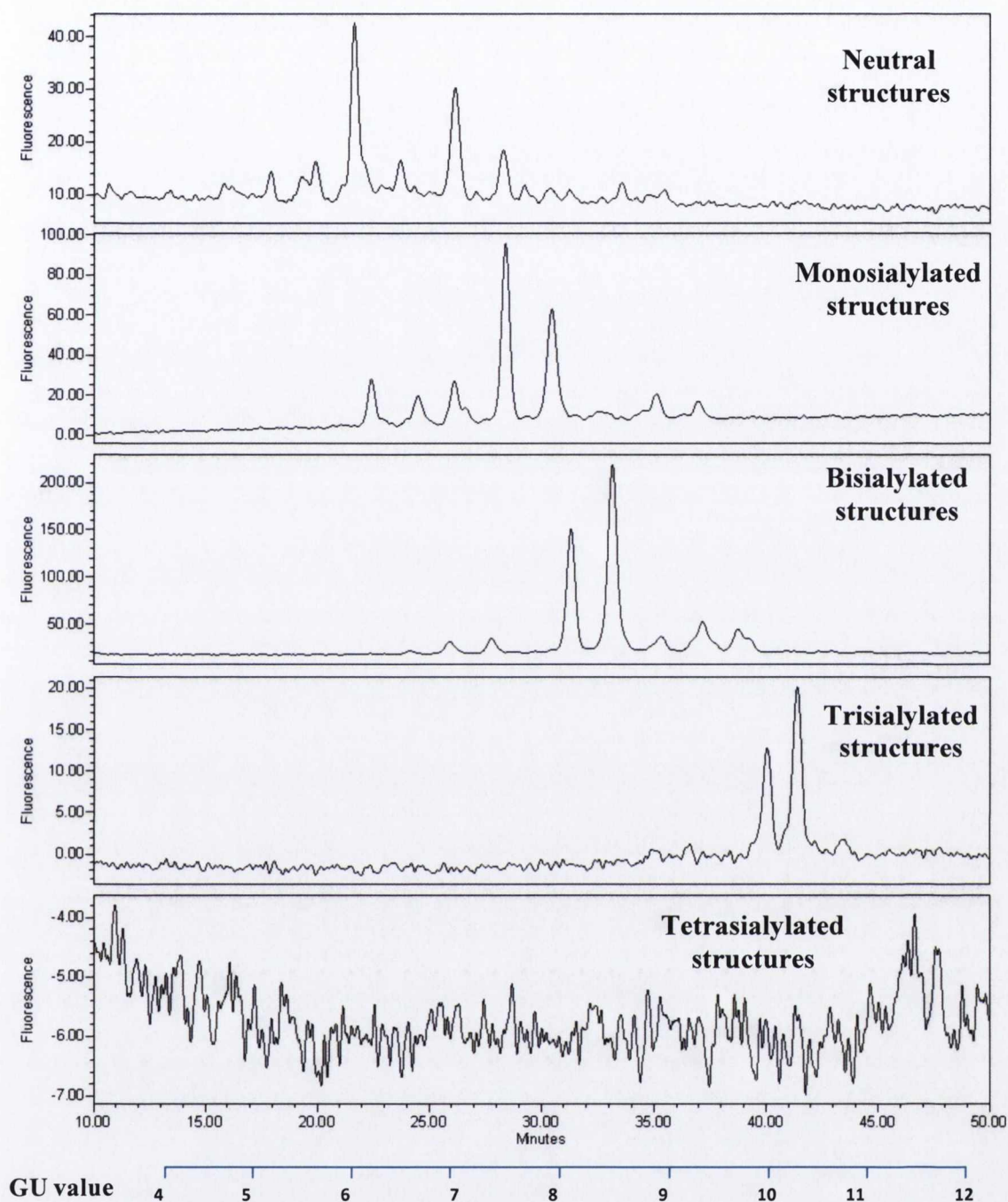


Figure 6.23 NP-HPLC of WAX separated bioengineered HCG fractions showing the increased level of trisialylated *N*-glycan structures.

The neutral, mono-, bi-, tri- and tetra-sialylated fractions were processed as described in Section 6.3.16.7. In the bioengineered HCG, the level of bi- and monosialylated structures is relatively high. There are some trisialylated structures present in the pool of *N*-glycans.

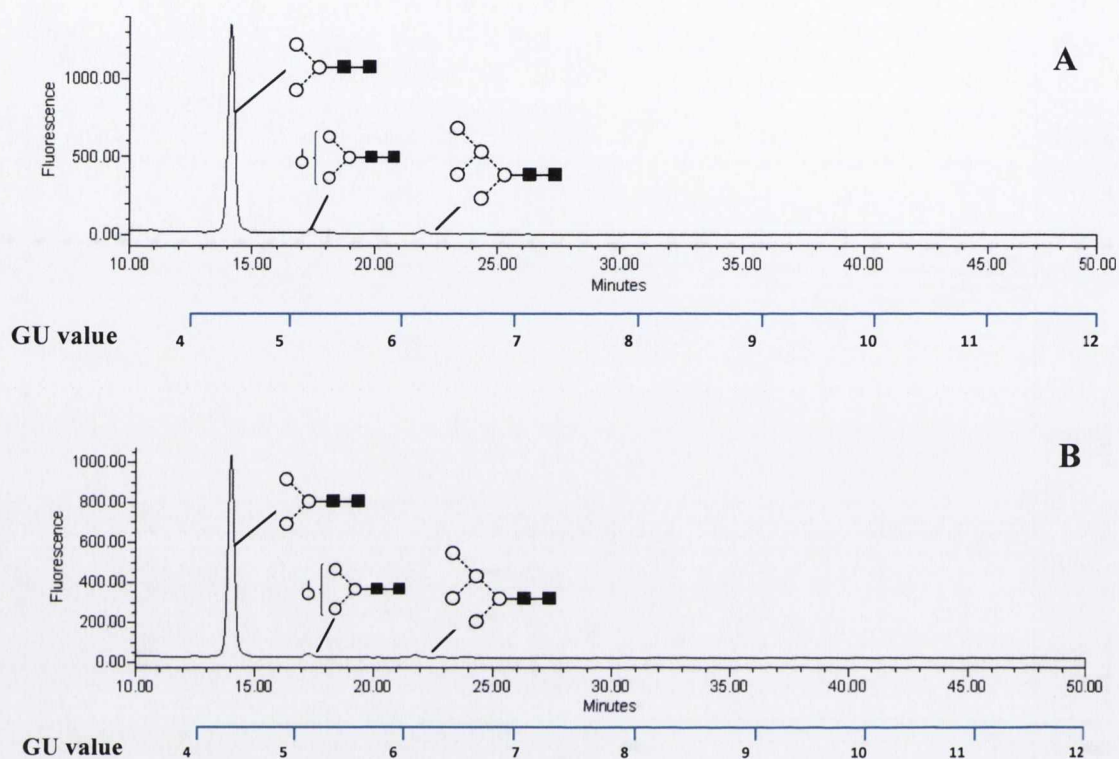


Figure 6.24 Sialidase A, galactosidase, α -fucosidase and hexosaminidase digest pattern with accompanying structure assignment.

The total pool of *N*-glycans from the control (**A**) and the bioengineered HCG (**B**) was digested with exoglycosidases ABS, BTG, BKF and GUH. Digests were carried out as in Section 6.3.16.6 and glycan structures were assigned with reference to GlycoBase (Version 2.0).

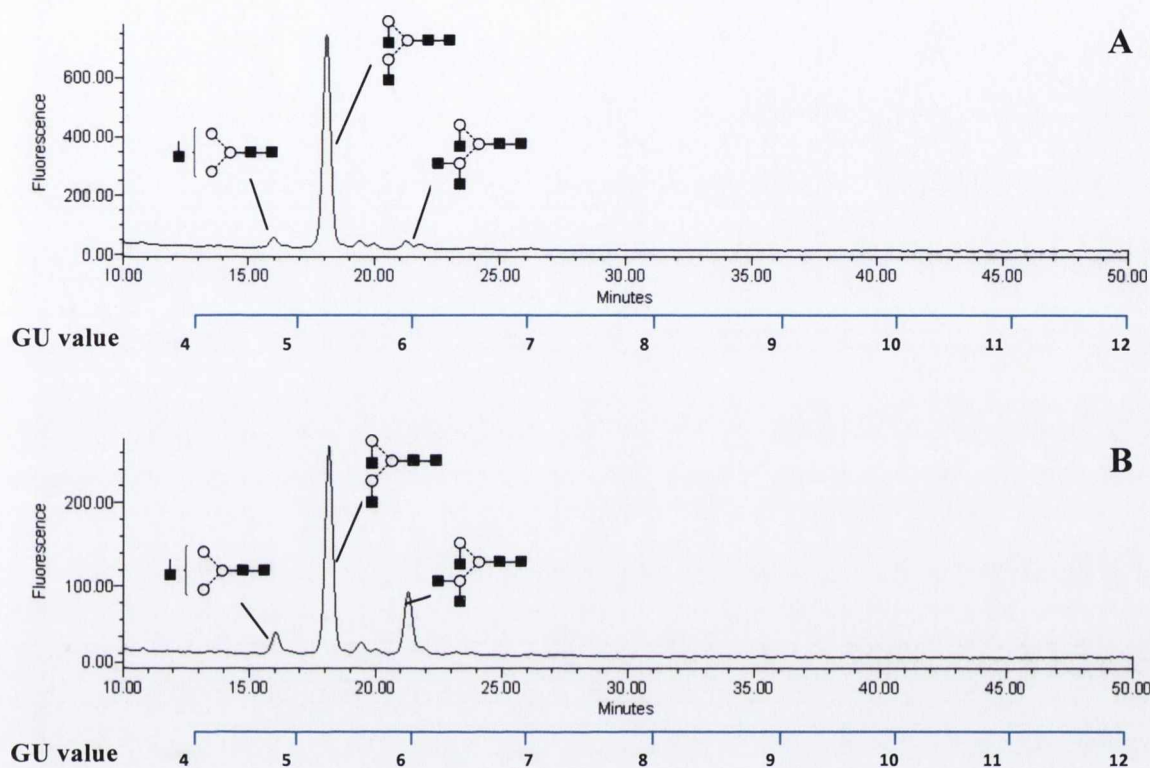


Figure 6.25 Sialidase A, galactosidase and α -fucosidase digest pattern with accompanying structure assignment.

The total pool of *N*-glycans from the control **(A)** and the bioengineered HCG **(B)** was digested with exoglycosidases ABS, BTG and BKF. Digests were carried out as in Section 6.3.16.6 and glycan structures were assigned with reference to GlycoBase (Version 2.0).

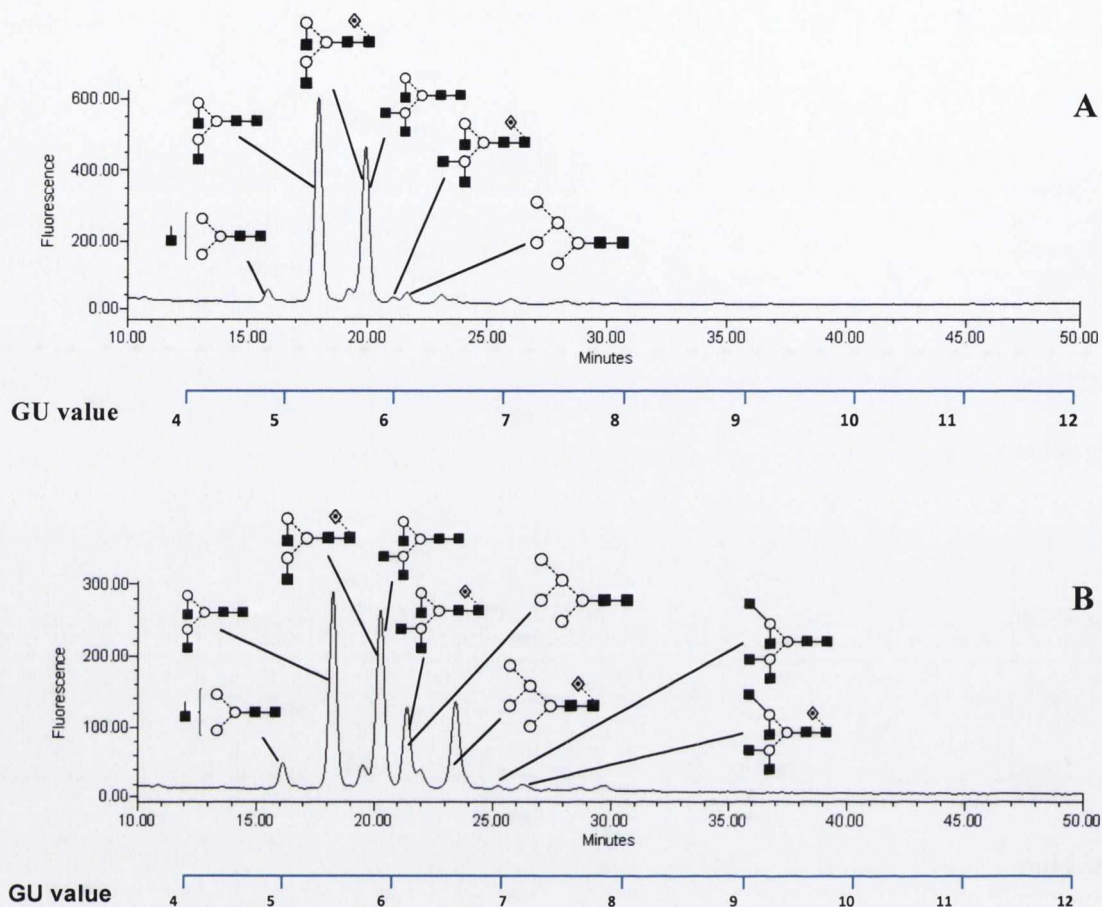


Figure 6.26 Sialidase A and galactosidase digest pattern with accompanying structure assignment.

The total pool of *N*-glycans from the control (**A**) and the bioengineered HCG (**B**) was digested with exoglycosidases ABS and BTG. Digests were carried out as in Section 6.3.16.6 and glycan structures were assigned with reference to GlycoBase (Version 2.0).

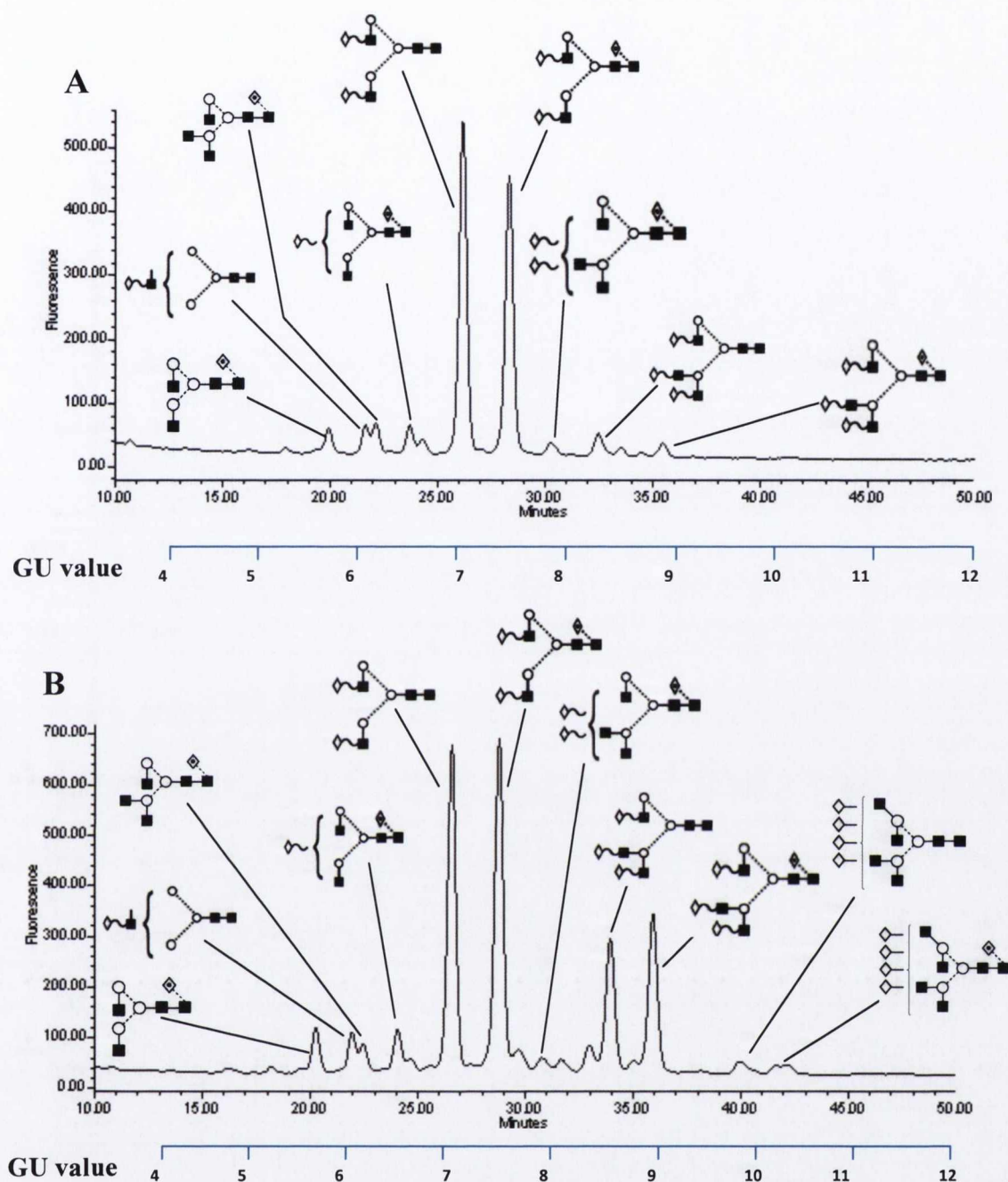


Figure 6.27 Sialidase A digest pattern with accompanying structure assignment.

The total pool of *N*-glycans from the control (A) and the bioengineered HCG (B) was digested with ABS. Digests were carried out as in Section 6.3.16.6 and glycan structures were assigned with reference to GlycoBase (Version 2.0).

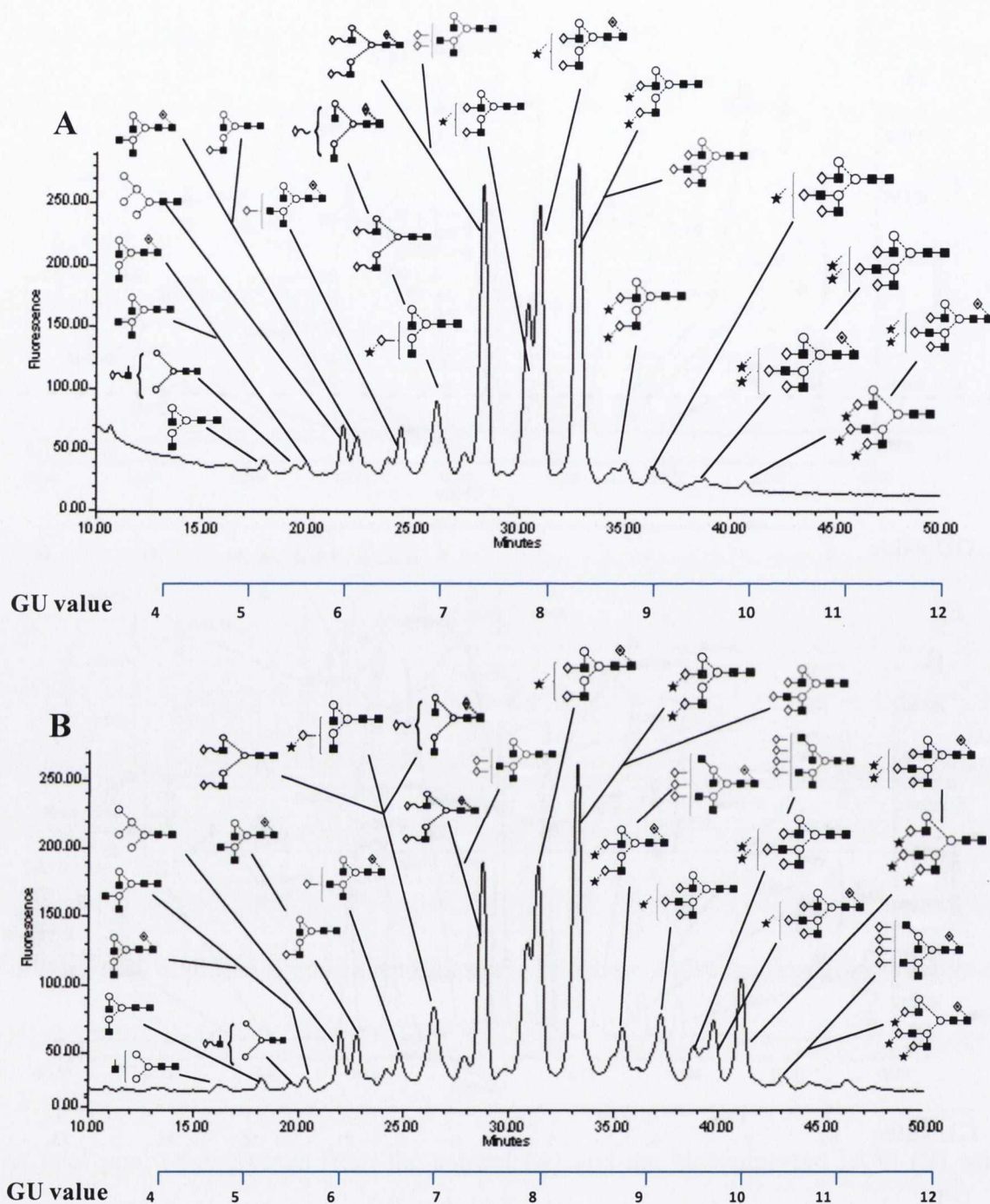


Figure 6.28 NP-HPLC trace of total *N*-glycan pool of HCG.

Structures were assigned with reference to WAX-HPLC and digest results for the control (A) and the pool of *N*-glycans from bioengineered HCG (B). The GU values for each peak with accompanying structure symbols are represented in Table 6.15 and 6.16.

Table 6.15 Composition of each peak of the *N*-glycan pool of control HCG.

Peak No.	GU value	% Area	Structure
1	4.89	0.25	A1
2	5.35	0.69	A2
3	5.56	0.6	A1G1
4	5.84	0.81	A3, FA2
5	6.10	1.18	M5
6	6.28	1.45	FA3, A2G1
7	6.58	0.52	M5A1
8	6.71	1.16	A3G1
9	7.05	5.83	FA2G1, A2G2, A2G1S1
10	7.35	1.27	FA2G1S1
11	7.51	19	A3G2, FA2G2
12	7.93	9.14	A2G2S1
13	8.05	24.41	FA2G2S1
14	8.42	24.74	A2G2S2, A3G3
15	8.88	2.51	FA2G2S2
16	-	-	-
17	9.68	1.52	A3G3S2, FA3G3S1
18	-	-	-
19	10.14	1.22	A3G3S3, FA3G3S2
20	-	-	-
21	-	-	-
22	-	-	-

Table 6.16 Composition of each peak of the *N*-glycan pool of bioengineered HCG.

Peak No.	GU value	% Area	Structure
1	4.85	0.53	A1
2	5.35	0.69	A2
3	5.67	0.6	A1G1
4	5.82	0.81	A3, FA2
5	6.19	3.76	M5
6	6.36	4.0	A2G1, FA3
7	6.65	3.9	M5A1
8	6.78	2.65	A3G1
9	7.13	7.3	A2G2, A2G1S1, FA2G1
10	7.41	2.65	FA2G1S1
11	7.58	11.03	A3G2, FA2G2
12	8.01	9.49	A2G2S1
13	8.11	12.7	FA2G2S1
14	8.49	19.55	A2G2S2, A3G3
15	8.94	6.67	FA2G2S2, FA4G3
16	9.32	8.42	A3G3S1
17	9.65	3.37	A4G4, A3G3S2
18	9.85	7.99	FA3G3S1
19	10.14	10.76	A3G3S3, FA3G3S2
20	10.57	2.01	FA4G4, FA3G3S3
21	10.84	1.16	A4G4S4
22	11.23	1.14	FA4G4S4

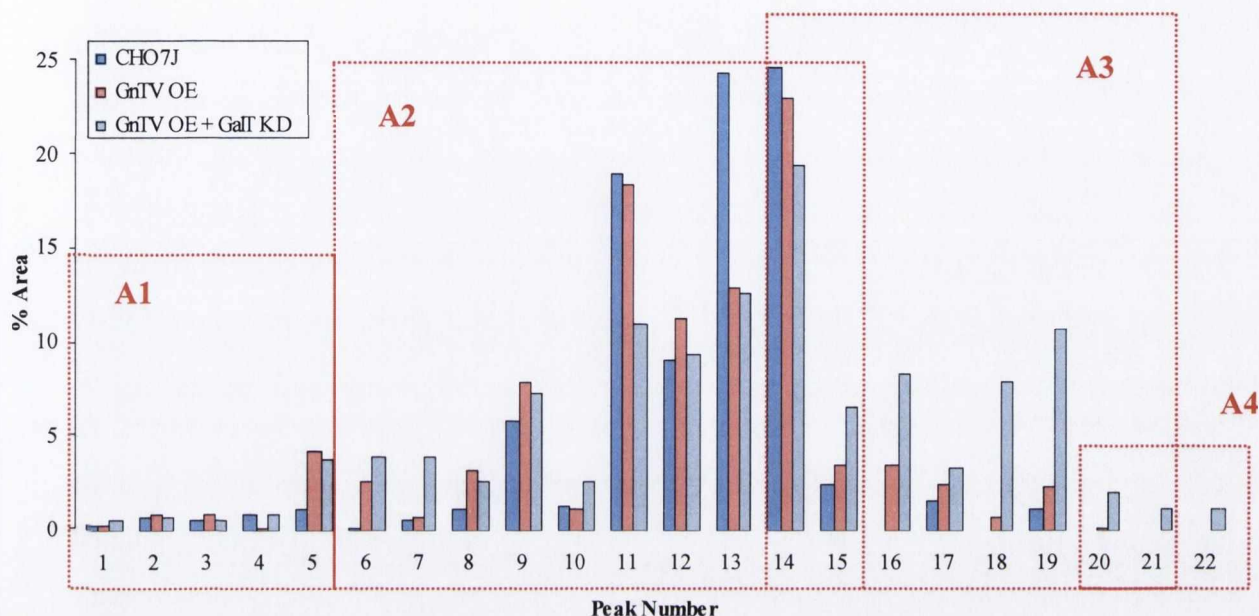


Figure 6.29 Overexpression of GnTV and the knockdown of GalT4 decreases biantennary and increases tri- and tetra-antennary glycans in CHO cells.

The graph shows the estimate of *N*-glycan antennarity based on the GU values and corresponding % areas for each peak obtained from the final NP-HPLC (see Table 6.15 and 6.16). The control values are presented as “CHO7J” whereas “GnTV OE” denotes the stable cell line that overexpresses GnTV – GnTV-CHO7J. The *N*-glycans from the GalT4 knockdown in GnTV overexpressing cell line is represented by “GnTV OE + GalT4 KD”. A1 (monoantennary), A2 (biantennary), A3 (triantennary) and A4 (tetra-antennary) frames illustrate the main distribution areas for specific antennarity of *N*-glycans.

6.5 Discussion

6.5.1 Computer model predictions, molecular cloning and the stable cell line

In this study, the computer model prediction of *N*-glycans attached to recombinant HCG facilitated the selection of enzymes from the glycosylation pathway, whose genetic modification could possibly increase the level of tri- and tetra-antennary structures. The model assumed the 25-fold increase in the level of GnTV mRNA and the knockdown of GalT enzymes to 10% of their original amount. The combination of these two modifications was expected to increase the amount of triantennary *N*-glycan structures (from 3% to 62.9% of the total pool of *N*-glycans) with some alteration in the level of tetra-antennary *N*-glycans (from 0% to 5%). The effect of GnTV overexpression without the subsequent knockdowns of galactosyltransferases on human interferon- γ was previously reported by Fukuta *et al.* in 2000. According to their experiments, the increased activity of GnTV resulted in the increased amount of triantennary sugar chains by up to 55.7%. In addition, co-expression of GnTIV and GnTV in parental CHO cells increased the level of tetra-antennary structures by 56.2 %. These results were in agreement with the predicted values from the computer model designed by Dr. Andrew McDonald and it was necessary to verify them experimentally by establishing a CHO7J cell line that harboured two genetic modifications, the overexpression of GnTV together with the RNAi-induced knockdown of galactosyltransferases.

According to QPCR, genetic manipulations on CHO7J cells expressing HCG resulted in the 11-fold increase in the GnTV mRNA and the diverse knockdown levels (up to 60%) of GalT enzymes. Unfortunately, no conclusions could be drawn with respect to the importance of GalT1 to the glycosylation profile of HCG and this results from an insignificant, 3%, silencing of this particular enzyme. Clearly, variable GalT knockdown levels are possibly the result of different transduction efficiencies for each lentiviral titer harbouring siRNA as well as the efficiency of mRNA silencing upon lentiviral transduction.

It should also be emphasized whether the RNAi-induced knocked-down of individual GalTs can be compensated by any other enzyme from the same family. The possible up-regulation can be easily verified by QPCR however the increased mRNA level may not reflect the actual protein accumulation in the Golgi apparatus.

6.5.2 Analysis of *N*-glycans attached to HCG

The 2-AB labelled *N*-glycans from immunopurified preparations of HCG were analyzed by the combination of normal phase HPLC (NP-HPLC), exoglycosidase digestion, weak-anion exchange HPLC (WAX-HPLC). To summarize, the combination of the GnTV overexpression with the RNAi-induced knockdown of CHO galactosyltransferases significantly increased the ratio of partially or fully sialylated and galactosylated triantennary structures with a potential fucose compared to the control pool of *N*-glycans. In addition, the simultaneous decrease in the level of biantennary glycans could be observed.

From the digestion profile, no differences were seen between the sialidase S (NAN1) and ABS digests, indicating that all sialic acids linkages were α 2,3 and α 2,8 and not α 2,6. A sialyltransferase that transfers sialic acid onto a terminal galactose residue using an α 2,6 linkage is expressed normally by many tissues of humans and other species (Paulsen *et al.*, 1989), but not by CHO cells (Lee *et al.*, 1989; Harduin-Lepers *et al.*, 2001). It is currently unknown whether the presence of α 2,6- as well as α 2,3-linked sialic acids have any significance in terms of the biological or pharmacodynamic properties of glycoproteins.

6.5.3 Summary

Chinese hamster ovary (CHO) cells are widely used for production of recombinant glycoproteins developed as therapeutics for a spectrum of diseases. The pattern of *N*-glycan structures in this particular cell line exhibit features characteristic of glycans naturally occurring on human proteins. Pharmacokinetic properties of many therapeutic glycoproteins are strongly dependant on the post-translational modifications with glycosylation being one of the most important.

In this study, the genetic modifications of the CHO7J cell line producing recombinant HCG and the subsequent *N*-glycan analysis suggest that the branching of sugar chains may be controlled by intracellular activities of certain enzymes (e.g. GnTV and galactosyltransferases) that take part in the glycosylation pathway. This technology can produce glycoforms out of natural occurrence, which should enlarge the potency of glycoprotein therapeutics.

CHAPTER 7

General Discussion

7.1 Discussion

Surprisingly, even 114 years after the original Camillo Golgi discovery, we know very little about this organelle. Found in both plant and animal cells, the Golgi is an extremely dynamic structure of a very limited space. Enzymes within the cisternae are able to modify the proteins by addition of carbohydrates (glycosylation) and phosphates (phosphorylation). The biological, developmental, and physiological significance of the action of glycosyltransferases is highlighted by the consequences of deletion or knock-down of members of this protein family, which results in developmental anomalies as well as in congenital defects in glycosylation (CDG) in humans. Growing evidence for the biologic importance of glycosylation has fueled a remarkable expansion in the field of glycobiology.

This PhD project aimed to investigate a group of GlcNAc transferases (GnTs) from Chinese hamster ovary cells (CHO), which belong to a large family of glycosyltransferases residing in the Golgi apparatus. First, these enzymes were cloned and expressed in bacterial cells in order to generate polyclonal antibodies. The soluble GnTI was also tested for its *in vitro* activity toward ovalbumin using a spectrophotometric coupled assay. GnTs were correctly localised in the Golgi apparatus when overexpressed in wild type CHO cells and co-stained with specific Golgi markers, giantin and TGN46. Finally, CHO7J cells expressing human chorionic gonadotropin (HCG) were used for bioengineering study in order to increase antennarity of *N*-glycans attached to this hormone.

Specific antibodies have always been of a great scientific value with a broad range of applications in medicine, biotechnology industry and research. In Chapter 3, all experiments were design to obtain full-length and soluble or insoluble GnTs in order to generate polyclonal antibodies raised in rabbits. At the start of this project there was no sequence information about CHO GnTII and three isoforms of GnTIV. The sequence information of missing cDNAs was obtained using existing data due to the high sequence identity ($\geq 84\%$) between enzymes from different species. All CHO GnT sequences were further cloned into expression vector that was compatible with *E.coli* expression system. Similarly to previous attempts to express and purify mammalian GnTs in *E.coli* (Nishiu *et al.*, 1995; Fujiyama *et al.*, 2001), full-length CHO GnTI, II and V were found in cytoplasmic granules (inclusion bodies) composed of insoluble protein aggregates.

The truncation of the membrane spanning region allowed for high-level expression of soluble GnTI and insoluble GnTIVB. The fact that only GnTI was expressed in a soluble form can be explained by the lack of potential glycosylation sites on this enzyme. All other GlcNAc transferases are post-translationally modified by glycosylation which is probably the reason for their accumulation in inclusion bodies. Polyclonal antibodies were generated against purified, insoluble CHO GnTII, IVB and V as well as soluble GnTI that were recovered from SDS-PAGE gel slices. The specificity of antibodies was further tested by Western blotting and gave positive signal for purified GnTI, II and IVB, and also for *myc*-tagged GnTs that were overexpressed in CHO cells. However antibodies were not successful in recognizing endogenous GnTs from CHO cell lysates probably due to their low expression levels. In the case of the anti-GnTV polyclonal antibody that was disregarded from further experiments due to lack of signal, epitopes on the misfolded antigen were probably not effectively exposed to the immune system and the antibody was not successful in recognizing the protein.

In addition to custom made polyclonal antibodies against full-length proteins generation of anti-peptide antibodies was an alternative. In this case, rabbits were injected with a mixture of three peptides that were carefully selected from each GnT protein sequence. Unfortunately, despite numerous attempts the anti-peptide antibodies failed to recognize endogenous or *myc*-tagged GnTs that were overexpressed in CHO lysates. This can be explained by the fact that synthetic peptide sequences may not be part of an epitope or epitopes are composed of different parts of the polypeptide chain that are brought into spatial proximity by the folding of the protein.

The aim of Chapter 4 was to investigate kinetic parameters of recombinant CHO GnTI and the spectrophotometric coupled enzyme assay proved to be a valuable method for measuring the activity of this enzyme. In this assay, the UDP produced by GnTI was coupled to NADH oxidation to NAD^+ via pyruvate kinase (PK) and lactate dehydrogenase (LDH). The decrease in absorbance at 340 nm was the basis for measurement of the enzyme activity. Since K_m values for acceptor substrates can vary dramatically, the kinetic data obtained from *in vitro* assays may not reflect the actual values from intracellular compartments. The K_m values of recombinant CHO GnTI for UDP-GlcNAc and $\text{Man}_5\text{GlcNAc}_2 \{M_5\}$ from ovalbumin appeared to be comparable to the values obtained from several animal sources (e.g. rabbit liver preparation, CHO extracts). Several factors

affected the activity of recombinant GnTI, including pH and temperature. In addition, GnTI as a member of glycosyltransferase family showed an absolute requirement for divalent metal ions (Mn^{2+}), which were also added to activate pyruvate kinase contained in the assay.

Transient overexpression of *myc*-tagged GnTs in CHO cells (Chapter 5) resulted in their correct targeting to the Golgi apparatus that was co-localised with specific Golgi markers (giantin and TGN46). Unfortunately, localisation studies of CHO GnTs showed that no distinction between specific Golgi compartments can be made using standard confocal microscopy and higher resolution, for instance electron microscopy, has to be applied. New insights into the morpho-functional organization of the Golgi can be provided by recent techniques, such as fluorescence resonance energy transfer (FRET), correlative light-electron microscopy (CLEM) and electron microscopy tomography (EMT). The main feature of those is the combination of life cell imaging and spatial resolution beyond the limits of conventional microscopy. Clearly, more work is required in order to gain some insight into this unique Golgi structure and its function.

In Chapter 6, genetic modifications of GnTV and galactosyltransferase levels in CHO7J was found to increase the sialylation, fucosylation and galactosylation of the *N*-linked glycans attached to HCG while increasing the proportion of triantennary and tetra-antennary structures (see Table 6.15 and Figure 6.29). It has been previously reported that pharmacokinetic properties of many therapeutic glycoproteins are strongly dependent on the post-translational modifications with glycosylation being one of the most important. Such modifications make them extremely hydrophilic and significantly enhance their circulatory half-life and consequently *in vivo* bioactivity. Incomplete synthesis, inconsistent sialylation and galactosylation of these structures can result in variable clearance of these proteins via specific receptors, such as the asialoglycoprotein receptor, which have evolved in the liver and in macrophages to bind and internalize glycoproteins devoid of terminal sugar moieties (Shenkman *et al.*, 1997). In addition to the stability, glycosylation is responsible for the intracellular folding and assembly, which have a profound influence on the secondary and tertiary protein structure (Jeffries and Lund, 1996; Elliott *et al.*, 2003). One of the major aims to the pharmaceutical industry is to control glycosylation of recombinantly produced proteins such that they are equipped with complex glycans and do not initiate adverse immune responses. Chinese hamster ovary

(CHO) and human embryonic kidney 293 cells (HEK293) are widely used for production of recombinant glycoproteins developed as therapeutics for a spectrum of diseases. The pattern of *N*-glycan structures in these particular cell lines exhibit features characteristic of glycans naturally occurring on human proteins (Jenkins and Curling, 1994; Liu, 1992). To summarize, it is possible to engineer glycoproteins bearing specific oligosaccharide structures by genetically modifying the genome of a cell. New technologies that control glycosylation and deliver glycoengineered protein are urgently required and are of huge commercial relevance.

7.2 Future work

Numerous questions related to the Golgi processing, regulation, specificity of glycosylation enzymes and how these enzymes are directed to, and retained in, the sub-compartments of the Golgi organization await investigation. The synthesis of particular oligosaccharide structures would seem to demand a unique biologic regulation. The current view concludes that the expression of enzymes involved in the glycosylation pathway is controlled by transcription factors on the early stage of gene expression (Robbins, 1991; Comelli *et al.*, 2006; Alison *et al.*, 2008). Several lines of evidence indicate that the process is also controlled by hormones and cellular regulators including cAMP (Konral and Merz, 1994). Are there any other key control points, e.g. specific chaperones or other cellular regulators for individual glycosyltransferases to be found? Understanding how the glycosylation process is regulated remains an important question in cell biology.

Several recent publications show unusual subcellular localisations of glycosyltransferases, which normally are localised to the Golgi apparatus. The majority of localisation studies on glycosyltransferases have been performed with common cell lines like CHO or HEK293 cells. What new features of glycosyltransferase organization will become evident when tissues and more exotic cell sources are utilized? There must be mechanisms to account for the different distributions of particular glycosylation enzymes across the Golgi stack and, further, such mechanisms must also explain the basis for the overlapping gradients of enzymes across the stack. Immunolocalisation, followed by confocal imaging or electron microscopy, provides a snap-shot of the distribution of a glycosyltransferases at any moment in time and thus represents a steady-state distribution, however, it gives no information on whether the proteins are actively retained or constantly moving. What are

the processes occurring within the cisternae that control targeting and retention of glycosyltransferases in the Golgi apparatus?

As previously stated, glycosylation enzymes represent an overlapping distribution and demonstrate cell-type-specific Golgi subcompartmentation (Rabouille *et al.*, 1995). In order to initiate the glycosylation process, the Golgi imports nucleotide sugars from the cytosol. What is the relationship between the organization of sugar-nucleotide transporters and glycosyltransferases? Is the presence or absence of nucleotide sugars another controlling point in the glycosylation? How is this process regulated by the Golgi apparatus?

GnTs transfer a GlcNAc residue from nucleotide sugars to saccharide acceptors, which are usually attached to polypeptides. In each case, the product of one transferase reaction serves as a substrate for transfer of the next monosaccharide. In many instances the same oligosaccharide has the potential to be an acceptor for more than one glycosyltransferase (Roseman, 1970). There are some questions that arise as regards peptide- as well as oligosaccharide-specific glycosyltransferases. For instance, what are the characteristics of peptide recognition sites? Can one transferase recognize multiple peptide sequences? Is peptide recognition dependent on continuous primary sequence or is the recognition site more complex? How many recognition sites are required when multiple oligosaccharides are present? How low must the apparent K_m be to assure efficient modification of specific target glycoproteins? What is the error rate for glycosyltransferases that display peptide specificity? Most importantly, how do kinetic parameters measured *in vitro* relate to the synthetic outcome *in vivo*? Detail analysis of many glycosylation enzymes revealed that the answers to these questions are far more complex than previously thought.

The sequences involved in the peptide recognition sites appear to be distant from glycosylation sites. It is also possible that glycosyltransferases have multiple recognition motifs. For many enzymes, recognition requires the maintenance of native tertiary structure. This requirement has made it difficult to define precisely the features of the recognition site. Modifications that alter the spatial relationship of amino acids usually result in a loss of recognition. Clearly, further studies have to be carried out to obtain valuable data.

Considering the fact that multiple transferases and glycosidases compete for modification of the same oligosaccharide, differences in K_m , V_{max} , and localisation must be sufficient to assure the outcome. The glycosyltransferases have their apparent K_m values in the low micromolar to the low millimolar range. An apparent K_m in the low micromolar range appears to assure both good fidelity and efficiency of transfer. Expression of equal levels of these enzymes could result in modification on glycoproteins lacking many oligosaccharide structures. The potential advantage of this would be to coordinate the expression levels of a specific glycosyltransferase so that the enzyme is near saturation for the higher-affinity substrate. As more is learned about the specificity of these enzymes, the ability to modulate the kinetic constants for transfer of monosaccharides to oligosaccharide acceptors will allow us to control glycosylation *in vivo*.

The analysis of sugar chains attached to recombinant HCG following genetic manipulations revealed significant changes that may have an impact on the overall pharmacokinetics and potency of a drug. However, in order to further increase the antennarity of complex *N*-glycans, several other approaches can be undertaken including the overexpression of GnTIV or simultaneous overexpression of GnTIV and V in combination with the knockdown of galactosyltransferases. Normal CHO cells do not have high activities of GnTs and it seems obvious that the overexpression can provide highly branched, possibly novel *N*-glycan structures. Clearly, generation of novel, genetically modified cell lines would be considered useful for investigating the potency of remodeling of sugar chains to improve functions of glycoproteins.

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