IMMUNODETECTION AND REGULATION OF \( \beta 1,4\)-GALACTOSYLTRANSFERASE IN B LYMPHOCYTES

Jeremy Joel Hugo KEUSCH

Department of Immunology
University College London Medical School
University College London

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Abstract

β1,4-galactosyltransferase (β1,4-GalTase) is a glycosyltransferase localized in the trans Golgi of most mammalian cells where it is involved in the galactosylation of glycoconjugates, transferring galactose from UDP-galactose to the non-reducing end of exposed GlcNAc residues. This project examined β1,4-GalTase expression in various B cells and its ability to galactosylate different acceptor substrates in vitro and in situ.

B cells, isolated from the peripheral blood of patients with rheumatoid arthritis (RA) and controls, were assayed for β1,4-GalTase activity in a newly developed ELISA-based assay which utilized the neoglycoprotein, GlcNAc-pITC-BSA, as the acceptor substrate. The previously reported decrease in B cell β1,4-GalTase activity from patients with RA was not apparent with the GlcNAc-pITC-BSA substrate, but could be detected using ovalbumin as an acceptor.

A number of anti-β1,4-GalTase monoclonal antibodies (mAbs) were produced against soluble human milk β1,4-GalTase. These mAbs were purified, conjugated and extensively characterized enabling the immunodetection of β1,4-GalTase in different assay systems. The binding affinities of the anti-β1,4-GalTase mAbs were measured against several different types of purified β1,4-GalTase using surface plasmon resonance and were all found to be of moderately high binding affinity (K_B approximately 10^8 M^-1, against human milk β1,4-GalTase). All the anti-β1,4-GalTase mAbs cross-reacted with bovine milk β1,4-GalTase although to different extents. Two anti-β1,4-GalTase mAbs against non-competing epitopes were used to develop a sensitive (>1 ng/ml) β1,4-GalTase protein quantification ELISA which could measure β1,4-GalTase protein from cell lysates and extracellular sources. Preliminary data suggested that there was no apparent difference between the β1,4-GalTase protein levels in the B cells from controls and patients with RA.

B cells which differed in their levels of β1,4-GalTase activity were produced following the stable transfection of IgG-secreting B cells, from the same cell line, with human β1,4-GalTase cDNA in the sense and antisense orientations. The β1,4-GalTase sense transfected B cells had higher levels of β1,4-GalTase expression and secreted IgG with more galactosylated structures than those B cells transfected with the antisense. However, factors other than β1,4-GalTase expression levels influenced the galactosylation of IgG.

Experiments and results presented in this thesis were discussed with particular reference to the under-galactosylated IgG observed in patients with RA.
"Ah, la belle chose que de savoir quelque chose"

Molière
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Abbreviations

ASpcDNA3  pcDNA3 with GalTase cDNA in the antisense orientation
BIC      50 mM bicarbonate buffer, pH 9.6
BSII     Bandeiraea simplicifolia II
BSA      bovine serum albumin
CD       cluster of differentiation
CMV      cytomegalovirus
Con A    Concanavalin A
DMSO     dimethylsulphoxide
E. coli  Escherichia coli
EBV      Epstein Barr virus
ECL      enhanced chemiluminescence
EDTA     ethylenediaminetetraacetic acid
ELISA    enzyme-linked immunosorbent assay
ELLA     enzyme-linked lectin assay
Endo H   endo-β-N-acetylglucosaminidase H
FBS      foetal bovine serum
FITC     fluorescein isothiocyanate
GalTase  galactosyltransferase
β1,4-GalTase β1,4-galactosyltransferase
GlcNAc   N-acetylglucosamine
GlcNAc-pITC-BSA N-acetylglucosamine-phenylisothiocyanate-bovine serum albumin
hCG      human chorionic gonadotropin
HBSS     Hanks’s buffered saline solution
HRP      horseradish peroxidase
5-IAF    5-iodoacetamidofluorescein
Ig       immunoglobulin
IgG G0   IgG lacking galactose residues
KDa      kiloDalton
LB       Luria broth
mAbs     monoclonal antibodies
ONPG     o-nitrophenyl-β-D-galactopyranoside
OPD      o-phenylenediamine
pAbs     polyclonal antibodies
PAGE     polyacrylamide gel electrophoresis
PB       peripheral blood
PBMC  peripheral blood mononuclear cells
PBS   0.15 M phosphate-buffered saline, pH 7.4
PBS-T 0.15 M phosphate-buffered saline, pH 7.4, 0.05% Tween 20
PEG   polyethylene glycol
PNA   peanut agglutinin
PNPP  p-nitrophenyl phosphate
RA    rheumatoid arthritis
RCAI  *Ricinus communis* agglutinin I
RF    rheumatoid factor
rhGalTase recombinant human β1,4- GalTase
rhGalTase^{116} recombinant human β1,4- GalTase from amino acid 116
*S. cerevisiae*  *Saccharomyces cerevisiae*
SD    standard deviation
sdH₂O sterile-distilled water
SDS   sodium dodecyl sulphate
SEM   standard error of the mean
SNA   *Sambucus nigra* agglutinin
SpcDNA3 pcDNA3 with GalTase cDNA in the sense orientation
SPR   surface plasmon resonance
TCR   T-cell receptor
TBE   89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3
TE    10 mM Tris-HCl, pH 7.5, 1 mM EDTA
TSSS  rhGalTase with a four point mutation downstream from the proposed UDP-galactose binding site
UDP   uridine diphosphate
UV    ultraviolet
X-gal  5-bromo-4-chloro-3-indoly-β-galactopyranoside

**Additional abbreviations**

*CV*  coefficient of variation
*ddH₂O* double-distilled water
*DTT*  dithiothreitol
*Nc*  nitrocellulose
*s.c.*  subcutaneous
*SIGAL* α2,6-sialyltransferase
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1. General Introduction
1.1 Introduction

Beta 1,4 galactosyltransferase (β1,4-GalTase) is one of a number of glycosyltransferases involved in the biosynthesis of glycoconjugates (Dinter and Berger, 1995b). Within the lumen of the trans Golgi, β1,4-GalTase galactosylates appropriate N-acetylglucosamine (GlcNAc) residues on oligosaccharide chains. This galactosylation function occurs at low levels in almost all eukaryotic cells and β1,4-GalTase is therefore thought of as a housekeeping gene. Although the reactions of many glycosyltransferases have been elucidated, their cellular regulation remains largely unknown.

In some disease states, such as rheumatoid arthritis (RA), reduced galactosylation of the N-linked oligosaccharides in IgG (IgG G0) has been demonstrated (Parekh et al., 1989). Apart from being a useful marker of disease activity (Young et al., 1991) and prognostic indicator (van Zeben et al., 1994), the IgG G0 appears to be pathogenic (Rademacher et al., 1994; Rademacher et al., 1995). The molecular basis for IgG G0 production is unclear, though it seems to be a pre-secretory event (Mullinax et al., 1976; Bodman et al., 1992). There have been some reports detecting reduced β1,4-GalTase activity in the B lymphocytes from RA patients but it is unclear if this leads to the production of IgG G0 (Axford et al., 1987; Furukawa et al., 1990; Wilson et al., 1993).

The research presented in this thesis was aimed at examining the relationship between β1,4-GalTase expression in B cells, from RA patients, controls and cell lines, and the galactosylation of different substrates.

1.2 Glycosyltransferases

The biosynthesis of oligosaccharide chains in eukaryotes occurs through the sequential activities of multiple glycosyltransferases and glycosidases (Roth, 1987; Moremen et al., 1994). The glycosylated products may consist of highly branched structures connected by many different types of linkages. This is in contrast to the synthesis of proteins and nucleic acids which utilize template-guided mechanisms. N-linked glycosylation commences in the endoplasmic reticulum following the 'en-bloc' transfer of the mannose/GlcNAc oligosaccharide chain to nascent proteins with a suitable asparagine found in the consensus sequence Asn-X-Ser/Thr/Cys, where X can be any amino acid except proline (Kornfeld and Kornfeld, 1985; Grinnell et al., 1991). The oligosaccharide chain then undergoes further trimming by glycosidases, elongation and terminal glycosylation by specific glycosyltransferases as it translocates through the
endoplasmic reticulum and Golgi. O-linked glycosylation is a much later post-translational event occurring in the Golgi. The synthesis of the oligosaccharide chain is usually initiated by the transfer of N-acetylgalactosamine to Ser/Thr via the hydroxyl group. Elongation of the sugar chain then takes place through sequential glycosyltransferase reactions, resulting in several different core structures compared to the common pentasaccharide core found in N-linked oligosaccharides (Figure 1.4). There is no unique consensus sequence for O-linked glycosylation (Hansen et al., 1995a), however programs are becoming available to predict potential sites of O-glycosylation (Hansen, 1995b). Further, recently developed techniques allows the identification and quantification of O-linked sugars at particular proteins sites (Gooley and Williams, 1997). The biological roles the oligosaccharides confer to their acceptor substrates are variable in both significance and function, and usually difficult to predict (Kobata, 1992; Hounsell and Davies, 1993; Lis and Sharon, 1993; Varki, 1993; Dwek, 1995). Another type of O-linked glycosylation occurs in the cytoplasm, where a single GlcNAc residue is attached to Ser/Thr, sometimes acting reciprocally with phosphorylation, and may regulate many cellular processes (Hart et al., 1995).

Glycosyltransferases consist of a family of enzymes which transfer, in a specific anomic linkage, the glycosyl moiety from nucleotide-, or dolichol-, activated phosphoglycosides (donor) to other carbohydrates or aglycones, such as a protein or lipid (acceptor), often requiring the presence of divalent cations as co-factors (Schachter and Roseman, 1980). Glycosyltransferases are very specific in their recognition of acceptor substrate which has given rise to the central dogma in glycobiology of 'one glycosidic-linkage, one enzyme'. However, there are a few exceptions to this rule including the Lewis blood group dependent α3/4-fucosyltransferase, Fuc-T III. This enzyme can transfer fucose residues to GlcNAc in both 3- or 4- positions in type 1 (Galβ1,3GlcNAc) and type 2 (Galβ1,4GlcNAc) structures. Conversely, it is possible, at least in vitro, for a single linkage to be synthesized by several distinct glycosyltransferases such as with the human α3-fucosyltransferases: Fuc-T III, Fuc-T IV, Fuc-T V and Fuc-T VI which form Galβ1-4(Fucα1-3)GlcNAc, the LeX product. Differences in the acceptor substrate specificities of these Fuc-T are found, for example Fuc-T IV will only transfer to non-sialylated Galβ1-4GlcNAc, whereas Fuc-T V and Fuc-T VI are also able to synthesize sialyl LeX, Neu5Aca2,3Galβ1-4(Fucα1-3)GlcNAc. In fact Fuc-T III, Fuc-T V, Fuc-T VI and Fuc-T VII can all form the sialyl LeX structure (Natsuka and Lowe, 1994).

Considering the vast array of oligosaccharides found in Nature, the central dogma predicts that there are about 150 glycosyltransferases responsible for their biosynthesis. Progress in the cloning of glycosyltransferases has until quite recently been an arduous
task, as the majority of the glycosyltransferases are only present in tiny quantities. Hence the isolation of glycosyltransferases and subsequent identification of their amino acid sequence was only possible for those enzymes available in high amounts. However, with the advent of expression cloning for glycosyltransferases (Larsen et al., 1989; Fukuda, 1996), the identification of the cDNA was possible without the need to isolate the enzyme. This has led to an increase in the number of glycosyltransferases cloned (for reviews see Schachter, 1994; Field and Wainwright, 1995; Dinter and Berger, 1995b; Wilson, 1997).

Despite the fact that many glycosyltransferases recognize identical donor or acceptor substrates, there is very little primary amino acid sequence homology. However, all the cloned Golgi glycosyltransferases do share the same topology of type II membrane-bound proteins (Paulson and Colley, 1989). These single chain polypeptides have a short, positively-charged cytosolic amino-tail linked to a hydrophobic transmembrane domain. The enzyme extends into the lumen of the Golgi, via a potentially glycosylated stem region followed by the largest portion, the globular carboxyl-terminal catalytic domain (Figure 1.1). The stem region is one of the most heterogeneous domains between glycosyltransferases. It has an unusually high number of glycine and proline residues to give it a flexible structure and includes a number of N-linked glycosylation consensus sites. Proteolytic cleavage in the stem region can occur to release a soluble and enzymatically active transferase protein. In contrast, the ER-resident glycosyltransferases show diverse protein domain structures (Shaper and Shaper, 1992).

1.3 β1,4 Galactosyltransferase (β1,4-GalTase)
(UDP-Gal:GlcNAc-R β1,4-Galactosyltransferase, EC 2.4.1.38/90)

1.3.1 Catalytic activity of β1,4-GalTase

\[ \text{UDP-Gal} + \text{GlcNAc-R} \xrightarrow{\text{Mn}^{2+}} \text{Galβ1-4GlcNAc-R} + \text{UDP} \]

β1,4-GalTase is located in the trans Golgi (Roth and Berger, 1982) where it is involved in glycan biosynthesis, specifically the formation of N-acetyllactosamine structures. The work in this thesis deals with the N-acetyllactosamine synthetase activity of β1,4-GalTase and is referred to throughout as β1,4-GalTase activity for the sake of brevity. The β1,4-GalTase reaction involves the transfer of galactose from UDP-galactose onto the 4-position of non-reducing terminal end of N-acetylglucosamine (GlcNAc).
Figure 1.1

Topology of human β1,4-GalTase. The type II membrane-bound glycoprotein is divided up into several domains with relevant amino acid positions noted:  
1) Short positively-charged N-terminal cytoplasmic tail.  
2) Transmembrane domain required for Golgi retention.  
3) Stem region which contains an N-linked consensus site (in green), and can be cleaved by proteases (grey arrow) to release a catalytically active soluble enzyme.  
4) Globular C-terminal catalytic domain containing a disulphide bond (in red) necessary for activity.  
Speculative phosphorylation sites of serine residues (blue circles). The SPHK consensus phosphorylation site for cdc2 kinases is located within the disulphide loop.  
Potential locations of palmitation (in pink).
The GlcNAc may be a free residue, part of an isolated oligosaccharide or attached to a protein or lipid, though the specificity and efficiency of the reaction does vary according to the aglycone acceptor substrate (Geren et al., 1976; Rao and Mendicino, 1978; Do et al., 1995; Portner et al., 1996). Other glycosyltransferase have also been shown to efficiently use glycosides linked to hydrophobic aglycones, which facilitated separation of the products on C-18 cartridges (Palcic et al., 1988; Palcic et al., 1990). β1,4-GalTase from rat liver Golgi and bovine milk can also specifically galactosylate 6-O-sulphated GlcNAc with a similar $K_m$ to the non-sulphated GlcNAc (Spiro et al., 1996).

β1,4-GalTase activity has conventionally been measured using a radiochemical assay (Verdon and Berger, 1983), though more recently a number of ELISA-based assays have been developed (Stults and Macher, 1990; Zatta et al., 1991; Keshvara et al., 1992; Taki et al., 1994; Keusch et al., 1995) (see chapter 3).

β1,4-GalTase exhibits a preference for galactosylation of the α1,3 arm of biantennary oligosaccharide chains over the α1,6 arm in vitro (Blanken et al., 1984; Paquet et al., 1984; Morita et al., 1988). A bisecting GlcNAc will reduce the rate of galactosylation but not affect the branch specificity (Narasimhan et al., 1985). However, IgG oligosaccharides isolated from serum revealed a higher frequency of galactose on the α1,6 branch (Figure 1.4). As the N-linked oligosaccharides on IgG are sequestered within the Fc domain, it is possible that the formation of the interchain disulphide bond on IgG affects the degree of glycosylation (Rademacher et al., 1996a). There is also the suggestion that the protein moiety per se influences the presentation of the acceptor substrate to β1,4-GalTase (Fujii et al., 1990; Lund et al., 1993b).

An unusual characteristic of β1,4-GalTase is the modification of its acceptor specificity following interaction with a regulator protein, α-lactalbumin. This 1:1 reversible complex between β1,4-GalTase and α-lactalbumin forms the enzyme, lactose synthase (EC 2.4.1.22). This interaction results in a decreased $K_m$ for glucose (Glc) by about three orders of magnitude, from 2M to 2 mM (Brew et al., 1968) thus creating an effective substrate at physiological concentrations of glucose and the synthesis of lactose:

\[
\text{UDP-Gal} + \text{Glc} \xrightleftharpoons{\text{Mn}^{2+}} \text{Mn}^{2+} \rightarrow \text{Galβ1,4Glc} + \text{UDP}
\]

The synthesis of α-lactalbumin only occurs in the epithelial cells of the mammary glands during late pregnancy and lactation, under the control of various hormones including insulin, glucocorticoids, prolactin and progesterone (Brew, 1970). Hence, the β1,4-GalTase activity as part of the lactose synthetase complex only occurs in vivo in
the lactating mammary gland. Inositol may also be galactosylated by lactose synthetase, though slowly, to produce galactinol (White et al., 1982).

Strong homologies exist between α-lactalbumin and mammalian and avian egg-white lysozyme with respect to amino acid sequence, genomic organization and chain-folding structure (Brew, 1970; Nitta and Sugai, 1989). However, lysozyme cannot substitute α-lactalbumin in the lactose synthase reaction, nor does α-lactalbumin possess lysozyme activity (Marshall, 1994). Lysozyme is a bacteriolytic enzyme which catalyses the hydrolysis of a β1,4 glycosidic linkage between N-acetylmuramate and GlcNAc residues in bacterial cell wall peptidoglycans.

It has been proposed that there are two distinct clusters of aromatic amino acid residues in α-lactalbumin, one involved in the binding of β1,4-GalTase and the other in the formation of the glucose substrate binding pocket (a "monosaccharide bridge"), with both sites in close proximity (Grobler et al., 1994). Different β1,4-GalTase interact with α-lactalbumin from different species. Non-mammalian β1,4-GalTase, in the form of chicken β1,4-GalTase, can also be modified by α-lactalbumin to carry out the lactose synthetase reaction (Hathaway et al., 1991). Further, the acceptor substrate specificity of the pond snail, Lymnaea stagnalis, β1,4 N-acetylgalactosaminyltransferase can be altered from GlcNAc to glucose following interaction with α-lactalbumin (Neeleman and van den Eijnden, 1996). These results are indicative of the α-lactalbumin binding site being conserved through evolution. However, there are examples of β1,4-GalTase exhibiting variable interaction and modulation by α-lactalbumin. IM-9 B lymphoblast β1,4-GalTase, whose activity was affected by α-lactalbumin could not be purified using an α-lactalbumin affinity column (Wilson, 1991). β1,4-GalTase isolated from porcine trachea (Sheares and Carlson, 1984) and from the adult worms of Schistosoma mansoni (Rivera-Marrero and Cummings, 1990) were found to be α-lactalbumin-independent. Further, the regulation of β1,4-GalTase acceptor substrate by α-lactalbumin is also dependent on the type of acceptor substrate (Furukawa, et al., 1990), and its concentration in the reaction (Brew, et al., 1968; Klee and Klee, 1970) since α-lactalbumin has surprisingly been shown to increase β1,4-GalTase activity towards AsAg-biantennary sugar chains (Morita, et al., 1988).

Recently, it was discovered that the interaction of α-lactalbumin with β1,4-GalTase could induce the enzyme to efficiently use UDP-GalNAc as a donor substrate and transfer the GalNAc in a β1,4 linkage to GlcNAc. This transfer was also observed to several chitin oligomers, with decreasing efficiency as the acceptor size increased, although no transfer to Glc was possible (Do, et al., 1995). It is interesting to note that p-nitrophenyl derivatives of GlcNAc were better substrates than free GlcNAc, in the presence or absence of α-lactalbumin (Do, et al., 1995), possibly due to the hydrophobic moiety substituting for the aromatic cluster of amino acids in α-lactalbumin (Grobler, et al., 1994) and enhancing β1,4-GalTase to use UDP-GalNAc.
1.3.2 Purification of β1,4-GalTase

The purification of β1,4-GalTase was facilitated by appreciable quantities being present in various extracellular fluids, most notably milk. β1,4-GalTase has been purified from many sources including: colostrum; amniotic fluid; mammary glands; serum; sperm; liver; and trachea from a number of species (Kleene and Berger, 1993). Exploitation of β1,4-GalTase specific interaction with its donor, acceptor and its regulator protein α-lactalbumin were crucial purification steps (Barker et al., 1972). The use of GlcNAc-derivatized agarose in the purification reduced the extent of proteolytic breakdown of bovine β1,4-GalTase (Barker, et al., 1972).

Human milk β1,4-GalTase purification resulted in a single glycoprotein band of approximately 55 kDa by SDS-PAGE (Gerber et al., 1979). However, when this band was subjected to isoelectric focusing, at least 13 different isoenzymes were apparent which were all enzymatically active (Gerber, et al., 1979). Part of the heterogeneity was due to phosphorylation and sulphation, but not to sialylation (Gerber, et al., 1979; Strous, 1986; Strous et al., 1987). The bovine colostrum was a much richer source of β1,4-GalTase than milk, resulting in a single glycoprotein component of 51 kDa compared to the multiple forms usually obtained from the bovine milk purification (Magee et al., 1974b), which were probably due to proteolytic degradation (Powell and Brew, 1974; Magee et al., 1976). Since the cloning of human β1,4-GalTase, the cDNA has been used in a variety of prokaryotic and eukaryotic expression systems including large-scale production in Saccharomyces cerevisiae, with the recombinant enzyme showing similar characteristics to the human milk β1,4-GalTase (Krezdorn et al., 1993; Herrmann et al., 1995; Malissard et al., 1996). Recombinant production of β1,4-GalTase may soon be successful in obtaining sufficiently good quality crystals for structural studies.

1.3.3 Genomic structure of β1,4-GalTase

Bovine β1,4-GalTase was the first mammalian glycosyltransferase gene to be cloned (Shaper et al., 1986). Monospecific polyclonal antibody to the affinity-purified bovine milk β1,4-GalTase was used to screen a λgt11 bovine kidney expression library which resulted in the isolation of a partial clone (1.3 kb) (Shaper, et al., 1986). Shortly after this first report, another group isolated several clones from a bovine mammary gland cDNA library using synthetic oligonucleotides as hybridization probes, based on the amino acid sequence of bovine milk β1,4-GalTase (Narimatsu et al., 1986). Both
groups had isolated partial cDNA clones encoding the carboxyl portion of β1,4-GalTase, with very good correlation between the sequences and only conservative amino acids changes found. Overlapping cDNA clones from bovine liver cDNA libraries spanning 1.7 kb, revealed a 1.2 kb open-reading frame that encoded the full-length membrane-bound form of bovine β1,4-GalTase of 402 amino acids (D'Agostaro et al., 1989).

A partial human cDNA was isolated from a human liver cDNA library which encoded the carboxyl-terminal region (Appert et al., 1986b). This fragment was used to probe a human placental cDNA library which resulted in the identification of overlapping clones spanning the full-length coding sequence (1.4 kb) for human β1,4-GalTase (Masri et al., 1988). HeLa cell cDNA coding for β1,4-GalTase was over 99% homologous to the placental cDNA. Apparent differences included two conservative amino acid substitutions in the transmembrane domain and an additional serine residue in the cytoplasmic tail (Watzele and Berger, 1990).

Full-length murine β1,4-GalTase has also been cloned (Shaper et al., 1988a) and mapped to the centromeric region on chromosome 4 (Shaper et al., 1987). Human β1,4-GalTase was encoded by six exons spanning more than 50 kb bases of genomic DNA (Mengle-Gaw et al., 1991) on human chromosome band 9p13-p21 (Duncan et al., 1986; Shaper et al., 1986). Although the human β1,4-GalTase gene appeared to be a single gene, a second weak hybridization signal on chromosome 22q13 was also noted, possibly indicating a homologous gene or a pseudogene sequence (Shaper et al., 1986). The chicken genome contains two functional non-allelic β1,4-GalTase genes (Shaper et al., 1995). Very recently, studies on β1,4-GalTase gene knock-outs, expression cloning and CHO cell glycosylation mutants, suggest the existence of more than one β1,4-GalTase gene in several different species (Asano et al., 1997; Lu et al., 1997; Sato et al., 1997) (Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, USA, personal communication).

A similar genomic structure was observed for murine (Hollis et al., 1989) and human (Mengle-Gaw, et al., 1991) β1,4-GalTase (Figure 1.2). Exon 1 encoded the 5' untranslated region, N-terminal cytoplasmic tail, transmembrane domain and the first 93 amino acids of the stem region in the carboxyl domain. The remainder of the β1,4-GalTase carboxyl domain was encoded by exons 2-6. The largest exon, exon 6, contained a TAG stop codon and a very long (2.9 kb) 3' untranslated region which included a polyadenylation site. Another common structure between human, murine and bovine β1,4-GalTase genes were the two transcription initiation sites present in exon 1 separated by approximately 200 bp. Murine β1,4-GalTase expressed two transcripts of 4.1- and 3.9 kb which translated a long- (399 amino acid) and short- β1,4-GalTase (386 amino acids) protein respectively, and differed only by a 13 amino acid
long cytoplasmic tail (Shaper et al., 1988b) (see Figure 1.2). The long transcript was produced by initiation upstream (-190 to -145, relative to the translation initiation codon) of the first in-frame ATG start codon and the short transcript was initiated between the two ATG codons, 14-24 bp downstream from the first ATG (Harduin et al., 1993). A similar process also occurred in bovine β1,4-GalTase (Russo et al., 1990). When the 4.1 kb mRNA transcript was translated in vitro only the long form of β1,4-GalTase was produced and not both sizes of β1,4-GalTase (Russo et al., 1990). Although the two transcription sites in the human β1,4-GalTase gene exist, it is unclear if both transcripts exist as Mengle-Gaw et al (1991) could only detect a single transcript in HeLa mRNA which corresponded to short β1,4-GalTase. However, Pouncey et al (1991) detected both transcripts in HeLa cells and both sizes of precursor polypeptides, indicative of both transcripts being translated (Strous et al., 1988).

Another transcriptional site, located within exon 1, has been found in the murine male germ cell. In the transcripts of pachytene spermatocytes and round spermatids an additional 560 bp of 5' untranslated sequence was present, compared to the 4.1 kb transcript of somatic cells and spermatogonia (Harduin et al., 1992). The additional sequence contained a germ cell specific start site (Shaper et al., 1994) which gave rise to transcripts containing the same open reading frame as the 4.1 kb transcripts but had truncated 3' untranslated ends due to use of alternative polyadenylation sites (Harduin et al., 1992). Further, all murine male germ cells produced the long β1,4-GalTase protein as the proximal transcriptional start site was not utilized (Shaper et al., 1990) (see Figure 1.2).

There was a lack of classic eukaryotic promoter sequences 5' of the transcription start sites such as TATA and CAAT. Sp1 transcription factor was found located in the region just upstream from the 4.1 kb start site, which contains several GC boxes (Rajput et al., 1996). Four of these sites were exactly conserved in human, bovine and murine β1,4-GalTase gene (Harduin et al., 1993). Other conserved sites included that of a mammary cell activating factor (MAF), a half palindromic CTF/NF1 element, and a negative element which repressed the transcription of the 3.9 kb transcript. There were also some AP2 biding sites, a cAMP-responsive regulatory element CREB and three half palindromic glucocorticoid response elements (GRE) much further upstream (between -1060 and -1160) (Harduin et al., 1993). The lack of TATA and CAAT sequences and the presence of the GC boxes, along with the different transcriptional start sites is more typically associated with constitutive genes. The ability of β1,4-GalTase to be upregulated in certain cells can be controlled at the transcriptional level as described below.
Genomic structure and mRNA transcripts of murine β1,4-GalTase. Coding sequences are divided into six exons (colour boxes) spanning more than 50 kb on chromosome 4 (Hollis, et al., 1989). Untranslated sequences are represented by open boxes. Bent arrows indicate the germ cell (Gc), the 4.1 kb and 3.9 kb transcriptional start sites (Shaper, et al., 1988a; Shaper, et al., 1994). A negative element (black square) represses the transcription of the 3.9 kb transcript (Rajput, et al., 1996). The dotted lines in the 3' untranslated region represent alternative polyadenylation sites (Shaper, et al., 1990). The transmembrane domain, encoded by sequences within exon 1, is represented by a black block in the folded protein.
1.3.4 Protein structure of β1,4-GalTase

1.3.4.1 Biosynthesis of β1,4-GalTase
In HeLa and HepG2 cells, β1,4-GalTase is synthesized as two polypeptides of 45 kDa and 47 kDa from two different mRNAs (Strous, et al., 1988) (see section 1.2.2). There was always a higher ratio of the larger molecular weight band, which reflects the preferential use of the 4.1 kb transcriptional start site in these cells (Strous, et al., 1988). Twenty minutes after synthesis in the endoplasmic reticulum, the β1,4-GalTase is translocated to the Golgi where it is retained as a 54 kDa glycoprotein (Strous, et al., 1988). Proteolysis of the Golgi membrane-bound β1,4-GalTase released a soluble 52 kDa form from the cell (Strous and Berger, 1982). β1,4-GalTase was found to have a total cellular half-life of about 19 hours (Strous and Berger, 1982). Glycosylation, palmitation and phosphorylation account for the increase in molecular weight in the mature β1,4-GalTase.

1.3.4.2 Glycosylation of β1,4-GalTase
As described above β1,4-GalTase is a type II membrane-bound Golgi glycoprotein (Figure 1.1). The type and extent of glycosylation varies depending on the source of β1,4-GalTase. Bovine β1,4-GalTase has two N-linked consensus sequences compared to the single sites conserved sites in human and murine β1,4-GalTase (Figure 1.3). The two chicken β1,4-GalTase genes are each predicted to encode for one and five N-linked glycosylation sites (Shaper, et al., 1995). O-linked sugars have been described in bovine milk β1,4-GalTase (Lehman et al., 1975) and human milk β1,4-GalTase, with blood group determinants expressed in accord with the blood types of the donors (Amano et al., 1991) The blood group-related carbohydrate structures on human milk β1,4-GalTase are immunogenic in rabbits (Childs et al., 1986). Human serum β1,4-GalTase contains approximately 11% carbohydrates but does not contain GalNAc residues which are commonly associated with O-linked glycosylation (Fujita-Yamaguchi and Yoshida, 1981). A higher degree of sialylation had been seen in β1,4-GalTase isolated from pooled human amniotic fluid and malignant ascites compared to pooled human milk (Gerber, et al., 1979). Increased sialylation had also been observed in embryonic chicken liver β1,4-GalTase compared to the adult form (Furukawa and Roth, 1985), yet it is unclear if the sialylation is on N-linked and/ or O-linked sugars. The many sialylated isoenzymes of β1,4-GalTase exhibited similar enzymatic activities to a variety of acceptor substrates (Gerber, et al., 1979). Also the carbohydrate moiety on β1,4-GalTase was not involved in its catalytic activity nor in the binding of α-lactalbumin (Wong et al., 1983).
Figure 1.3

Comparison of the amino acid sequences of human placental (Hu, Masri, et al., 1988), murine mammary (Mu, Shaper, et al., 1988a and 1988b) and bovine liver (Bo, D’Agostaro, et al., 1989) β1,4-GalTases. Identical amino acids to the human sequence are shown by a (-). Potential N-linked glycosylation sites are in green. All seven conserved cysteine residues are in red. Repetitive elements in the stem region are in blue. The transmembrane domain is over- and under-lined. Known cleavage sites in human β1,4-GalTases are indicated by ~. Amino acids are divided into six exons by « ». Missing amino acids are marked by *. Differences in the human sequence have been seen at position 11 where an extra serine found in HeLa β1,4-GalTase (Watzle and Berger, 1990). The ygt1 at position 211 was not found in two other reports (Watzle and Berger, 1990; Mengle-Gaw, et al., 1991).
Further, human β1,4-GalTase cDNA may be expressed in *Escherichia coli* in a catalytically active non-glycosylated form (Aoki et al., 1990; Chatterjee, 1991; Nakazawa et al., 1993). Soluble human β1,4-GalTase expression in *S. cerevisiae* has been possible in glycosylated and non-glycosylated (via site-directed mutagenesis) forms with enzyme kinetics comparable to the human milk β1,4-GalTase (Malissard, et al., 1996). In addition the carbohydrate moiety on β1,4-GalTase was not required for its solubility or its thermostability. The function of the carbohydrates on β1,4-GalTase is unknown. They may serve in the correct folding of β1,4-GalTase, as a lower expression was achieved with the non-glycosylated β1,4-GalTase compared to its glycosylated form (Malissard, et al., 1996). Further, the expression levels of active β1,4-GalTase in *E. coli* were low (Chatterjee, 1991; Nakazawa, et al., 1993).

### 1.3.4.3 Soluble β1,4-GalTase

The soluble form of β1,4-GalTase is derived from its membrane-bound form by proteolytic cleavage (Smith and Brew, 1977; Strous, 1986). Different cleavage sites have been identified in human milk β1,4-GalTase as well as heterogeneity at the amino terminal end (Gerber, et al., 1979; Appert, et al., 1986a). Other glycosyltransferases also exist as soluble forms. A cathepsin D-like protease has been suggested to cleave the membrane-bound ST6Gal I to release the soluble enzyme (Lammers and Jamieson, 1988). Whilst the function of the soluble β1,4-GalTase is unknown, further research on this phenomenon is warranted as large amounts can be found in the extracellular fluid (Kim et al., 1972b; Strous, 1986). It is unlikely to be simply a means of controlling the cellular levels of β1,4-GalTase as this would be an energetically expensive process. ST6Gal I is secreted as part of an acute phase response (Kaplan et al., 1983). Although the soluble form of β1,4-GalTase is catalytically active, suitable concentrations of UDP-galactose are unlikely to exist in the extracellular fluid for the β1,4-GalTase reaction to occur *in vivo*. It may be possible for the soluble β1,4-GalTase to function as a lectin-like molecule, binding to suitably exposed GlcNAc residues.

### 1.3.4.4 Stem region of β1,4-GalTase

The cleavage site has been found within the stem region at amino acids 42 (Gerber, et al., 1979), and Arg77 (Appert, et al., 1986a) in human milk β1,4-GalTase and at 76 and 96 in bovine milk β1,4-GalTase (D'Agostaro, et al., 1989). The terminal amino acid found in the pooled human β1,4-GalTase at position 42 varied, including tyrosine, leucine, valine, lysine and arginine (Gerber, et al., 1979) (Prof. Eric Berger, University of Zurich, Switzerland, personal communication). Thus cleavage is possible towards the luminal-end of the transmembrane domain, probably via a protease with broad hydrophobic specificity. At present it is unclear if this degree of heterogeneity is due to
differences within the sample source and/or between the samples. Although the stem region exhibits the most diversity between the different species of β1,4-GalTase, there are common features such as the consensus sites for N-glycosylation and an unusually high abundance of proline and glycine residues with some repetitive elements which indicate flexibility.

1.3.4.5 Substrate binding sites on β1,4-GalTase

As the soluble form of β1,4-GalTase is catalytically active for both β1,4-GalTase and lactose synthetase activities, the cytoplasmic tail, transmembrane domain and stem region, up to amino acid 129 (Boeggeman et al., 1993; Wang et al., 1994), are not required for enzymatic activity per se. N-terminal deletion mutants of β1,4-GalTase showed very similar apparent $K_m$ values for GlcNAc, chitobiose and chitotriose as those for bovine milk β1,4-GalTase (Boeggeman et al., 1995). The essential binding sites for the donor, acceptor and Mn$^{2+}$ must therefore reside within the soluble form of β1,4-GalTase. Chemical modifications of β1,4-GalTase has identified key amino acid residues involved in the binding of substrates. Ultraviolet photo-inactivation of β1,4-GalTase, and inactivation with 2-hydroxy-5-nitrobenzyl bromide, revealed the importance of tryptophan in the β1,4-GalTase—Mn$^{2+}$—UDP-galactose interaction (Clymer et al., 1976). Aromatic residues were also shown to be involved in the same β1,4-GalTase—Mn$^{2+}$—UDP-galactose interaction complex as detected using circular dichroism (Geren et al., 1975). Incorporation of $^{125}$I into β1,4-GalTase resulted in modification of tyrosines with loss in enzyme activity to various degrees. Tyrosines could be protected by α-lactalbumin or UDP-galactose, however, combinations including α-lactalbumin, UDP and GlcNAc afforded the greatest protection (Silvia and Ebner, 1980). Further differential spectroscopic studies have also shown the importance of tyrosine and tryptophan in the β1,4-GalTase—Mn$^{2+}$—UDP-galactose interaction (Takase and Ebner, 1981). Chemical modification with a periodate-cleaved UDP and trace acetylation analysis, identified Lys$^{341}$, located next to one of the seven cysteines in β1,4-GalTase, and Lys$^{351}$ in the binding of UDP-galactose (Yadav and Brew, 1990). Even though cysteine residues are important in β1,4-GalTase enzymatic function as demonstrated by inhibition with a variety of sulphhydryl reagents, the inhibition was not complete as approximately 5-15% of the original activity remained (Magee and Ebner, 1974a; Wong and Wong, 1984). The binding sites on bovine milk β1,4-GalTase for α-lactalbumin and UDP-galactose appear to be separate as suggested by photo-affinity labelling with a UDP-galactose analogue (Lee et al., 1983). The α-lactalbumin interacts in the N-terminal portion of the molecule between residues 93 and 250 (Yadav and Brew, 1991), whilst UDP-galactose binds in the carboxyl end of β1,4-GalTase from amino acid 275 (Yadav and Brew, 1990).
Since the availability of the β1,4-GalTase cDNA, recombinant DNA technologies have been used in structure and function studies. Boeggeman et al (1995) have localized the binding sites on β1,4-GalTase for GlcNAc and UDP-galactose to distinct domains. Bovine cDNA mutants were expressed in E. coli and binding sites for GlcNAc and UDP-galactose (via Mn^{2+}) were found to occur in the N-terminal (residues 130-257) and C-terminal (residues 258-402) regions respectively (Boeggeman, et al., 1995). GlcNAc was also found to bind to the C-terminal fragment, but only the interaction with the N-terminal fragment exhibited similar binding characteristics to catalytically active β1,4-GalTase protein (residues 130-402). However, using human cDNA mutants obtained through site-directed mutagenesis and expressed in E. coli, substrate binding sites were found to be in a much closer proximity to each other. The binding area centred around a hydrophobic pocket and included residues Tyr^{284}, Tyr^{309} and Trp^{310} involved in GlcNAc binding with Tyr^{309} also involved in the binding of UDP-galactose (Aoki, et al., 1990). Further studies around this hydrophobic area identified Phe^{305}Pro^{306}Asn^{307}Asn^{308} residues involved in the UDP-galactose binding site, though they did not affect the binding of Mn^{2+} (Zu et al., 1995). The apparently contradictory data on substrate binding sites in β1,4-GalTase may be explained if the catalytic domain of β1,4-GalTase was accessible enough to allow weak binding of substrates. The interaction of UDP-galactose and acceptor substrate may then induce a conformational change which would allow a very close association between the substrate binding regions to permit the transfer of galactose and the formation of the glycosidic bond. Hence the binding of substrates on separate β1,4-GalTase fragments containing N-terminal (130-257) or C-terminal (258-402) residues, would not cause such a conformational change. Similarly, if substrate analogues were able to bind to the substrate binding sites on β1,4-GalTase but unable to induce the required conformational change, then no β1,4-GalTase reaction would occur. It has been suggested that β1,4-GalTase may have two GlcNAc binding sites. Synthetic divalent spacer substrates, containing two molecules of GlcNAc separated by different length spacer molecules, were found to give an optimal rate of galactosylation when 10 spacer atoms were used (Ats et al., 1994). The lipophilic linker may have also increased the β1,4-GalTase activity to a certain extent due to a previously observed hydrophobic effect (Geren, et al., 1976; Portner, et al., 1996). Another study reported that ovalbumin and the acceptor, benzyl-β-glucosamide, resulted in non-competitive inhibition of the β1,4-GalTase activity (Polentz et al., 1995). These results may be explained as two active sites being present on β1,4-GalTase, but equally β1,4-GalTase may only have one active site per molecule yet exist as a dimer (Malissard, et al., 1996). The N- and C-terminal β1,4-GalTase mutants produced by Boeggeman et al. (1995) show that the N-terminal fragment binds GlcNAc much tighter than the C-terminal fragment, but it is
unclear from this data if there are two GlcNAc binding sites on β1,4-GalTase (Dr. Pradman Qasba, National Institute of Health, Maryland, USA, personal communication).

In all the cloned β1,4-GalTases there are seven conserved Cys residues (Figure 1.3). It has been predicted that there is a disulphide bond Cys\(^{129}\)•••Cys\(^{245}\) in human β1,4-GalTase that is essential for catalytic activity (Wang, et al., 1994). A similar observation has been made with the equivalent (Cys\(^{134}\)•••Cys\(^{247}\)) bond in bovine β1,4-GalTase (Boeggeman, et al., 1995). Interestingly, recombinant inactive β1,4-GalTase mutants lacking the Cys\(^{134}\) will still bind their substrates, albeit with a reduced affinity for the UDP compared to the catalytically active enzyme (Boeggeman, et al., 1995). Mutation of the carboxyl end Cys\(^{340}\) to a serine yielded a catalytically active β1,4-GalTase with a much higher \(K_m\) for UDP-galactose, further Cys\(^{340}\) is the only cysteine residue that reacts with sulphydryl reagents (Wang, et al., 1994).

The limited degree of homology between the primary sequences of the glycosyltransferases has been restricted to small areas of functional importance. Comparison of a short peptide in β1,4-GalTase, Cys\(^{340}\)(X)\(^{5,6}\)Lys/ArgAspLysLysAsn(Asp/Glu), with that found in with mouse UDP-Gal:Galβ1,4GlcNAc-R α1,3-GalTase, Cys(X)\(^{5,6}\)GlnAspLysLysHisAsp, has led to the suggestion that this region may form the UDP-galactose binding site (Joziasse et al., 1989; Yadav and Brew, 1990), a binding function both these transferases share. Yadav et al (1990) also found some correlation between these two transferases at sites further upstream of the hexapeptide that were involved in UDP-galactose binding. This extended region was very close to the hydrophobic pocket identified as the likely UDP-galactose binding site (Zu, et al., 1995). Comparisons of functional domains have been made between β1,4-GalTase and the glycosyltransferase UDP-GlcNAc: GlcNAcβ-R β1,4-N-acetylglucosaminyltransferase from Lymnaea stagnalis which both transfer sugars onto GlcNAc residues in a β1,4 linkage (Bakker et al., 1994). High conservation of amino acids between these two transferases have been found in the area surrounding the hydrophobic domain (Bakker, et al., 1994), previously identified as involved in the catalytic site (Aoki, et al., 1990; Zu, et al., 1995). A number of the cysteines were also conserved between the two enzymes (Bakker, et al., 1994). Other glycosyltransferases, distinct from β1,4-GalTase but which share limited sequence homology and/ or function, have been identified (Bakker, et al., 1994; Neeleman and van den Eijnden, 1996). This opens up the possibility for the existence of a GalTase gene family. It is interesting to note that β1,4-GalTase can accept a variety of donor substrates, including UDP-Glc and UDP-GalNAc but not UDP-GlcNAc, yet at less than 0.4% of the rate for UDP-Gal (Palcic and Hindsgaul, 1991). Moreover, it has recently been demonstrated
that α-lactalbumin could induce β1,4-GalTase to efficiently use UDP-GalNAc in its transfer to GlcNAc, with the transfer of GalNAc only occurring to GlcNAc and not to Glc (Do, et al., 1995).

Divalent cations are an essential requirement for the β1,4-GalTase reaction, with Mn$^{2+}$ as the most efficient (Navaratnam et al., 1986). It has been shown that there are two binding sites for the cations, a high affinity site I requiring micromolar concentrations of Mn$^{2+}$ (apparent K$_m$ of approximately 10-20 μM) and a low affinity site II requiring millimolar concentrations of Mn$^{2+}$ (apparent K$_m$ of approximately 800 μM). β1,4-GalTase has approximately 70% full activity when site I is occupied and site II is empty, but is not active with only site II occupied (Kuhn et al., 1991). However, these requirements probably exceed the likely physiological concentration of Mn$^{2+}$ by 2-4 orders of magnitude. When β1,4-GalTase was assayed within sealed Golgi membrane vesicles, apparent K$_m$ values towards Mn$^{2+}$ of 0.1-0.2 μM were observed with either added glucose or endogenous acid-precipitable acceptor (Kuhn, et al., 1991). The use of chelators in these vesicles permeabilized with A23187 almost abolished lactose synthetase activity, and was only adequately restored by Mn$^{2+}$. Lysis of the vesicles caused the apparent K$_m$ at site I to increase to 10 μM. Thus it appears that site I is probably occupied by Mn$^{2+}$ in vivo (Kuhn, et al., 1991). Site II on the other hand can be stimulated by basic proteins such as histone and clupeine, as well as by small organic cations such as spermidine and spermine (Navaratnam, et al., 1986; Navaratnam et al., 1988). Treatment of bovine lymphocytes and the epithelium of mouse small intestine with inhibitors of polyamine biosynthesis resulted in swelling of the Golgi apparatus and a decrease in β1,4-GalTase activity (Sakamaki et al., 1989). Addition of spermine or spermidine, restored the Golgi structure and also stimulated β1,4-GalTase activity (Sakamaki, et al., 1989). Whilst activation of β1,4-GalTase is achieved at both sites I and II, only site II stabilizes β1,4-GalTase (Kuhn et al., 1992).

1.3.5 Subcellular localization of β1,4-GalTase

1.3.5.1 Golgi β1,4-GalTase

β1,4-GalTase has been localized predominantly to the trans Golgi where it was found to codistribute with thiamine pyrophosphatase (Roth and Berger, 1982; Strrous, 1986; Ulrich et al., 1986; Watzele et al., 1991; Taatjes et al., 1992; Berger et al., 1993). However, in some cells the β1,4-GalTase distribution has been found to overlap into the trans Golgi network (Geuze et al., 1985; Taatjes et al., 1987; Nilsson et al., 1993b; Rabouille et al., 1995). The oligosaccharides on β1,4-GalTase contain sialic acids.
which also suggests that there is some overlap between the glycosyltransferases and/or the biosynthesis of β1,4-GalTase involves some sort of retrieval trafficking from the trans Golgi network to the trans Golgi. In several cell types the colocalization of β1,4-GalTase and ST6Gal I to the trans Golgi cisternae could be distinguished into separate compartments following treatment with monensin and to a lesser extent with chloroquine, suggesting discrete subcompartmentation (Berger, et al., 1993). Chloroquine has been shown to inhibit sialylation, but not affect galactosylation, of immunoglobulins in chloroquine-treated plasma cells (Thorens and Vassalli, 1986). The ability of individual glycosyltransferases to concentrate within certain Golgi locales requires a retention mechanism(s) (Shaper and Shaper, 1992; Machamer, 1993; Pelham and Munro, 1993; Colley, 1997). All the cloned Golgi glycosyltransferases are type II membrane-bound proteins, yet share little sequence homology. Therefore the retention signals are most likely to be general conformations rather than exact peptide sequence.

At present there are two potential Golgi retention mechanisms being tested which may work together: 1) the bilayer thickness model, which proposes that the shorter transmembrane domain of Golgi proteins prevent them from entering the cholesterol-rich vesicles for targeting to the plasma membrane (Bretscher and Munro, 1993; Masibay et al., 1993; Munro, 1995). It is thought that a higher concentration of cholesterol exists across the Golgi stack (Orci et al., 1981) 2) The oligomerization/kin recognition model, which proposes that the oligomerization of Golgi proteins blocks their entry into transport vesicles (Nilsson et al., 1993a; Gleeson et al., 1994; Rabouille, et al., 1995; Nilsson et al., 1996).

Golgi retention of ST6Gal I indicated that retention may be attributed to several domains including the transmembrane domains, flanking sequences and residues present within the stem region (Munro, 1991; Colley et al., 1992; Dahdal and Colley, 1993; Masibay, et al., 1993). Recently, it has been found that the stem region of N-acetylglucosaminyltransferase I (GnT I) was required and sufficient for kin recognition of mannosidase II and Golgi retention (Munro, 1995; Nilsson, et al., 1996). Studies with β1,4-GalTase deletion mutants and chimeras have identified the major role the transmembrane domain plays in anchoring β1,4-GalTase to the trans-Golgi (Nilsson et al., 1991; Aoki et al., 1992; Teasdale et al., 1992; Masibay, et al., 1993; Teasdale et al., 1994; Yamaguchi and Fukuda, 1995).

Comparison of the sequences in the transmembrane domain and the area flanking the N-terminal side, show a very high degree of homology between mammalian and avian β1,4-GalTases. This conserved sequence may be relevant to Golgi retention (Shaper and Shaper, 1992). Point mutations of Cys²⁹ to Ser and His³² to Leu within the
transmembrane domain of β1,4-GalTase resulted in the loss of some Golgi retention and appearance on the cell surface (Aoki, et al., 1992). Further, stable expression of β1,4-GalTase was sensitive to deletions in the transmembrane domain, as deletion of the first five amino acids, up to the cysteine residue, in this region abolished activity (Masibay, et al., 1993). However, Nilsson et al (1991) found that chimeric constructs using the human invariant chain as a reporter protein achieved Golgi retention with just the luminal half of the transmembrane domain of β1,4-GalTase (which excluded Cys\textsuperscript{29} and His\textsuperscript{32}). Other Golgi proteins show no primary sequence homology between their transmembrane domains. Additional studies have indicated that β1,4-GalTase as well as other Golgi localized enzymes exist as dimers (Navaratnam, et al., 1988; Bendiak et al., 1993; Fleischer et al., 1993; Colley, 1997). Overexpression of β1,4-GalTase-transferrin chimeras resulted in a small percentage of β-mercaptoethanol-resistant homo-dimers which required the Cys\textsuperscript{29} and His\textsuperscript{32} residues in the transmembrane domain (Yamaguchi and Fukuda, 1995). Even less dimer formation was apparent with the β1,4-GalTase wild-type constructs. However, full-length β1,4-GalTase stably transfected into murine L cells was found to exist almost entirely as high molecular weight oligomers, which may indicate a role in Golgi retention (Teasdale, et al., 1994). Purified soluble β1,4-GalTase from different sources has been shown to exist as a dimer (Powell and Brew, 1974; Malissard, et al., 1996). β1,4-GalTase isolated from the rat liver Golgi membranes was found to form large oligomers which were dependent upon a 66 N-terminal amino acid sequence (Bendiak, et al., 1993). This data would suggest that β1,4-GalTase has some inherent capacity to self-associate without the need for additional molecules. Although the intracellular dimer formation may well be different from that seen with the purified form, in that additional complexes with other Golgi proteins, lipid bilayer and cytoskeletal components are involved (Nilsson et al., 1994; Slusarewicz et al., 1994; Nilsson, et al., 1996). It has been found that the Golgi enzymes β1,4-GalTase, ST6Gal I, uridine diphosphatase and α-mannosidase II are all functionally active as dimers (Moreman et al., 1991; Fleischer, et al., 1993). However, another disulphide-bonded dimer of ST6Gal I, representing about 30% of the enzyme in rat liver, was found to be catalytically inactive yet could still bind galactose (Ma and Colley, 1996). Rat hepatoma cells stimulated with dexamethasone synthesized homodimers of ST6Gal I \textit{in vivo} which constituted approximately 20 % of the total immunoprecipitated ST6Gal I from these cells (Bosshart and Berger, 1992).

The cytoplasmic tail may play an accessory role in the retention process (Nilsson, et al., 1991; Evans et al., 1995). Nilsson's work suggested that the cytoplasmic tail, provided either by β1,4-GalTase or an isoform of the human invariant chain, Iip31, in combination with the transmembrane domain sustained complete retention of β1,4-GalTase.
GalTase in the Golgi. In contrast, Shur and colleagues have attributed the role of the cytoplasmic tail in β,4-GalTase to its cell surface expression.

1.3.5.2 Cell surface β,4-GalTase

β,4-GalTase expression is also found on the plasma membrane of certain cell types using biochemical and immunodetection techniques. It is unlikely that cell surface β,4-GalTase galactosylates structures in vivo as there is no evidence of sufficient concentrations of UDP-galactose found extracellularly. However, the β,4-GalTase still has the capacity to bind GlcNAc terminating glycoconjugates and could therefore act like a lectin with other cell surface molecules or cell matrix molecules exposing the appropriate substrates (Shur, 1991). The functional roles of cell surface β,4-GalTase have included the following possibilities: fertilization of murine ova (Miller et al., 1992); a receptor for polymeric IgA (Aicher et al., 1992); cell migration (Eckstein and Shur, 1989; Shur, 1993), developmental growth (Hinton et al., 1995); neurite outgrowth (Huang et al., 1995); and a signal transducing receptor (Gong et al., 1995).

Whist the detection of β,4-GalTase at the cell surface is acknowledged, the molecular mechanism(s) responsible for this localization is contested. Very recently two similar liver ST6Gal I have been identified which differ by a single amino acid Cys/Tyr in the catalytic domain at position 123. Low levels of the ST6Gal I Tyr^{123} was found on the cell surface of transfected COS cells and high levels secreted into the tissue culture medium, whereas the ST6Gal I Cys^{123} was retained within the Golgi (Ma et al., 1997). Differential transcription of the β,4-GalTase gene can result in a long- or short- β,4-GalTase protein which are identical except for a 13 amino acid cytoplasmic tail extension in the long form (see section 1.2.2 and Figure 1.2). Most somatic cells were found to produce low levels of the long β,4-GalTase, suggesting that the transmembrane domain is of prime importance for Golgi retention (Harduin et al., 1993). Murine sperm only express the long β,4-GalTase (Shaper et al., 1990) which is localized to the plasma membrane (Lopez et al., 1985). Stable transfections of the long and short form β,4-GalTase cDNA suggest that the long form β,4-GalTase preferentially locates to the plasma membrane (Lopez et al., 1991), which has been confirmed in both F9 and 3T3 cells using antibodies against the 13 amino acid tail extension (Youakim et al., 1994a). Overexpression of the long form of β,4-GalTase in COS-1 cells lead to increased protein in both the Golgi and the plasma membrane, whereas the short β,4-GalTase only targeted to the Golgi (Dinter and Berger, 1995a).

Variable cellular locations has been found in different cells expressing constructs at different levels (Teasdale et al., 1994; Dinter and Berger, 1995a) (see chapter 5). Consistent with the cell surface localization of β,4-GalTase was a post-translational modification, likely to have occurred in the trans Golgi network (Teasdale et al., 1994). Further, it was thought unlikely that any of the cell surface β,4-GalTase was recycled
to the Golgi (Teasdale, et al., 1994). Others have shown that overexpression of proteins can lead to retention in the ER but not saturate the Golgi and cause a leakage of the excess protein onto the cell surface (Nilsson, et al., 1991; Russo et al., 1992). Further, other groups have shown no preferential localization of the long form of β1,4-GalTase to the plasma membrane in stably transfected CHO cells (Russo, et al., 1992) or when highly expressed in COS-1 (Teasdale, et al., 1992), COS-7 cells (Masibay, et al., 1993), or HeLa cells (Nilsson, et al., 1991). Possible explanations for this conflicting data may be due in part to different experimental approaches such as the reporter proteins used in chimeras, variations in the transfection efficiencies, the detection methods used and the particular cell types which utilize different localization signals (Tang et al., 1995). A potential caveat in studying retention signals with chimeric molecules or mutants proteins may be the creation of retention signals or cause the subsequent loss of a transport mechanism (Low et al., 1994; Low et al., 1995; Wahlberg et al., 1995).

Masibay et al. (1993) found that increasing the number of hydrophobic residues (using isoleucines) in the transmembrane region of β1,4-GalTase led to an increase in its cell surface location on COS cells. Similar results have been seen for other Golgi proteins (Munro, 1991; Burke et al., 1994), though it was not an all-or-nothing process suggesting that other sequences or retention mechanisms are involved. There is some indication that both of the above models may be involved in localizing β1,4-GalTase to the trans Golgi. At present it is unclear if the disruption in Golgi retention is mainly due to an increased number of transmembrane residues or if the folding and recognition signal of amino acids has been perturbed. Replacement of the transmembrane domain in GnT I with leucines did not alter its kin recognition of mannosidase II or its Golgi retention but severely disrupted the Golgi structure (Nilsson, et al., 1996). Very recently two similar liver ST6Gal I have been identified which differ by a single amino acid Cys/Tyr in the catalytic domain at position 123. Low levels of the ST6Gal I Tyr123 was found on the cell surface of transfected COS cells and high levels secreted into the tissue culture medium, whereas the ST6Gal I Cys123 was retained within the Golgi (Ma, et al., 1997).

During the biosynthesis of β1,4-GalTase it undergoes palmitation, probably at or just before entry in the Golgi complex (Strous, 1986). Addition of fatty acid groups on other integral membrane proteins has been located near to or within the transmembrane region and linked to either a serine, threonine or cysteine residue (Strous, 1986; James and Olson, 1990). Such residues exist in this region in β1,4-GalTase and it would be interesting to speculate if fatty acylation played a role in membrane targeting (Masibay, et al., 1993) and/or cell signalling pathways (Molenaar et al., 1988). Post-translational
phosphorylation of β1,4-GalTase may also participate in Golgi localization (Strous, 1986).

1.3.6 Regulation of β1,4-GalTase activity

1.3.6.1 Transcriptional and translational regulation of β1,4-GalTase

The structure and amount of β1,4-GalTase transcripts is regulated during murine spermatogenesis (Shaper, et al., 1990; Harduin, et al., 1992; Shaper, et al., 1994). The primitive germ cells, spermatogonia, only express the 4.1 kb mRNA transcript, which is identical to the 4.1 kb transcript found in most somatic cells (Shaper, et al., 1990). As the diploid spermatogonia enter meiosis and develop into pachytene spermatocytes, the expression of β1,4-GalTase reaches very low levels (Shaper, et al., 1990). Following differentiation to the haploid round spermatids the expression of the 4.1 kb transcript is replaced with higher levels of two shorter transcripts of 3.1- and 2.9 kb (Shaper, et al., 1990). These truncated transcripts encode the same open reading frame as the 4.1 kb transcript but differ in their 5' and 3' untranslated regions (Shaper, et al., 1990). The shorter transcripts result from the use of alternative polyadenylation sites in the 3' untranslated region (Shaper, et al., 1990). The haploid round spermatids have an extra transcriptional start site (-732 bp) in the extended 5' untranslated region compared to spermatogonia and somatic cells (Shaper, et al., 1994).

Thus the murine β1,4-GalTase gene has been shown to have three transcriptional start sites: i) The distal one that is germ cell specific and found only in pachytene spermatocytes and early round spermatids (Shaper, et al., 1994); ii) The intermediate start site used predominantly in almost all somatic cells and spermatogonia (Harduin, et al., 1992) and; iii) The proximal start site which is highly upregulated in mid- to late-pregnant and lactating mammary gland (Harduin, et al., 1993). Further, there is some evidence to suggest that tissue-specific and housekeeping promoters are regulating the expression of the different transcripts (Shaper, et al., 1990; Harduin, et al., 1993; Rajput, et al., 1996).

Two β1,4-GalTase mRNA transcripts of 4.1 kb and 3.9 kb, coding for the long and short forms of β1,4-GalTase respectively, have been found in many tissues from different species (Shaper, et al., 1988a; Russo, et al., 1990; Shaper, et al., 1990). Pouncey, et al (1991) also identified the 4.1 kb transcript, sometimes with a smaller sized 3.0 kb transcript. Cells with varying levels of β1,4-GalTase expression were analysed for their transcriptional start sites (Harduin, et al., 1993) and regulatory factors of transcription (Rajput, et al., 1996). Sp1 transcription factor bound to regions just upstream from the 4.1 kb start site (Rajput, et al., 1996). Different DNA-binding
proteins were located close to the 3.9 kb start sites including AP2 (in the lactating mammary gland) a mammary gland form of CTF/NF1, Sp1 and a negative regulatory factor (NRF) which repressed transcription from the 3.9 kb start site (Rajput, et al., 1996). A model of transcriptional regulation proposed that the housekeeping promoter was utilized in the expression of the 4.1 kb transcript, whilst transcription from the 3.9 kb start site was controlled by an adjacent tissue-specific promoter. In somatic cells expressing low levels of β1,4-GalTase such as brain tissue, the neuroblastoma cell line N18TG2 and spermatogonia (Shaper, et al., 1990) only the 4.1 kb transcript was synthesized (Harduin, et al., 1993) as NRF would be bound (no 3.9 kb mRNA produced) and only low levels of Sp1 transcription factor available to bind to the GC boxes upstream from the 4.1 kb start site. Those cells of intermediate β1,4-GalTase expression which included almost all somatic cells, would have more Sp1 present and a less stable interaction of the NRF resulting in about 5 times more of the 4.1 kb- than the 3.9 kb- mRNA. The mammary glands from mid to late pregnant mice and from lactating mice, which have very high expression of β1,4-GalTase, showed a much greater level of the 3.9 kb- compared to the 4.1 kb-mRNA (10:1) (Harduin, et al., 1993). These tissues would not have any interaction of the NRF, lots of AP2 and a mammary gland specific form of CTF/NF1 bound between the 4.1 kb and 3.9 kb start sites and similar amounts of Sp1 as in the cells expressing intermediate levels of β1,4-GalTase (Rajput, et al., 1996). In addition, the predicted secondary structure in the 5' untranslated region of the 4.1 kb mRNA transcript is extensive and stable, possibly resulting in poor translation compared to the 3.9 kb mRNA (Kozak, 1991; Harduin, et al., 1993). Elements for such regulation also occur in the 5' end of the bovine (Masibay and Qasba, 1989) and human (Masri, et al., 1988) β1,4-GalTase genes.

β1,4-GalTase is thought of as a housekeeping gene as it is present in most eukaryotic cells and expressed constitutively at low levels. Contrary to this proposed role are studies looking at β1,4-GalTase expression in embryonic development and cell cycle. The expression of β1,4-GalTase was shown to vary in murine embryogenesis with a peak apparent in 13 day old post-coitus embryos and low levels found at days 16 and 19. Re-expression of β1,4-GalTase was apparent in the adult mouse tissues (Pouncey et al., 1991). The developmental regulation of β1,4-GalTase mRNA was very similar to that of p58GTA mRNA, which expresses a kinase that can activate β1,4-GalTase (Pouncey, et al., 1991). Recent studies on β1,4-GalTase knock-out mice demonstrated that these mice were born normally and were fertile, thus indicating that β1,4-GalTase expression was not required for fertilization or embryonic development (Asano, et al., 1997; Lu, et al., 1997). These β1,4-GalTase-null mice had stunted growth, pituitary deficiencies, dysregulation of epithelial cell proliferation and differentiation, and high mortality (Asano, et al., 1997; Lu, et al., 1997). β1,4-GalTase mRNA was upregulated
in chicken embryos during corneal development (Cai et al., 1996). Treatment of mouse F9 teratocarcinoma cells with retinoic acid or retinoic acid plus dibutyryl cyclic AMP causes the cells to differentiate into primitive endoderm and parietal endoderm-like cells respectively with concomitant increases in β1,4-GalTase expression (Lopez et al., 1989; Kudo and Narimatsu, 1995). The accumulation of β1,4-GalTase mRNA in the F9 differentiated cells was due to post-transcriptional stabilization rather than transcriptional activation (Kudo and Narimatsu, 1995). Much higher levels of β1,4-GalTase enzyme activity compared to β1,4-GalTase mRNA were expressed in the differentiated cells, possibly as a result of post-translational regulation (Kudo and Narimatsu, 1995).

Serum stimulation of quiescent mouse 3T3 fibroblast cells (Masibay et al., 1991) and human HeLa cells (Pouncey, et al., 1991) led to an increase in the β1,4-GalTase mRNA, activity and protein levels. β1,4-GalTase gene expression is regulated in a similar way to early response genes during re-entry into the cell cycle from G0. During the cell cycle, β1,4-GalTase expression peaks at late G1, S and early G2 phases (Pouncey, et al., 1991). Superinduction of β1,4-GalTase mRNA was seen in the presence of inhibitors of protein synthesis (Masibay, et al., 1991; Pouncey, et al., 1991). The time course of β1,4-GalTase mRNA expression did not parallel that of β1,4-GalTase protein (Masibay, et al., 1991). Induction of the message peaked at 2-4 hours (early-gene) post-serum stimulation then declined, whereas the level of β1,4-GalTase protein increased steadily, reaching a maximum at 8 hours and remained stable until the cell divided (Masibay, et al., 1991). Further, the 4-fold upregulation in β1,4-GalTase protein only resulted in a 2-fold increase in β1,4-GalTase activity (Masibay, et al., 1991). Both the Golgi and cell surface β1,4-GalTase were increased following serum stimulation. A slightly greater increase occurred in the population at the cell surface, supporting an additional function for this differentially regulated β1,4-GalTase species (Masibay, et al., 1991; Hinton, et al., 1995). Similar experiments were conducted using RNase protection assays to distinguish between the long and short β1,4-GalTase transcripts, indicating that the long transcript was expressed in a biphasic pattern with peaks at 0.75 hours and at 4 hours post-stimulation whilst the short transcript only peaked at 4 hours (Hinton, et al., 1995). Further, an increase in cell surface β1,4-GalTase expression on mouse 3T3 fibroblasts, correlated with an increased number of cells arrested in the G1 phase of cell cycle (Hinton, et al., 1995). The above studies correlating surface β1,4-GalTase expression with cell growth would argue that β1,4-GalTase is a cell cycle dependent gene.

The extent of galactosylation in vivo will not only depend upon the β1,4-GalTase expression levels, but also its substrate requirements and activities of glycosyltransferases competing for the same acceptor substrate such as the bisecting GnT III (Fujii, et al., 1990; Nishiura et al., 1990). NIH 3T3 fibroblasts stably
transfected with N-ras-proto-oncogene were induced with dexamethasone to express the ras gene (Easton et al., 1991). These cells were then able to increase the activities of several glycosyltransferases including the bisecting GnT III and branching GnT V and the elongating enzymes β1,4-GalTase and β1,3 GnT resulting in higher molecular weight N-linked glycans at the cell surface (Easton, et al., 1991). Although dexamethasone can induce ST6Gal I activity via increased transcription (Wang et al., 1989), the effects seen in these transfected cells were not due to the action of dexamethasone alone (Easton, et al., 1991).

Poor correlation between in vivo (human tissues) and in vitro (transformed cell line) particular glycosyltransferase mRNA levels have been found (Li et al., 1995). Sometimes changes in glycosylation may result in little effect in vitro yet have profound consequences in vivo (Varki, 1993). For example cells lacking GnT I, which is a key enzyme in converting high mannose N-linked oligosaccharides into complex and hybrid chains, are viable (Stanley et al., 1975). However, a lack of GnT I activity in vivo results in embryonic death by day 10.5 (Ioffe and Stanley, 1994; Metzler et al., 1994).

Obviously the subcellular localization of β1,4-GalTase will influence its functional role. It has been proposed that Golgi and plasma membrane β1,4-GalTase are two independently regulated pools of cellular β1,4-GalTase (Eckstein and Shur, 1989; Lopez, et al., 1991; Masibay, et al., 1991; Evans, et al., 1995). If the long form of β1,4-GalTase does truly locate preferentially to the plasma membrane (see section 1.2.4) then transcriptional control could alter its subcellular location. Different levels of β1,4-GalTase expression have been found in various tissues as has the differential use of promoters (see above).

1.3.6.2 Post-translational regulation of β1,4-GalTase activity

Post-translational modifications of β1,4-GalTase include glycosylation, sulphation, palmitation, and phosphorylation (Strous, 1986). The oligosaccharide moieties on β1,4-GalTase were shown to have no effect on the enzymatic activity (see section 1.2.3). Sulphation has not been studied in relation to β1,4-GalTase activity but contributes to the charge heterogeneity (Strous, 1986). Palmitation was shown to occur on the two precursor and mature β1,4-GalTase polypeptides (Strous, 1986). Although the role of palmitation on β1,4-GalTase has received little interest, one report indicated that palmitate and also phosphatidylcholine could upregulate human kidney β1,4-GalTase activity, involved in the synthesis of lactosylceramide (Chatterjee and Ghosh, 1990). Similar effects were also observed on the soluble bovine milk β1,4-GalTase, whilst other acid lipids, such as phosphatidylinerine and phosphatidic acids, inhibited the activity (Mitranic et al., 1983). A much shorter fatty acid chain, n-butyrate, a known
inducer of gene expression (Kruh, 1982), has been shown to have variable effects on glycosyltransferases, causing a 2.5-fold increase in β1,4-GalTase activities (Li, et al., 1995).

Phosphorylated serine residues in β1,4-GalTase has been described which contribute to the charge heterogeneities seen in β1,4-GalTase (Strous, et al., 1987) and resulted in higher enzymatic activities (Bunnell et al., 1990a). The possibility of O-linked sugars being phosphorylated was not discounted (Strous, et al., 1987). The phosphorylated forms of β1,4–GalTase were detected primarily in Golgi-associated β1,4-GalTase in HeLa and HepG2 cells, with very little found in secreted soluble β1,4-GalTase, suggesting that the negatively charged groups may be involved in cell retention of the β1,4-GalTase (Strous, et al., 1987). Humphreys-Beher et al (1986) isolated a cDNA clone from a human expression system using an anti-β1,4-GalTase antibody. This clone was thought (wrongly) to code for human β1,4-GalTase. Further studies showed that this clone encoded a calmodulin-regulated serine/threonine protein kinase, p58GTA, which showed a high degree of sequence homology with the p34cdc2 kinases, a family of cell division control kinases. (Bunnell et al., 1990c; Kidd et al., 1991). The p58GTA is also involved in the cell cycle of CHO cells and in apoptosis (Bunnell, et al., 1990c; Lahti et al., 1995). High level expression of p58GTA in COS cells retarded cells in the early G1 phase of cell cycle (Bunnell, et al., 1990c) and caused a concomitant increase in the β1,4-GalTase activity post-translationally, but no effect on ST6 activity was observed (Bunnell, et al., 1990a). Conversely, a decreased level of p58GTA kinase expression in CHO cells correlated with increased DNA synthesis and with low β1,4-GalTase activity (Bunnell et al., 1990b). However in one study, no correlation was found between β1,4-GalTase activity and what is now known to be the p58GTA cDNA, in particular no mRNA p58GTA transcript was detected from mouse lactating mammary gland, a very rich source of β1,4-GalTase expression (Lopez and Shur, 1988). Nonetheless, p58GTA was able to phosphorylate β1,4-GalTase in vitro with an approximately three-fold increase in activity, whereas incubation in the presence of phosphatase resulted in about a six-fold decrease in activity (Bunnell, et al., 1990a).

Another calmodulin-dependent protein kinase isolated from isoproterenol-stimulated proliferating rat parotid acinar cells was also able to phosphorylate serine residues on β1,4-GalTase, but this had no effect on β1,4-GalTase activity (Purushotham et al., 1992). β1,4-GalTase contains an SPHK phosphorylation consensus sequence used by this cdc2 kinase family which is located within the soluble portion of β1,4-GalTase (Figure 1.1). This site could be phosphorylated when β1,4-GalTase is located within the Golgi, possibly even involved in retention, and dephosphorylated on the secreted β1,4-GalTase as the cleaved enzyme does not contain much phosphorylation (Strous, et al., 1987).
It would be interesting to see if β1,4-GalTase contained any O-GlcNAc which could be potential reciprocal phosphorylation sites as a means of regulating protein activity and/or function (Hart, et al., 1995). Soluble bovine milk β1,4-GalTase does not contain any O-GlcNAc (Doris Snow, University of Alabama, USA, personal communication), but as the majority of these post-translational modifications occur in the cytoplasm, the cytoplasmic tail of β1,4-GalTase which contains two serines on the long form and one serine on the short form, may be modified.

1.3.6.3 Manipulative regulation of β1,4-GalTase activity

There are a number of mammalian cells which produce defectively glycosylated proteins as they lack some functional part(s) in their glycosylation machinery, including defective glycosyltransferases, glycosidases, nucleotide-sugars or their transporters, oligosaccharide dolichol synthesis or various combinations of these (Brandli, 1991; Stanley, 1993). The Lec8 mutant CHO cell line has a reduced transport of UDP-galactose, resulting in a reduced galactosylation of glyco-proteins and lipids (Deutscher and Hirschberg, 1986). Depletion of manganese from mammalian cells in culture resulted in an inhibition of O-and N-linked glycosylation (Kaufman et al., 1994). Other modulators of β1,4-GalTase activity have already been mentioned including those of α-lactalbumin and cationic peptides (see sections 1.2 and 1.2.3). It is possible to vary the number of functional copies of specific glycosyltransferases via sense/antisense oligonucleotide approaches (Kukuruzinska et al., 1994; Yoshimura et al., 1995; Zeng et al., 1995; Hiraiwa et al., 1996) (see chapter 5). However, it is sometimes difficult to relate the level of glycosyltransferase expression to the observed glycosylated protein. Overexpression of β1,4-GalTase in F9 embryonal carcinoma cells did not result in a concomitant increase in glycoprotein biosynthesis (Youakim and Shur, 1993). Mice transgenic for β1,4-GalTase were found to have a large excess of β1,4-GalTase on the surface on sperm, which unexpectedly resulted in poor egg binding (Youakim et al., 1994b). It was thought that the transgenic sperm bound more epididymal glycosides, thus blocking β1,4-GalTase and inhibiting interaction with the egg-coat protein ZP3 (Youakim, et al., 1994b). Recent data using β1,4-GalTase knock-out mice show that β1,4-GalTase is not required for fertilization (Asano, et al., 1997; Lu, et al., 1997).

Some inhibitors of glycosyltransferase have been obtained by using deoxygenated (at the reactive hydroxyl group) acceptor analogues (Hindsgaul et al., 1991). These inhibitors had a similar Ki values to their corresponding acceptor Km and could thus be used as competitive inhibitors. However, this type of inhibitor was not effective against β1,4-GalTase or several other glycosyltransferases tested (Hindsgaul, et al., 1991). Substitution of the 4-hydroxyl group on the acceptor with a cationic group to interfere
with a putative basic group in the active site of β1,4-GalTase only resulted in weak inhibition (Field et al., 1994b). Recently, a carbocyclic analogue of UDP-galactose was synthesized and found to be an effective competitive inhibitor for bovine milk β1,4-GalTase (Yuasa et al., 1995). Mono-O-methylated UDP-galactose analogues, especially UDP-3-OMe-galactose significantly inhibited β1,4-GalTase activity as did UDP-fucose and UDP-mannose (Endo et al., 1996).

1.3.7 β1,4-GalTase and disease

1.3.7.1 Rheumatoid arthritis
Rheumatoid arthritis (RA) is a chronic inflammatory joint disease with systemic involvement. An unknown aetiological agent(s) initiates an autoimmune disease. Inflammation of the joint and destruction of the cartilage ensues resulting in joint deformity. RA primarily affects females and an increased risk has been associated with human leukocyte antigen complex, HLA-DR4 (Jawaheer et al., 1994).

1.3.7.2 Rheumatoid factor and immune complexes
Many of the clinical features associated with RA are mediated through humoral responses. Common with other autoimmune diseases are the increased levels of serum gamma globulins. In RA there is an increased secretion of immunoglobulin by both peripheral blood B cells and synovial fluid B cells. About 20% of patients with RA produce antinuclear antibodies yet more than 90% have high titres of rheumatoid factor (RF). RF, are autoantibodies which bind to part of the heavy chain on IgG, usually the Fc domain. Despite the name, RF also occur in other autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren's syndrome. The presence of RF in patients with RA generally indicates a more severe disease. Further, the RF can be of any isotype, though IgM and IgG are the most common, the latter indicating T cell help in immunoglobulin class switching (Munthe and Natvig, 1972). Since IgG can act as both the antigen and antibody, self-association can lead to the formation of immune complexes, which can also contain other immunoglobulins and complement. Large aggregates of immune complexes can activate both the classical and alternative complement pathways with subsequent production of neutrophil chemotactic factors. Immune complexes containing IgG are phagocytosed by macrophages and neutrophils which secrete cytokines and proteases, resulting in an amplified inflammatory response. As the majority of B cells in the synovium produce RF, it is unsurprising that the synovial joints are major sites for inflammation and tissue destruction (Youinou et al., 1984; Moynier et al., 1992). B cells can also act as antigen presenting cells. They are
capable of endocytosing IgG-immune complexes via their FcγR, their surface bound IgG specific receptors and there are B cells expressing surface RF. Presentation of antigen by these B cells to T cells may lead to an enhanced antigen specific immune response but also in the necessary T cell help required for increased antibody production, including RF. RA patients have higher numbers of a B cell sub-population expressing the CD5 surface antigen. Although the CD5 marker is commonly found on all mature T cells and most thymocytes, it has also associated with B cell chronic lymphocytic leukaemia and a small percentage of B cells from healthy individuals. It has been demonstrated that CD5+ B cells from control subjects are able to produce RF which are polyreactive (Casali et al., 1987; Hardy et al., 1987). In RA patients the CD5+ B cell population contains cells which secrete the polyreactive RF and also cells which produce higher affinity monoreactive RF with greater specificity to the IgG Fc fragment (Burastero et al., 1988).

1.3.7.3 IgG glycosylation
IgG N-linked glycosylation consists of two biantennary complex oligosaccharides (1 and 2, one on each heavy chain at Asn297) located within the Fc domain (Deisenhofer, 1981). The carbohydrate chains are not thought to be required for keeping the two Cγ2 domains apart (Rudd et al., 1991). N-glycans have also been detected in approximately 25% of Fab fragments and 15% of the light chains isolated from human myeloma proteins. This glycosylation occurred in the variable region, with the glycosylation site at Asn95 utilized the most frequently (Wright and Morrison, 1993). The X-ray crystallography data on (rabbit) IgG Fc domain indicated that the pentasaccharide core (Figure 1.4 D) in both oligosaccharide chains interacted with residues along the surface on the Cγ2 domain (Phe241, Val264 and Asp265) (Sutton and Phillips, 1983). The Manα1,6 branch of both chains were in contact with Cγ2 surface residues (Phe243, Pro246 and the terminal galactose on chain 2 also in contact with Thr260, with space beyond to accommodate a potentially mobile sugar). The galactose on the Manα1,6 arm of chain 1 was positioned away from the surface of the protein towards the opposite Cγ2 domain. The Manα1,3 arm on chain 2 did not contact the Cγ2 domain but its GlcNAc interacted with the GlcNAc-Man core of the opposing oligosaccharide, chain 1. All the electron density maps for the chains described thus far were well defined, indicating relative immobility. The density map for Manα1,3 arm on chain 1 however was weaker the further the chain extended, suggesting mobility and the possibility of a sialic acid residue. There was no contact between the Manα1,3 arm of chain 1 and any part of chain 2 or the Cγ2 surface domain. The pairing of oligosaccharide chains suggested that there was only sufficient space within the Fc domain if half the molecules were deficient in galactose on their Manα1,3 arms (Sutton and Phillips, 1983). However,
there have since been several reports of hypergalactosylated (human) IgG molecules (with up to 80% G2 structures) (Lund et al., 1993a; Kumpel et al., 1994). Variable terminal glycosylation gives rise to the many IgG glycoforms (Parekh et al., 1985) (Figure 1.4). In RA and a restricted number of other diseases including juvenile chronic arthritis, tuberculosis and Crohn's disease, a decrease in the level of galactose on IgG compared to the age-matched control population has been established (Parekh, et al., 1989). However, other diseases involving inflammation were found to have normal levels of IgG galactosylation. IgG completely lacking galactose (agalactosylated or IgG G0) results in the oligosaccharides terminating in GlcNAc. IgG G0, has proved to be a useful diagnostic marker of the disease and could also predict the onset of the disease when assayed for in patients with early synovitis (Young, et al., 1991; van Zeben et al., 1993; Tomana et al., 1994; van Zeben, et al., 1994; Bodman et al., 1994b). A combination of rheumatoid factor and IgG G0 gave a test with 94% positive predictive value (Young, et al., 1991). Moreover, the biological activity of IgG is affected by its glycosylation status.

1.3.7.4 Biological activity of agalactosyl IgG (IgG G0)

RA patients have increased levels of IgG G0 present in all IgG subclasses, but not significantly so in IgG3 (Tsuchiya et al., 1994). The effector functions of IgG, are subclass dependent, and require the presence of the oligosaccharide chains in the Fcγ domain. IgG produced in vitro in the presence of tunicamycin, resulted in IgG lacking N-glycosylation which had reductions in the following biological activities: binding to FcγR on a number of cells; antibody dependent cellular cytotoxicity (ADCC); complement activation; and rapid clearance of antigen-antibody complexes from the circulation (Heyman et al., 1985; Leatherbarrow et al., 1985). Deglycosylated IgG showed no change in antigen binding, protein A binding or recognition by IgM RF compared to the normally glycosylated IgG. Some of these effects were also found with the IgG G0 glycoform following the treatment of intact IgG with β-galactosidase from Streptococcus strain 6646K. Specifically IgG G0 exhibited a decreased binding to complement component C1q and to the FcγRI on the monocyte cell line U937 (Tsuchiya et al., 1989). Similar results have been reported using monoclonal IgG with different levels of galactose content obtained from EBV-transformed B cell lines (Cant et al., 1994; Kumpel, et al., 1994). Autoantibodies are generally of low affinity and directed against few epitopes, and as a result the small complexes formed will be slowly removed, via the low affinity FcγRIII receptor, from the circulation (Male et al., 1991). If reduced binding of the IgG G0 glycoform to FcγRII receptors also occurs then the normal negative immunoregulatory signal to B cells may be lacking (Male, et al., 1991), potentially resulting in the up-regulation of IgG synthesis and increased production of the IgG G0 glycoform.
Figure 1.4

Four types of N-linked complex biantennary oligosaccharides associated with human IgG. (A) is the most extensively glycosylated chain which occurs only in the Fab domain. Microheterogeneities in the terminal sugar residues can be seen in (B) G2, in (C) G1 and (D) G0 structures. These structures may also contain fucose and bisecting GlcNAc as depicted in (A). The common pentasaccharide core found in all N-linked oligosaccharides is outlined in green.
Cryoglobulins may play a potential role in the tissue damage of some autoimmune diseases including RA and SLE (Brouet et al., 1974; Izui et al., 1993). These immunoglobulins self aggregate and precipitate at temperatures below 37°C. One possible mechanism for the association of cryoglobulins has been attributed to the glycosylation on the heavy chain variable regions (Middaugh and Litman, 1987). It is interesting to note that a higher GlcNAc content was found in the immune complexes from RA patients' sera compared to other disease groups (Bond et al., 1995). Immune complexes isolated from the sera of MRL-lpr/lpr mice, an animal model of arthritis, also contained more GlcNAc than the soluble IgG fraction (Bond et al., 1990). Autoaggregation of IgG immune complexes can occur via Fab glycosylation (Hymes et al., 1979), possibly interacting with exposed sites in the Fc domain (Rademacher et al., 1988). There are of course immune complexes which occur through the binding of rheumatoid factor to the Fc domain of IgG G0. Some types of RF have shown increased recognition (Soltys et al., 1994; Newkirk, 1996) and affinity (Randen et al., 1992) to the agalactosylated IgG form compared to the normally glycosylated immunoglobulin.

Moreover, additional effector functions of the IgG G0 glycoform have been suggested. It has been proposed that IgG G0 can activate the classical complement pathway via a Clq-independent route by interaction with mannose-binding protein (Malhotra et al., 1995). Both IgG G0 and mannose-binding protein are found in the serum and synovial fluid from RA patients, indicating that the chronic inflammation of the synovial membrane in RA patients may be attributable, at least in part, to their activation of complement (Malhotra, et al., 1995). It has been proposed that the lack of galactose residues in the Fc domains, unveils a lectin-like pocket to which the sugar residues in the Fab domain of IgG G0 may bind resulting in the self-association of IgG G0 RF and immune complex formation (Rademacher, et al., 1988). The most persuasive evidence for IgG G0 playing a role in the immunopathogenesis of RA has come from a study using a murine collagen-induced model of arthritis. Antibodies from these mice were treated with β-galactosidase from Streptococcus strain 6646K and injected into T cell-primed mice (by injection of heat-denatured collagen in complete Freund's adjuvant). The IgG G0 antibodies were found to more pathogenic than the untreated-IgG, causing an earlier and more severe onset of arthritis (Rademacher, et al., 1994). These experiments have also been repeated using monoclonal anti-collagen antibodies with similar results (Rademacher, et al., 1995). The mice which received the GO glycoform of IgG2b anti-type II collagen monoclonal produced their own antibodies of isotypes IgG1 and IgG2a. One possible mechanism for the action of the agalactosylated IgG may be to block the antibody negative feedback signal. Other interactions involving IgG G0 may occur through the GlcNAc-binding receptors present on cells. It would seem likely
that the pathogenic effects of the IgG G0 glycoform in autoimmunity may only occur if the specificity of this antibody is directed against an autoantigen. This would explain why other diseases such as tuberculosis and Crohn's disease which have raised IgG G0 do not always have joint pathology (though this can occur). In addition, β1,4-GalTase knock-out mice do not develop arthritis, indicating that the glycosylation defect alone is not sufficient for the development of arthritis (Asano, et al., 1997). In other words the specificity of the IgG G0 autoantibody directs the site of autoimmune attack.

1.3.7.5 Production of IgG G0
The regulation of IgG G0 production is unknown. The in vitro culture of B cells from RA patients produce more agalactosyl IgG than those from controls, indicating that the cause is likely to be pre-secretory (Bodman, et al., 1992). No evidence of increased serum β-galactosidase activity in RA patients exists, in fact there was significantly less activity in the disease state (Mullinax, et al., 1976). There have been a few reports of decreased B cell β1,4-GalTase activity from RA patients with differences found depending upon the acceptor substrate used (Axford, et al., 1987; Furukawa, et al., 1990; Wilson, et al., 1993) (see chapter 3). One of these studies found the β1,4-GalTase from the RA B cells had a higher $K_m$ for UDP-galactose than the control group (Furukawa, et al., 1990). There is still some debate as to whether this reduction in β1,4-GalTase activity is responsible for the large increase in IgG G0 glycoforms seen in RA. One report has found no correlation between β1,4-GalTase activity levels and IgG galactosylation (Kumpel, et al., 1994). However, it is possible to alter the cellular β1,4-GalTase activity with concomitant changes in glycoprotein glycosylation (see chapter 5). Other factors such as the culture conditions may influence the degree of IgG galactosylation (Goochee and Monica, 1990; Patel et al., 1992).

The in vivo production of IgG G0 occurs naturally and varies with age (Parekh et al., 1988a). Increased levels of IgG G0 in RA patients have also been found in more than 50% of the healthy spouses of these patients (Sumar et al., 1991), possibly suggestive of an environmental factor(s) influencing IgG galactosylation. It is interesting to note that female patients with RA often go into remission during pregnancy, have less of the IgG G0 glycoform present, but flare up post-partum with a concomitant rise in the level of IgG G0 (Pekelharing et al., 1988; Rook et al., 1991b). A similar fluctuation is also observed in healthy women with corresponding changes in the serum β1,4-GalTase levels (Lacord et al., 1988). This data would suggest some sort of hormonal control in glycosylation.

There are animal models of arthritis which exhibit similar immunological and biochemical changes to those seen in human RA (Trentham, 1982; Cohen and Eisenberg, 1991). MRL-lpr/lpr mice spontaneously develop an autoimmune disease
characterized by RF, immune complexes and swelling of the joints (Cohen and Eisenberg, 1991). There is also a lack of galactose on IgG which is age-dependent (Bond, et al., 1990; Bodman et al., 1994a) and a decrease in the peripheral B cell β1,4-GalTase activities (Axford et al., 1994b). The cause of the IgG G0 production in mice is also unclear. The low levels of IgG galactosylation was accompanied by a change in the amount of total spleen lymphocytic β1,4-GalTase mRNA expression (Jeddi et al., 1994), but no change in the splenic B cells was observed (Jeddi et al., 1996). Further, animal models of induced-arthritis exhibit similar physiological fluctuations in IgG G0 levels during pregnancy (accompanied by remission) and birth (flare-up) (Rook, et al., 1991b; Thompson et al., 1992). It would appear that the pregnancy-associated IgG glycosylation changes in mice are not controlled solely by β1,4-GalTase expression levels (Jeddi et al., 1997). Pregnancy hormones such as prolactin are able to regulate glycosyltransferase activities including β1,4-GalTase, at least in the mammary gland. Prolactin receptors are present on B and T cells, however, the short-term culture of human peripheral blood lymphocytes in the presence of prolactin, insulin and hydrocortisone had no effect on β1,4-GalTase mRNA expression (Jeddi, 1996). An enriched B cell population which was stimulated by anti-μ and IL-2 showed a slight increase in β1,4-GalTase mRNA when prolactin was also added to the culture, yet there was no rise in the IgG galactosylation levels (Jeddi, 1996). Further there was no correlation found between IgG G0 and prolactin levels in a large collection of sera (Pilkington et al., 1996). Oestrogen has been shown to increase the number of Ly1B+ (CD5+ B) cells resulting in increased production of autoantibodies (Ahmed et al., 1986).

Pro-inflammatory cytokines such as TNFα and IL-6 are elevated in RA and are able to affect glycosylation. TNFα stimulates monocytes/macrophages to secrete IL-6 which has pleitropic effects including a major role as a terminal differentiation factor. IL-6 is a growth factor for B and plasma cells, stimulates T cells and induces the production of acute phase proteins in the liver (Van Snick, 1990). Non-RA patients with raised levels of IL-6, such as Crohn's disease and Castleman's disease, often have increased amounts of the agalactosyl IgG (Nakao et al., 1991; Rademacher, 1991). Mice transgenic for IL-6 also secrete increased amounts of agalactosyl IgG, though they do not get arthritis (Rook et al., 1991a). It is probable that for agalactosyl IgG to have a role in pathogenesis it also has to be an autoantibody. An intraperitoneal injection of pristane, a non-metabolizable component of mineral oil, in mice causes a rise in the serum IL-6 levels. Three weeks later, a subsequent single challenge in these mice with native collagen type II in complete Freund's adjuvant resulted in the onset of arthritis (day 40). The arthritis correlated with a second rise in serum IL-6 and the appearance of anti-collagen autoantibodies (Rademacher et al., 1996b). As IL-6 correlates with agalactosyl IgG glycoform (Rademacher, et al., 1995) it is thought that autoantibodies produced in
an IL-6 rich environment are pathogenic. A different collagen-induced arthritis protocol, used two challenges of native collagen type II in complete Freund's adjuvant given three weeks apart. This resulted in an earlier onset of arthritis. Further, the appearance of anti-collagen antibodies preceeded the onset of the arthritis and only those which were of the IgG G0 glycoform coincided with the arthritis (Rademacher, et al., 1994). High IL-6 serum levels have been reported to rise with age in man and mice, as does the IgG G0. The IL-6 levels could be controlled by the administration of dehydroepiandrosterones (Daynes et al., 1993). Pregnancy sera has been described as inhibiting the IL-6 mediated proliferation of an IL-6 dependent B cell line, with a greater inhibition observed using serum from the late stages of pregnancy, a time when a decline in IgG G0 is apparent (Pilkington, et al., 1996). IL-6 can affect glycosyltransferases activities including the reduction of β1,4-GalTase in a B cell line (Nakao et al., 1990). However, no significant changes in splenic β1,4-GalTase mRNA or activity levels were observed in mice transgenic for IL-6, even though increased agalactosylated IgG was apparent (Jeddi et al., manuscript in preparation). It has been suggested that raised IgG G0 is due to T cell-mediated inflammation in combination with an acute phase response as observed in RA, Crohn's disease and tuberculosis (Filley et al., 1989). However, this scheme does not account for the raised IgG G0 in a number of healthy people in RA family studies or that IgG G0 may be present in the absence of inflammation.

The IgG biosynthetic route has also been suggested as a potential mechanism for the production of agalactosyl IgG (Sutherland et al., 1972; Rademacher, et al., 1996a). Terminal Fc glycosylation in IgG may be partly controlled by the folding pathway IgG takes in the assembly of its two heavy and two light chains. The intermediates which will exist will depend upon the one of two major assembly pathways as to whether the first disulphide bond formed occurs between the two heavy chains (pathway 1) or between a heavy and a light chain (pathway 2). Pathway 1 will sequest the oligosaccharides attached to the heavy chains within the Fc domain making them less accessible to β1,4-GalTase resulting in higher amounts of the agalactosyl IgG glycoforms produced (Sutherland, et al., 1972). Pathway 2 would allow β1,4-GalTase to act upon the exposed oligosaccharide of the disulphide bonded heavy-light chains complex and result in less of the agalactosyl IgG structures formed. Each cell is thought to have the capacity to use both of these pathways (Baumal et al., 1971) which may be under the control of hormones and cytokines (Rademacher, et al., 1996a).

1.3.7.6 β1,4-GalTase and cancer

Increased β1,4-GalTase activity has been observed in various tumours, however the identification of a specific cancer-associated β1,4-GalTase has been inconclusive, due
to the large number of naturally occurring β1,4-GalTase isoforms secreted (Whitehead et al., 1979; Davey et al., 1984). However, the production of mAbs against a β1,4-GalTase associated with tumour may be more successful (Uejima et al., 1992; Uemura et al., 1992). The growth-stimulatory effect has on the parotid glands of rats and mice is accompanied by a large increase in β1,4-GalTase activity in these tissues (Humphreys-Beher, 1984; Marchase et al., 1988). The majority of the increased expression of β1,4-GalTase was localized to the plasma membrane (Marchase, et al., 1988) and also an increased secretion of the soluble β1,4-GalTase form into the saliva (Humphreys-Beher, 1984). Incubation of the parotids with anti-β1,4-GalTase antibodies or the donor substrate UDP-galactose inhibited the isoproterenol-induced hypertrophy. The UDP-galactose causes cell surface β1,4-GalTase to catalyse the galactosylation of appropriate glycoconjugate substrates on other cell surfaces, possibly also signalling a negative growth signal, and subsequently releases the β1,4-GalTase specific cell-cell contact (Shur, 1991).

1.3.7.7 β1,4-GalTase and HEMPAS
Defective N-glycosylation, in particular the lactosaminoglycans, of plasma membrane glycoproteins, occurs in the genetic disorder HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test). There are various forms of HEMPAS, which have been attributed to either a defective Golgi α-mannosidase II gene (Fukuda et al., 1990) or to reduced GlcNAcT II activity (Fukuda et al., 1987). Another variant gave a HEMPAS-like disease but no positive acidified serum test was observed. This patient had low levels of membrane-bound β1,4-GalTase, yet high levels of soluble β1,4-GalTase, with no difference in I/i glycan structures (Fukuda et al., 1989).

1.3.7.8 β1,4-GalTase and heart failure
Recently a rat model of heart failure was screened for differential gene expression between failing hearts and controls using subtractive hybridization. The isolated cDNA was 86% homologous to the murine β1,4-GalTase sequence and the β1,4-GalTase gene expression and activity were significantly increased in spontaneously hypertensive rats with failing hearts (Humphries et al., 1996).
2. Materials and Methods
2.1 Reagents

2.1.1 Subjects
Peripheral blood (PB) was obtained from patients with RA who fulfilled the revised criteria of the American Rheumatism Association (Arnett et al., 1988). Control PB was donated by healthy volunteers and non-RA patients. Study one consisted of 11 patients with RA (eight females, mean age 56.6 years, range 34-78; three males, mean age 59.3, range 49-72) and ten healthy controls (three females, mean age 40.7 years, range 26-64; seven males, mean age 41.4 years, range 27-60). Between 20-30 ml of blood was collected per donor.

Study two consisted of 11 patients with RA (ten females, mean age 60.7, age range 49-73; one 64 year old man) and 12 non-RA controls (six females, mean age 60, age range 50-74; six males, mean age 64.7, age range 58-77). Two patients in the control group had lumbar spondylosis, four had osteoarthritis and the remainder had no arthritis. Despite collecting larger samples (between 40-50 ml of blood) for the second study, there was still insufficient material in some cases for use in all assays (see figures 3.8 and 3.9). It has previously been shown that β,1,4-GalTase activity is not age-related (Furukawa, et al., 1990).

2.1.2 Source of β1,4-GalTase proteins
Bovine milk β1,4-GalTase, (Sigma G-5507, Poole, Dorset, UK). Prof. Eric Berger, University of Zurich, kindly provided several forms of human β1,4-GalTase: human milk β1,4-GalTase which was purified as described previously (Gerber, et al., 1979); recombinant human β1,4-GalTase (rhGalTase) and mutated rhGalTase which had the Asn in the single N-glycosylation site mutated to Asp (N-deglycosylated rhGalTase) were both expressed in Saccharomyces cerevisiae and purified as described (Malissard, et al., 1996). Dr Hubert Appert, Medical College of Ohio, generously supplied several recombinant human β1,4-GalTase proteins which were expressed in E. coli as described (Zu, et al., 1995): two deletion mutants rhGalTase⁷⁸ and rhGalTase¹¹⁶, whose N-terminal amino acids were Thr⁷⁸ and Val¹¹⁶ respectively; and a quartet mutant generated by site-directed mutagenesis, a few amino acids downstream from the proposed UDP-galactose binding site, Glu³¹⁵Thr³¹⁶Ser³¹⁶Asp³¹⁷Ser³¹⁷Ser³¹⁸Ser (TSSS).

2.1.3 Source of anti-β1,4-GalTase antibodies
A number of monoclonal antibodies (mAbs) against β1,4-GalTase were produced in concert with the mAb group at UCL, during the course of this project (section 2.7.3). The characterization of these mAbs form part of the body of work presented in this
thesis. Another mAb, 2/36/118, was provided as a concentrated hybridoma supernatant by Prof. Berger for collaborative studies and has already been described elsewhere (Berger et al., 1986).

I had previously produced a rabbit polyclonal antibody (pAb) against bovine milk β1,4-GalTase whilst working with Prof. Pierre Youinou, University of Brest (section 2.7.1). Prof. Berger kindly provided another pAb which was raised against a non-glycosylated β-galactosidase-galactosyltransferase fusion protein expressed in *E. coli* and was antigen-affinity purified (Watzele, et al., 1991).

### 2.1.4 Mice

Six-week old Balb/c mice were used in the production of anti-β1,4-GalTase mAbs. The mice were individually housed within the animal house facilities at UCL.

### 2.1.5 Tissue culture media

Note that all solutions, containers and disposable equipment were sterile, having been either sterile-filtered, autoclaved or gamma-irradiated. Complete medium used in mAb production consisted of RPMI-1640 medium with sodium bicarbonate (Sigma R-7580) plus the following supplements: 15% heat-inactivated Myoclone super plus fetal bovine serum (Life Technologies, Paisley, Renfrewshire, UK), 5% heat-inactivated horse serum (TSH-free, Sigma H-1263), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Sigma 58636), 1 μg/ml fungizone (Life Technologies), 50 IU penicillin and 50 μg/ml streptomycin (Life Technologies).

HT medium was 0.5 mM hypoxanthine with 80 μM thymidine HT (Sigma H-0137) made up in the above complete medium. Selective HAT medium consisted of HT medium supplemented with 4 μM aminopterin (Sigma A-5159).

For the second limiting dilution and expansion of clones, the serum in the complete medium was replaced with a serum-free supplement of 1:100 (v/v) Nutridoma (Boehringer Mannheim, Lewes, East Sussex).

Complete medium used in the culture of established B cell lines consisted of RPMI-1640 with 25 mM HEPES and sodium bicarbonate and supplemented with 10% heat-inactivated fetal bovine serum (sometimes gamma globulin-free which was < 0.1 μg/ml IgG, Life Technologies), 2 mM L-glutamine, 50 IU penicillin and 50 μg/ml streptomycin. COS cell cultures were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) with sodium pyruvate and the same supplements used for B cell line complete medium.

### 2.1.6 Cell lines

Immortalized human B-cell line HB-1 was a kind gift from Keith James, University of Edinburgh Medical School. HB-1 was derived from a spontaneous transformant from
peripheral B cells donated by a healthy volunteer (IgG1 kappa anti-hepatitis B) (James et al., 1990). EBV-transformed B cell lines, derived from hyperimmune donors, were a kind gift from Belinda Kumpel, International Blood Group Reference Laboratory, Bristol. The cell lines produced monoclonals that were specific for rhesus-D antigen on human erythrocytes (Kumpel et al., 1989) JAC10 (IgG1 lambda, G1m(3)), 2B6 (IgG1 kappa, G1m(3)), BRAD3 (IgG3 kappa, G3m(21)).

2.1.7 Molecular biology solutions

Note that all solutions, containers and disposable equipment were sterile, having been either sterile-filtered, autoclaved or gamma-irradiated.

Luria Broth (LB): 1% NaCl, 1% Bactotryptone, 0.5% yeast extract, (Difco laboratories, East Molesey, Surrey, UK). 2X TY (5% NaCl, 1.6% Bactotryptone, 1% yeast extract). LB agar consisted of 1.5% Bacto-agar in LB medium. LB agar plates contained about 30 ml medium in 100 mm Petri dishes and any air-bubbles flamed away. These agar plates were stored for up to one month at 4°C. Neutralization solution, potassium acetate, pH 4.8, was prepared by adding 11.5 ml of glacial acetic acid and 28.5 ml ddH2O to 60 ml of 5M potassium acetate. This solution was 3 M with respect to potassium and 5 M with respect to acetate.

2.1.8 β1,4-GalTase cDNA

Full-length human β1,4-GalTase cDNA was kindly provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation, CA, USA. The complete β1,4-GalTase cDNA, pCT7-J20, was constructed by ligating a 5' portion of the CT7 insert and a 3' portion of the J20 insert and sucloned in Bluescript KS vector. The human β1,4-GalTase cDNA clones CT7 and J20 were isolated from a λgt11 human placenta cDNA (Masri, et al., 1988). CT7-J20 cDNA was inserted into Bluescript KS at EcoRI site in 3' to 5' direction. BamHI —→EcoRI —→5' end of β1,4-GalTase (amino terminus) —→internal Rsr II site —→internal Not I site —→3' end of β1,4-GalTase —→EcoRI —→Hind III.

2.1.9 Transfected oligonucleotides

Antisense human β1,4-GalTase 15 mer 5' AACGAGGGTGACGCC 3' and 21 mer 5' CTGTAGGGACGCCTGGCAT3' along with the respective sense 15 mer 5' GGCGTCACCCTCGTT 3' and 21 mer 5' ATGCCAGGCGTGCTCCCTACAG3' phosphorothioate oligonucleotides (S-oligos) were custom synthesized (Genosys Biotechnologies, Cambridge, UK). These S-oligos corresponded to sequences present in exon 1 of the human β1,4-GalTase gene: the 15 mer sense S-oligos encoding for part of the transmembrane region and the 21 mer sense corresponding to the second initiation start sequence (Masri, et al., 1988).
2.2 GlcNAc-pITC-BSA

2.2.1 Preparation of GlcNAc-pITC-BSA neoglycoprotein acceptor
The principle of this conjugation involves the use of a carbohydrate derivative, phenylisothiocyanate-N-acetylglucosamine (pITC-GlcNAc, Sigma A-5920), containing the phenylisothiocyanato functional group which serves to alkylate the amino terminal and lysyl ε-amino groups of a protein, in this case BSA. The specificity and efficiency of this coupling reaction has been well characterized with the reagent phenylisothiocyanate, also known as Edman reagent, used in protein sequencing (Edman, 1950). The method of McBroom et al. (1972) was followed with modifications. Briefly, 1 ml of a 5 mg/ml solution of GlcNAc-pITC in 0.3 M NaCl with 0.1 M NaHCO₃, pH 10.0, was added slowly with mixing to a 2 ml solution of bovine serum albumin (BSA, 69 mg, fatty acids and γ-globulin free), in the same buffer and left shaking overnight at room temperature. The aromatic pITC-GlcNAc is sparingly soluble in aqueous solution, though the reaction appears to proceed smoothly, possibly due to continued solubilization of the derivative over time. The reaction mixture was then extensively dialysed against distilled water and the neoglycoprotein, GlcNAc-pITC-BSA, lyophilized at -50°C, 5-10⁻¹ atmos. and stored desiccated at -20°C.

2.2.2 Determination of the number of GlcNAc residues incorporated per BSA molecule
The benzothiazolone hydrazone assay which is highly specific for bound amino sugars was used (Manzi and Varki, 1993). Duplicate glass tubes (5 x 0.8 cm) containing 100 μl of GlcNAc standard (3-50 nmol), sample and blanks each had 100 μl of 1 M HCl added. Tubes were capped, vortexed and heated for 2 h at 110°C. The tubes were cooled in a water bath to room temperature, and transferred to a fume hood. Four hundred μl of 2.5% (w/v) sodium nitrate was added and vortexed. The reaction was left for 15 min at room temperature. Two hundred μl of 12.5% ammonium sulfamate was carefully added, vortexed, and allowed to stand for 10 min at room temperature. After the brown nitrogen oxide fumes had dissipated, 200 μl of 0.25% 3-methyl-2-benzothiazolinone hydrazone hydrochloride was added, vortexed and incubated for 30 min at 37°C. Two hundred μl of 0.5% ferric chloride was added and incubated for 5 min at 37°C. Samples were cooled to room temperature and absorbances read at 650 nm against the blank. A standard curve of absorbance against nmoles of GlcNAc was used to calculate the amount of GlcNAc in the glycoprotein.
2.2.3 ELLA: lectin reactivity to GlcNAc-pITC-BSA

An enzyme linked lectin assay (ELLA) was used to determine the interaction of several lectins with GlcNAc-pITC-BSA. One hundred µl of GlcNAc-pITC-BSA or just BSA at 50 µg/ml in 0.15 M phosphate-buffered saline, pH 7.2 (PBS, 0.137 M NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$ and 1.5 mM KH$_2$PO$_4$) was coated onto Maxisorp (Nunc, Denmark) ELISA plates overnight at 4°C, washed four times with PBS-Tween 0.05%, PBS-T, (polyoxyethylene sorbitan-20 (v/v)), pH 7.2, then blocked for 1 h at 37°C with 200 µl PBS-1% BSA. After washing, 100 µl of biotinylated lectins *Sambucus nigra* (SNA), *Bandeiraea simplicifolia* II (BSII), *Ricinus communis* agglutinin I (RCAI), Concanavalin A (Con A) and peanut agglutinin (PNA) (Vector laboratories, Peterborough, UK) at 2 µg/ml in PBS-T with 0.1 mM Ca$^{2+}$ (except RCAI which was at 0.5 µg/ml in just PBS-T) were incubated for 1 h at 37°C. Following washing, the plates were incubated with 100 µl of 1:1000 streptavidin-horseradish peroxidase (HRP, Dako, Buckinghamshire, UK) in PBS-T for 1 h at 37°C. After washing, the plates were developed using 100 µl of 1 mg/ml o-phenylenediamine (OPD) in 0.1 M citrate phosphate buffer, pH 5.0 with 0.03% H$_2$O$_2$. The reaction was stopped with 50 µl 3N H$_2$SO$_4$ and absorbances read at 490 nm using a Dynatech MR5000 ELISA plate reader.

2.3 GalTase activity

2.3.1 ELISA: β1,4-GalTase assay

This assay measures the transfer of galactose from UDP-galactose (donor) onto the non-reducing end of GlcNAc (acceptor) in the presence of divalent cations acting as cofactors. The resulting Galβ1,4GlcNAc linkage is specifically detected by biotinylated RCAI. One hundred µl of GlcNAc-pITC-BSA, non-derivatized BSA or ovalbumin (chick egg albumin, grade VI, Sigma A-2512) at 50 µg/ml in PBS was coated onto Maxisorp ELISA plates, with PBS alone in the border wells, for 2 h at 37°C then overnight at 4°C. Plates were washed four times with PBS-T and then blocked with 200 µl of 1% BSA in PBS for 1 h at 37°C. Plates were washed and then doubling dilutions of bovine milk β1,4-GalTase (diluted in 0.1 M sodium cacodylate, pH 7.0, 0.5 M NaCl, 20 mM MnCl$_2$) or the test samples were loaded into the wells. The reaction was started by the addition of buffer to give a total incubation volume of 100 µl and containing final concentrations of 0.1 M sodium cacodylate, pH 7.0, 0.5 M NaCl, 20 mM MnCl$_2$, 0.5 mM ATP and 0.1 M UDP-galactose. Following incubation for 1 h at 37°C, the reaction mixture was washed out and the plates incubated for a further 1 h with 100 µl RCAI-biotin diluted to 0.5 µg/ml in PBS-T. In some assays, duplicate wells were also probed with 100 µl of BSII-biotin at 2 µg/ml in PBS-T with 0.1 mM Ca$^{2+}$ at various time points (Figure 3.3). Plates were then washed prior to the addition of 100 µl of
streptavidin-HRP at a 1:5000 dilution in PBS-T for 1 h at 37°C. Development and reading of the plates was as above (section 2.2.3). The samples were quantified using the bovine milk β1,4-GalTase standard curve and activity expressed in nmol/mg of protein/h. Inhibition assays were performed with either rabbit F(ab')2 anti-β1,4-GalTase (section 2.7.1), mouse IgG anti-β1,4-GalTase mAbs, section 2.7.3) or irrelevant isotype controls, all at 50 μg/ml in the β1,4-GalTase incubation buffer which contained either cell lysates or known amounts of β1,4-GalTase.

2.3.2 Radiochemical GalTase assay
This reaction detects the transfer of [3H]Galactose from UDP-[3H]Galactose (donor) onto the non-reducing GlcNAc (acceptor) in the presence of divalent cations. Cell lysates were incubated in Eppendorf tubes for 30 min at 37°C with final concentrations of 0.1M sodium cacodylate, pH 7.0, 0.5 M NaCl, 20 mM MnCl₂, 0.5 mM ATP, 10 mg/ml ovalbumin or GlcNAc-pITC-BSA, and 0.1 M UDP-[6-³H]-galactose (100 mCi/mmol) in a total volume of 100 μl. Where Kₘ studies were performed, doubling dilutions of acceptor substrate concentrations from 16 mg/ml were carried out (Figure 3.2). Reactions were stopped by the addition of 1 ml of ice-cold 2% phosphotungstic acid in 0.5 M HCl. The precipitate was centrifuged at 4°C for 10 min at 3000 g in a microfuge. The pellet was resuspended and washed a further two times, then dissolved in 1M NaOH. Samples were neutralised with glacial acetic acid and prepared for liquid scintillation counting in a Minaxi Tri-Carb 4000 beta counter (United Technologies, Packard, Berkshire, UK) following the addition of Ecoscint A scintillant (National Diagnostics, Atlanta, Georgia, USA). Enzyme activity was calculated from a β1,4-GalTase standard curve, as stated for the ELISA assay. Note that the GalTase activities with endogenous acceptors was minimal, invariably giving the same background values as just scintillant alone.

2.4 B lymphocytes

2.4.1 Isolation of PBMC and CD19+ B cells
Solutions used for density gradient centrifugation were pre-warmed to room temperature. PB was collected from donors (section 2.1.1) by venepuncture, transferred immediately into heparinized tubes (final concentration of 10 units/ml) and inverted several times. The blood was diluted with an equal volume of RPMI-1640. Up to 35 ml of the diluted blood was layered onto 15 ml of Lymphoprep (Nycomed Pharma, Olso, Norway) in a 50 ml Falcon tube (polypropylene, Grenier Laboratories) and centrifuged at 800 g for 30 min at room temperature. The interface, consisting of peripheral blood mononuclear cells (PBMC), was carefully removed with a sterile
disposable 3 ml Pasteur pipette and washed three times with RPMI-1640 at 350 g for 10 min at room temperature. PBMCs were resuspended in 10 ml of RPMI-1640 and counted. For the isolation of B cells, all the following solutions and PBMCs were kept at 4°C. B cells were estimated to be 15% of the total lymphocyte population and the number of anti-CD19-coated magnetic beads added according to the manufacturer's instructions (Dynabeads, Dynal, Wirral, UK). Briefly, anti-CD19 Dynabeads were pre-washed in RPMI-1640 1% FCS and collected using a magnetic separator. Ten μl of anti-CD19 Dynabeads (4 x 10^6 beads) was added per 10^6 of target B cells in a total volume of ≤1 ml in a 5 ml sterile Falcon tube, capped and sealed with Nescofilm. Following gentle rotation for 30 min at 4°C, positively separated B cells were isolated using a magnet. These cells were washed five times with RPMI-1640 1% FCS, then a further two times in RPMI-1640 and counted. Cells attached to the anti-CD19 Dynabeads and which excluded the trypan blue dye were regarded as viable, purified B cells. Cells were then spun down in Eppendorfs and washed with PBS.

2.4.2 Preparation of lymphocytic cell extracts
All cells were lysed with approximately 10^6 cells per 100 μl of ice-cold lysis buffer (PBS-0.2% Triton X-100) for 30 min at 4°C. Following centrifugation at 15 000 g in an IEC Centra-3RS microfuge for 1 h at 4°C, aliquots of the supernatants were collected and stored at -70°C. Prior to assaying for β1,4-GalTase activity, the lysates were centrifuged at 126 000 g for 5 min at room temperature in polyallomer tubes (5 x 20 mm, Beckman, Buckinghamshire, UK) using an A-100/30° fixed angle rotor in a Beckman CLS ultracentrifuge airfuge (Beckman).

2.4.3 Protein quantification
Protein concentrations in cell lysates were determined using the Bio-Rad (Hemel Hempstead, Hertfordshire, UK) protein micro-assay, with BSA as a standard (Bradford, 1976). Briefly, 100 μl of BSA protein standard from 50 μg/ml in doubling dilutions or sample diluted in H2O was pipetted into a microtitre well. Twenty-five μl of protein binding dye (Coomassie brilliant blue G-250) was added to each well, mixed thoroughly and left at room temperature for 10 min. Absorbances were read at 570 nm, and samples read off the BSA standard curve.

2.5 β-galactosidase assays

2.5.1 β-galactosidase assay with ONPG substrate
β-galactosidase catalyses the release of o-nitrophenol from the colourless substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), into a bright yellow product which can be
read at 410 nm. Fifty μl of cell lysate was pipetted into a microtitre plate and 110 μl of 0.1M citrate phosphate buffer at a range of pH 2-8 was added. Following addition of 50 μl of ONPG at 4 mg/ml the plates were incubated at 37°C until colour development. The reaction was stopped with 90 μl 1M NaCO₃ and the optical densities read at 410 nm. Purified jack bean β-galactosidase (Oxford Glycosystems, Oxford, UK) and E. coli β-galactosidase (Sigma G-6008) were used as positive controls.

2.5.2 β-galactosidase assay with X-gal reagent
In order to determine transfection (section 2.10.2) efficiencies, pCR3/LacZ (Invitrogen, Leek, The Netherlands), containing the β-galactosidase insert, lacZ, was used. The transfected cells were harvested at various time points and washed with PBS. Cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 10 min at room temperature, then washed twice in PBS. The cell pellet was resuspended in 250 μl of X-Gal reagent (1 mg/ml X-Gal, 5-bromo-4-chloro-3-indolyi β-galactopyranoside, in dimethylformamide, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide and 2 mM magnesium chloride hexahydrate in PBS, pH 7.4). Following an overnight incubation at room temperature, the cells were viewed under a light microscope. Positively transfected cells had turned blue in the presence of X-Gal.

2.6 IgG

2.6.1 ELISA: quantification of IgG
Maxisorp ELISA plates were coated with 100 μl of goat F(ab')2 anti-human IgG (Sigma 1-9885) at 5 μg/ml in PBS overnight at 4°C. Plates were washed four times with PBS-T, blocked with 200 μl PBS-1% BSA for 1 h at 37°C then washed. One hundred μl of human standard serum (Behring, Germany) in three-fold dilutions from 3 μg/ml or samples diluted in PBS-T was loaded onto the plates and incubated for 1 h at 37°C. Serum samples were usually diluted between 1:20 000 - 1:100 000, whereas cell culture supernatants were either tested neat or diluted up to 1:50. After washing, 100 μl of goat F(ab')2 anti-human IgG-alkaline phosphatase (Sigma A-3312) diluted 1:30 000 in PBS-T was incubated for 1 h at 37°C. The plates were then washed and developed with 100 μl of p-nitrophenyl phosphate (PNPP, Sigma 104-105) at 1 mg/ml in 50 mM bicarbonate buffer (BIC), pH 9.6, with 2 mM MgCl₂.6H₂O and absorbances read at 410 nm.

Where mouse IgG was quantified the following modifications were included at the above concentrations. Plates were coated with sheep F(ab')2 anti-mouse IgG as the capture antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The
standard IgG calibration curve was carried out using mouse standard serum of pre-
determined IgG concentration (The Binding Site, Birmingham, UK). Detection of
bound IgG antibodies was performed using sheep anti-mouse IgG-alkaline phosphatase
(Sigma A-3563).

2.6.2 ELLA: determination of IgG galactosylation

The IgG galactosylation status was assayed based on an enzyme linked lectin assay
(ELLA) previously described (Sumar et al., 1990). The ELLA measured
oligosaccharides terminating in one or two GlcNAc, whilst the IgG G0 structures were
determined biochemically measured oligosaccharides terminating in two GlcNAc
residues, with good correlation shown between two methods (r=0.82), (Sumar, et al.,
1990).

Duplicate Maxisorp flat-bottomed ELISA plates were coated with 100 µl of protein-G
(Sigma P-4689) at 2 µg/ml in PBS and were left overnight at 4°C. The plates were
washed four times with PBS-T then blocked with 200 µl of 1% BSA in PBS for 1 h at
37°C. Following washing, serum from patients or standards (containing known
amounts of IgG G0, determined by the Department of Biochemistry, University of
Oxford, using the hydrazinolysis method) was diluted to 2 µg/ml IgG in 0.1 M glycine,
0.159 M NaCl, pH 7.0 and 100 µl incubated per duplicate well for 2 h at 37°C. After
washing, 100 µl of PBS was added per well and the plates floated in a 90°C water bath
for 8 min in order to denature the IgG and expose the oligosaccharides. The PBS was
flicked out and the plates tapped onto paper towels and allowed to cool down to room
temperature. One hundred µl of biotinylated RCAI (which bound terminal galactose) at
1.25 µg/ml in PBS-T-1% BSA or biotinylated BSII (which bound terminal GlcNAc) at
20 µg/ml in PBS-T-1% BSA with 0.1 mM Ca²⁺ was added and incubated overnight at
4°C. Following washing, 100 µl of streptavidin-HRP diluted 1:3000 in PBS-T-1% BSA
was added and incubated for 1 h at 37°C. Plates were developed with 100 µl of
2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma A-1888) with
0.03% H₂O₂ and the reaction stopped with 100 µl of sodium fluoride at 2 mg/ml.
Absorbances were read at 410 nm. A standard curve of the absorbance ratios of
BSII/RCAI against the known amounts of IgG G0 was plotted and the sample
absorbances interpolated. As human IgG galactosylation is known to vary with age
(Parekh, et al., 1988b; Keusch et al., 1996), the IgG G0 results were scored in relation to
previously defined age-population curves determined for normal serum IgG (Sumar, et
al., 1990) and expressed as standard deviation units about the normal mean. It was
noticed that the storage of samples at 4°C lead to a decreased lectin binding when
compared to freshly thawed samples. Further the decrease in BSII binding was greater
than that of RCAI, hence all samples were assayed using freshly thawed samples.
2.7 Anti-β1,4-GalTase antibodies

2.7.1 Polyclonal anti-β1,4-GalTase antibodies
I produced these antibodies whilst working in Prof. Youinou's laboratory in the University of Brest, France. Rabbits were injected intramuscularly with 150 μg bovine milk β1,4-GalTase in complete Freund's adjuvant, and repeated at two weekly intervals in Freund's incomplete adjuvant. Blood sample ear-bleeds were obtained for testing the immune response via a direct-binding ELISA with bovine milk β1,4-GalTase coated onto the plate (section 2.7.6). When the titres were high enough, the rabbit antiserum was collected by exsanguination via cardiac puncture under a terminal anaesthetic (the procedure was kindly performed by the Department of Surgery, Brest University Hospital). Serum was collected, heat-inactivated and IgG isolated using a protein-G affinity chromatography column (section 2.7.11). Pepsin digestion was performed on the IgG fraction to generate F(ab')2 fragments (Hudson and Hay, 1989) which were then affinity purified on a bovine milk β1,4-GalTase-sepharose column (Lopez, et al., 1985). Verification of purity and specificity of these rabbit F(ab')2 anti-β1,4-GalTase antibodies was performed using SDS-PAGE (section 2.8.3) and Western blotting (section 2.8.4), respectively.

2.7.2 ELLA: analysis of the glycosylation states of anti-β1,4-GalTase antibodies
The same method as stated in section 2.2.3 was used except 100 μl of rabbit F(ab')2 anti-β1,4-GalTase or pre-immune rabbit F(ab')2 were coated onto a Maxisorp ELISA plate at 10 μg/ml in PBS for 2 h at 37°C.

2.7.3 Monoclonal antibody production: immunization
The services of The mAb Group, UCL, were employed to produce anti-β1,4-GalTase mAbs. Briefly, three Balb/c mice were bled, to obtain some pre-immune sera, then injected subcutaneously (s.c.) with 5, 10 or 20 μg of human milk β1,4-GalTase at 1 mg/ml in conjunction with Ribi adjuvant. Two weeks later a second s.c. injection, as above was given. Ten days after the second injection, mice were bled and sera tested for immune response. A third final booster of 15 μg of antigen in saline was given intra-venously two weeks after the second injection. Three days later two of the mice were sacrificed under halothane (Fluothane, ICI Pharmaceuticals, Macclesfield) anaesthetic. Spleens were removed under sterile conditions, by spraying 70% ethanol on the animal and collecting the spleen into a universal containing RPMI supplemented with penicillin, streptomycin and fungizone, warmed to 37°C. Animals were bled by cardiac puncture and the blood collected and allowed to clot at room temperature. The clot was carefully removed from the wall of the tube with a pipette tip to allow the clot
to form fully. Following centrifugation at 6500 g for 5 min the serum was collected, decomplemented at 56°C for 30 min, and stored in aliquots at -20°C.

2.7.4 Fusion between spleen cells and myeloma cell line

The mAb group, UCL, carried out the fusion between JK.Ag8:653 myeloma cells and the spleen cells (1.63 x 10^8, in a 1:4 ratio respectively) using a simplified polyethylene glycol (PEG) matrix fusion adapted from the classical method (Kohler and Milstein, 1975). Following the fusion, cells were cultured at between 6-8 x 10^5 cells/ml in complete medium with an equal volume of double strength HAT medium added. One hundred μl of cells were plated out into wells containing 100 μl of 10% hybridoma enhancing supplement (HES) of macrophage origin (Sigma H-8142) in HAT medium. The culture medium was changed at 4-7 day intervals from the day of fusion with fresh HAT medium. Unfused myeloma or unstable hybridomas died off in the HAT medium after about one week. Stable hybridomas then appeared, and the medium was changed at about every 4-7 day intervals until hybridomas were about 50% confluent (after approximately another week). Hybridoma culture supernatants could then be screened for positive clones (section 2.7.5). Screening was carried out at least twice and positive clones were then transferred to 24-well plates for the next stage of growth, using HES as a feeder supplement. When hybridomas had reached confluence, they were transferred to small flasks without feeder layer. After reaching confluence, approximately 1-5 x 10^6 hybridoma cells per vial were frozen down in liquid nitrogen.

2.7.5 Screening of hybridomas

Maxisorp ELISA plates were coated with 100 μl of 2.5 μg/ml human milk β1,4-GalTase (the same stock as used for immunogen) in PBS for 2 h at 37°C then overnight at 4°C. Plates were washed four times with PBS-T, then blocked for 1 h at 37°C with 200 μl of 1% dried-skimmed milk powder in PBS, some plates were coated with just blocking agent for background binding. Following washing of plates, 50 μl of hybridoma supernatant was added to 50 μl PBS-T per well and incubated for 1 h at 37°C. Plates were washed and incubated for 1 h with 100 μl of 1:2000 goat anti-mouse IgG/A/M alkaline phosphatase (Sigma A-0162) diluted in PBS-T. Plates were developed and read as stated in section 2.6.1. Positive wells were taken as absorbances > 0.4, which was more than five times the background absorbance. The second screening was carried out by The mAb Group, UCL, as described above but the anti-mouse Ig was ^125^I labelled. The radioactivity associated with each well was counted in a gamma counter.
2.7.6 ELISA: detection of antibody response in immunized animals

Immunized mice (section 2.7.3) were tested as described above (section 2.7.5), except that the pre- and post-immune mouse serum was diluted 1:500, 1:2500, 1:12500, 1:62500, and 1:312500. Immunized rabbits (section 2.7.1) were tested as stated above (section 2.7.5) with the following modifications: coated antigen was bovine milk β1,4-GalTase; doubling dilutions of pre- and post-immune rabbit serum from 1:500-1:256000; goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma A-7539) diluted 1:1000.

2.7.7 ELISA: isotyping of mouse monoclonal antibodies

Initially the isotyping of antibodies was carried out by the mAb group at UCL using immunodiffusion kits (The Binding Site, Birmingham, UK). However, this proved to be insensitive and I performed subsequent analysis using an ELISA-based isotyping kit (Sigma ISO-2). These reagents were used to detect the mouse monoclonals present in the hybridoma supernatants by a capture ELISA, an indirect ELISA and also the isotypes of the mAb recognizing β1,4-GalTase by a direct-binding ELISA.

The capture ELISA was carried out by coating maxisorp ELISA plates with 100 µl of goat anti-mouse Ig subclass specific antibodies, diluted 1:1000 in PBS, at incubated overnight at 4°C. Plates were washed four times with PBS-T and blocked with 1% dried-skimmed milk powder in PBS for 1 h at 37°C, washed again and incubated with 100 µl of concentrated (via centricon, to give between 1-20 µg/ml of immunoglobulin) hybridoma supernatant for 2 h at 37°C. After washing, 100 µl of goat anti-mouse IgG/A/M-alkaline phosphatase (Sigma A-1062) diluted 1:2000 in PBS-T was added for 1 h at 37°C. Plates were washed and developed as described in section 2.6.1.

The indirect ELISA was performed by coating 100 µl of (NH₄)₂SO₄ purified antibody (section 2.7.10), from the anti-β1,4-GalTase hybridomas, at 2 µg/ml in PBS on Maxisorp ELISA plates for 2 h at 37°C then overnight at 4°C. After washing four times with PBS-T and blocking with 200 µl PBS-1% BSA for 1 h at 37°C, plates were washed and incubated with 100 µl of goat anti-mouse Ig subclass specific antibodies diluted 1:1000 in PBS-T for 30 min at room temperature. Following washing, the plates were incubated for 15 min at room temperature with 100 µl of rabbit anti-goat IgG-HRP (Sigma A-4174) diluted 1:14000 in PBS-T then washed and developed as stated in section 2.2.3.

The direct binding ELISA was carried out by coating Maxisorp ELISA plates with 100 µl of human milk β1,4-GalTase at 0.5 µg/µl in PBS for 2 h at 37°C then overnight at 4°C. Plates were washed and blocked as for the indirect ELISA. One hundred µl of (NH₄)₂SO₄ purified antibody (section 2.7.10), from the anti-β1,4-GalTase hybridomas, at 5 µg/ml in PBS-T was added for 2 h at 37°C. All subsequent steps from the addition of the goat anti-mouse Ig subclass specific antibodies were as described for the indirect
ELISA. Cloned mAb anti-human chorionic gonadotropin antibodies, hCG 111 and hCG 51, were used as internal IgG1 isotype controls.

Light chain isotyping of the anti-\(\beta_1,4\)-GalTase mAbs was performed using protein-G purified IgG (section 2.7.11) in the direct binding assay as described above. After the incubation of the anti-\(\beta_1,4\)-GalTase mAbs and washing, 100 \(\mu\)l goat anti-mouse kappa or anti-mouse lambda antibodies (Southern Biotechnologies, kindly given by Joe Cambridge, UCL) were diluted 1:1000 in PBS-T and incubated for 1 h at 37°C. Following washing, plates were incubated with 100 \(\mu\)l rabbit anti-goat IgG-HRP conjugate diluted 1:15 000 in PBS-T for 1 h at 37°C and developed as described in section 2.2.3.

2.7.8 ELISA: specificity of anti-\(\beta_1,4\)-GalTase mAbs

One hundred \(\mu\)l of the following antigens: human milk \(\beta_1,4\)-GalTase; bovine \(\beta_1,4\)-GalTase; human \(\alpha\)-lactalbumin; bovine \(\alpha\)-lactalbumin; human IgG; and BSA were coated onto Nunc maxisorp ELISA plates at 5 \(\mu\)g/ml in PBS and incubated overnight at 4°C. Plates were washed four times with PBS-T and blocked with 200 \(\mu\)l PBS-1% BSA for 1 h at 37°C. After washing, 100 \(\mu\)l of protein-G purified (section 2.7.11) anti-\(\beta_1,4\)-GalTase mAbs at 1 \(\mu\)g/ml in PBS were incubated for 1 h at 37°C. Following washing, the plates were incubated with 100 \(\mu\)l of 1:30 000 sheep F(ab')2 anti-mouse IgG-alkaline phosphatase (Sigma A-3563) diluted in PBS-T for 1 h at 37°C. After washing, plates were developed as stated in section 2.6.1.

Unpurified periplasmic fractions of \(E.\ coli\) cells expressing mutant \(\beta_1,4\)-GalTase proteins were kindly supplied by Dr. Appert, Ohio, USA. 100 \(\mu\)l of these fractions containing rhGalTase\(^{78}\), rhGalTase\(^{116}\) and TSSS mutants at 15.39 ng/ml, 37.33 ng/ml and 6.2 ng/ml \(\beta_1,4\)-GalTase protein respectively (section 2.8.1) in a total protein concentration of 174 \(\mu\)g/ml, 656 \(\mu\)g/ml and 250 \(\mu\)g/ml respectively, were coated onto Nunc maxisorp ELISA plates. The remaining steps in the assay were as stated above.

2.7.9 ELISA: direct binding to \(\beta_1,4\)-GalTase protein

Human \(\beta_1,4\)-GalTase is known to be glycosylated and it has been proposed that these carbohydrate structures may themselves be antigenic (Childs, et al., 1986). Oxidation of sugars by treatment with sodium periodate, cleaves the sugar ring. The specificity of the anti-\(\beta_1,4\)-GalTase mAbs was tested before and after the oxidation of \(\beta_1,4\)-GalTase. Human \(\beta_1,4\)-GalTase was coated overnight at 4°C onto Maxisorp ELISA plates at 2.5 \(\mu\)g/ml in PBS, then washed four times with PBS-T. After blocking with 200 \(\mu\)l PBS-1% BSA for 1 h at 37°C, the plates were washed. Two hundred \(\mu\)l of ice-cold 50 mM sodium periodate in 0.1M citrate phosphate buffer, pH 5.0 was added for 5 min in the dark, then washed a further three times. Either anti-\(\beta_1,4\)-GalTase mAbs were added and processed as stated in section 2.7.5 or 100 \(\mu\)l of the following biotinylated lectins
were used: SNA-biotin at 4 μg/ml, RCAI-biotin at 1.25 μg/ml, BSII-biotin at 4 μg/ml in PBS-T (with 0.1 mM Ca^{2+} included with SNA-biotin and BSII-biotin lectins). Plates were washed and those incubated with biotinylated lectins were incubated for 1 h at 37°C with 1:1000 streptavidin-HRP in PBS-T and developed as stated in section 2.2.3.

2.7.10 Ammonium sulphate precipitation of IgG
Saturated (NH₄)₂SO₄ was made up several days before use by adding 500 g (NH₄)₂SO₄ to 500 ml ddH₂O and left stirring continuously at 4°C. A 50% precipitation was done by adding dropwise an equal volume of saturated (NH₄)₂SO₄ to clone supernatant and stirring gently. The mixture was left for a 1 h stirring at 4°C, before being centrifuged at 1000 g for 30 min at 4°C. Supernatants were carefully decanted and the pellet was washed three more times with 50% (NH₄)₂SO₄ then resuspended in a small volume of PBS, approximately 1:10 of the original volume. This solution was then transferred to a dialysis bag, prepared by boiling in 1 mM EDTA for 5 min, and extensively dialysed against at least 500 volumes PBS, with several changes of PBS, at 4°C. The IgG concentration was then assessed using the IgG quantification ELISA (section 2.6.1).

2.7.11 Protein-G purification of IgG
Individual 1 ml columns of protein G sepharose 4B fast flow (Sigma P-3296) were set up in 2 ml syringes for each monoclonal to be purified. Columns were pre-equilibrated to pH 7.0 with 20 mM phosphate buffer (20 mM NaH₂PO₄.H₂O pH to 7.0 with 20 mM Na₂HPO₄). The hybridoma supernatants were adjusted to pH 7.0 and recycled over the protein-G column overnight at 4°C. The column was then washed with phosphate buffer whilst monitoring the absorbance at 280 nm. IgG was eluted from the column with 0.1 M glycine-HCl pH 2.7 and 1 ml fractions collected in Eppendorf tubes containing 30 μl of 1 M NaOH to neutralize the protein. The absorbance of the eluted IgG was measured at 280 nm and later quantified via ELISA (section 2.6.1). After use the protein-G column was re-equilibrated with phosphate buffer then flushed through with 20% ethanol and stored at 4°C.

2.7.12 Biotinylation of IgG
Proteins were conjugated as described (Goding, 1986). The reaction with proteins involves a nucleophilic attack of the unprotonated ε-amino of lysine on a derivative of biotin, commonly the N-hydryosuccinimide ester, resulting in the displacement of N-hydryosuccinimide and the formation of an amide bond. As the reaction is more efficient at alkaline pH and conjugation is inhibited by extraneous amines, the protein samples were extensively dialysed against 0.1 M NaHCO₃, pH 8.3 before use. Protein was adjusted to 1 mg/ml. N-hydryosuccinimide ester biotin was dissolved in dimethyl
sulphoxide (DMSO) to 1 mg/ml, immediately before use. The ester was then added to
the protein at a 1:10 \( \frac{v}{v} \) ratio and mixed immediately. Following incubation at room
temperature for 2 h, the reaction went to completion and the biotinylated protein was
dialysed against PBS with 0.1% NaN\(_3\) overnight at 4°C. Mouse IgG1 MOPC-21 kappa
(Sigma M-9269) was biotinylated and used as an irrelevant isotype control.

2.7.13 Epitope mapping of β1,4-GalTase to analyse anti-β1,4-GalTase mAbs
To determine if any of the anti-β1,4-GalTase mAbs were binding to the same or
overlapping epitopes on human β1,4-GalTase, a competition ELISA was set up. ELISA
Maxisorp plates were coated with 100 µl of 2 µg/ml of human milk β1,4-GalTase in
PBS for 1 h at 37°C, then overnight at 4°C. Plates were washed four times with PBS-T,
blocked with 200 µl of 1% dried-skimmed milk powder in PBS for 1 h at 37°C.
Following washing of the plates, 100 µl of biotinylated anti-β1,4-GalTase mAbs at a
concentration which gave 50% maximum binding (ranged between 40-210 ng/ml for the
different mAbs) with or without unlabelled anti-β1,4-GalTase mAbs at 10 µg/ml in
PBS-T, were incubated for 2 h at 37°C. Plates were washed and incubated with 100 µl
of 1:5000 dilution of streptavidin-HRP in PBS-T for 1 h at 37°C. After washing, plates
were developed as described in section 2.2.3. Results were expressed as a percentage of
binding, taking 100% binding as the absorbance obtained with biotinylated anti-β1,4-
GalTase mAbs, in the absence of unlabelled anti-β1,4-GalTase mAbs.

2.7.14 Surface plasmon resonance
To further characterize these mAbs, binding affinities were assayed using surface
plasmon resonance (SPR) with the BIAcore instrument (Pharmacia Biosensor, Uppsala,
Sweden). This biosensor detects biological interactions in real time (Jonsson et al.,
1991; Malmqvist, 1993; Panayotou et al., 1993). It consists of a sensor chip where
biological interactions take place and are detected; integrated flow channels for
injection of samples and buffers; optics; and an on-line computer for interpretation of
the SPR signal. The sensor chip consists of a gold surface to which carboxymethylated
dextran is linked in a 100 nm thick layer. Such a hydrophilic environment minimizes
non-specific binding. A monochromatic light beam is focused on the sensor chip under
conditions of total internal reflection. Due to the phenomenon of SPR, a component of
the reflected light, called the evanescent wave, is able to penetrate into the dextran layer
and is used to probe refractive index changes, which in turn reflect changes in the
amount of bound macromolecules. The signal from the detector is expressed in
arbitrary units (Resonance Units, RU) and presented in a plot versus time (sensorgram).
The practical procedure was carried out in collaboration with Dr. George Panayotou at
the Ludwig Institute for Cancer Research, London. Briefly, a sensor chip was
equilibrated in running buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 3.4 mM EDTA,
0.005% Tween20) at a constant flow rate of 5 μl/min. The matrix was activated by injecting 80 μl of a 1:1 mixture of 11.5 mg/ml N-Hydroxysuccinimide and 75 mg/ml N-Ethyl-N'-(3-dimethylaminopropyl)-carbodiimide to enable it to covalently bind free amino groups of proteins. This was followed by a 80 μl injection of a 20 μg/ml solution of polyclonal rabbit anti-mouse IgG1 (Pharmacia) in acetate buffer, pH 4.5. Any remaining unreacted sites were blocked with an injection of 40 μl of 1M ethanolamine. Mouse antibodies were then captured on the immobilised anti-IgG1 by injection of a 100 μg/ml solution in running buffer. Saturation of all available sites was confirmed by injection of additional antibody until no further increase in the signal was observed. Note that a small amount of bound material dissociated with time, resulting in a drift of the baseline signal. This did not affect measurements significantly, especially since the response obtained with control, non-reactive antibodies (3E2 anti-hCG) could be subtracted. Antigen was then injected over the captured antibodies at a concentration of 0.3 μM. After the end of the protein injection (25 μl), running buffer resulted in dissociation of bound material, enabling the determination of the dissociation rate constant using evaluation software supplied with the instrument. The association rate constant could then also be determined from the sensorgram and the relative affinities of the various antibodies calculated (binding constant $K_B = k_{ass}/k_{diss}$). After each run the chip was regenerated with a 4 μl pulse of 20 mM HCl which resulted in complete dissociation of captured antibody. The same surface could be used repetitively for hundreds of injections.

### 2.7.15 Flow cytometry

Each stain was performed in Eppendorf tubes containing 2.5 x 10^5 cells which had been washed in PBS. The cell pellet was resuspended with 100 μl of test mAb or isotype control mAb at 20 μg/ml in wash buffer (PBS-0.1% BSA with 15 mM NaN₃) and incubated for 45 min on ice. Following two 1 ml washes, 50 μl of rabbit F(ab')₂ anti-mouse IgG FITC, diluted 1:10 in wash buffer, was used to resuspend the cell pellet and incubated for 45 min on ice. Cells were washed twice in wash buffer and resuspended in 250 μl PBS. Propidium iodide (Sigma P-4170), a fluorescent dye which binds nucleic acids, was added just prior to analysis at a final concentration of 5 μg/ml to assess cell viability. Stained cells were read (a minimum of 5000 events) the same day on a Beckton Dickinson FACScan. For intracellular staining, cells were fixed and permeabilized before being stained. The PermeaFix reagent (Ortho Diagnostic Systems, Buckinghamshire, UK) allowed access to the intracellular molecules whilst preserving the cell surface antigens and the overall cellular structure and morphology. Five hundred μl of working strength PermeaFix was used to resuspend a pellet of 1 x 10^6 washed cells. The cells were incubated at room temperature in the dark for 40 min,
then washed two times in 1 ml of wash buffer. Thereafter the staining procedure was as described above except that an additional 30 min wash step was included following staining with the primary antibody. Where cells were stained with biotinylated RCAI, 100 μl of 0.08 μg/ml RCAI-biotin diluted in wash buffer was added to 2.5 x 10^5 cells for 45 min on ice then washed as described above. Cells were then incubated with 50 μl of 1:100 diluted streptavidin-FITC (Serotec) for 45 min on ice, washed and analysed. Note that the RCAI was titrated (from 10 μg/ml) for different cell lines as oversaturation with the lectin altered the scatter profiles of the fixed and permeabilized cells. Cells analysed by microscopy were stained as indicated above. Glass microscope slides were prepared by coating with 10 mM poly-L-lysine in Tris-HCl, pH 7.2, and rinsed with ddH2O. Stained cells were mounted onto slides using a drop of fluorescent mounting medium (Dako). Coverslips were added and the edges were sealed with nail varnish. Slides were analysed using a Zeiss microscope fitted with epifluorescence for immunofluorescence work and a similar system linked up to a Bio-Rad MRC600 Confocal microscope for the confocal studies. Images were captured using Biosis software (Bio-Rad) and exported into NIH-Image 1.59 (http://rsb.info.nih.gov/nih-image/) for processing.

2.8 β1,4-GalTase Protein

2.8.1 ELISA: β1,4-GalTase protein quantification assay

Fifty μl of streptavidin (Sigma S-4762) at 5 μg/ml was coated overnight at 4°C, washed four times with PBS-T, then blocked with 100 μl of PBS-1% BSA for 2 h at 37°C. After washing, one half of the plate was incubated with 50 μl of biotinylated (section 2.7.12) isotype control MOPC-21 IgG1 (Sigma M-9269) at 5 μg/ml in PBS, with the other half of the plate incubated with 50 μl of a mixture of biotinylated anti-β1,4-GalTase mAbs, 1B6 and 1H11, each at 2.5 μg/ml in PBS. Plates were incubated for 2 h at 37°C and then overnight at 4°C. Following washing, the plates were incubated with 50 μl human milk β1,4-GalTase standard at doubling dilutions in PBS-T from 60 ng/ml, or with 50 μl of sample: cell lysate, serum or culture supernatant. Note that samples were assayed in duplicates on both sides of the plates. Plates were incubated for 2 h at 37°C, then washed and incubated with 50 μl affinity-purified rabbit anti-human β1,4-GalTase (from Prof. Berger) at 5 μg/ml in PBS-T-1% BSA for 1 h at 37°C. After washing, the plates were incubated with 50 μl of goat F(ab')2 anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories) at 1:2000 in PBS-T-1% BSA for 1 h at 37°C. Plates were washed and developed as described in section 2.2.3, except that reactions were stopped with 100 μl of 3N H2SO4. Sample absorbances were averaged, corrected
against their own blanks, then quantified by reading off the human \( \beta 1,4\text{-GalTase} \) standard curve.

### 2.8.2 Endoglycosidase H treatment of \( \beta 1,4\text{-GalTase} \)

The endo-\( \beta \)-N-acetylglucosaminidase H (Endo H, of \textit{Streptomyces plicatus} from a recombinant \textit{E. coli} strain, Boehringer Mannheim), preferentially hydrolyses N-glycans of the high mannose type by cleaving at the N-acetylglucosaminide -> N-acetylglucosaminide linkage. The reaction was performed by incubation of 1 mU Endo H per 2 \( \mu \)g of rhGalTase in 100 mM sodium citrate buffer, pH 5.4, for 2 h at 37°C.

### 2.8.3 SDS-PAGE

Maxi electrophoresis apparatus SE600 or the mini electrophoresis apparatus Mighty Small II SE250 (Hoefer Scientific Instruments, San Francisco, USA) in conjunction with an LKB Bromma 2197 power pack were used for SDS-PAGE analysis. Glass plates were cleaned thoroughly using a weak detergent solution, then rinsed extensively with ddH\(_2\)O and finally sprayed with absolute ethanol, which was left to evaporate. Gel cassettes were assembled using 1.5 mm thick spacers. For a 100 ml solution of 10 % separating gel, 33.3 ml of Protogel (National Diagnostics Georgia, USA), 25 ml of 1.5 M Tris-HCl, pH 8.8, 40 ml of ddH\(_2\)O and 150 \( \mu \)l of 10% ammonium persulphate were gently mixed and degassed. One ml of 10% SDS and 50 \( \mu \)l of TEMED were added and the mixture gently mixed up and down with a pipette. The solution was poured immediately into the gel cassettes to about 1 cm below the level of the well comb. Care was taken to avoid formation of air bubbles. About 0.5 ml of isobutanol was carefully layered onto each gel to allow the gel to polymerize with a smooth even finish. After 2-3 h, the gel had polymerized and the isobutanol was washed off with ddH\(_2\)O and replaced with 375 mM Tris-HCl, pH 8.8, 0.1% SDS to prevent dehydration, covered and left overnight. The separating gel contained 10% acrylamide, 2.7% cross-linked with methylene bisacrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8. Before use, the overnight buffer was removed and replaced with stacking gel mixture. For 30 ml of a 3% stacking gel, 3 ml of Protogel, 3.75 ml of 1 M Tris-HCl, pH 6.8, 22.75 ml ddH\(_2\)O and 150 \( \mu \)l of ammonium persulphate were gently mixed and degassed. Three hundred \( \mu \)l of 10% SDS and 50 \( \mu \)l of TEMED were added, the solution mixed gently and poured immediately. The sample well combs were set in place, any air bubbles eliminated and the gel allowed to polymerize for at least 2 h at room temperature. Combs were removed from the polymerized sample wells and the wells were flushed clear with ddH\(_2\)O. For reducing conditions, samples were added to an equal volume of 2x concentrated Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue in 125 mM Tris-HCl, pH 6.8), non-reduced samples were added in
the same buffer without any 2-mercaptoethanol. Rainbow molecular weight markers (Amersham International, UK) were always run as they provided an indication of how far to run the SDS-PAGE for the protein size of interest and also acted as a control for the migration of proteins during the semi-dry transfer. The samples were then boiled for 3 min. Electrode chambers were filled with running buffer (192 mM glycine, 25 mM Tris. 0.1% (w/v) SDS, pH 8.3), and the water cooling system switched on. Samples were loaded into the sample wells and the current set at a constant 8 mAmps or at 30 mAmps for an overnight or daytime run respectively.

2.8.4 Western blotting
Thirty min before the end of the gel electrophoresis migration, 0.45 µM pore nitrocellulose membrane (NC, Schleicher and Schuell) and six thick blotting pads (Schleicher and Schuell) were cut to the size of the gel and soaked in transfer buffer (150 mM glycine, 20 mM Tris and 20% methanol, pH 8.0). Following electrophoresis, the stacking gel was removed and the separating gel equilibrated in transfer buffer for 30 min. A sandwich of blotting pads (Hoefer Scientific Instruments, San Francisco, USA), NC membrane, and gel was made, any air bubbles eliminated and assembled in a Semi-Phor TE70 (Hoefer Scientific Instruments, San Francisco, USA) semi-dry transfer apparatus, so that the proteins were transferred from the gel to the NC membrane at a constant current of 0.8 mAmps/cm² of membrane for 3 h. The NC membrane was removed from the sandwich, briefly rinsed in ddH2O and stained with Ponceau-S solution to check for transfer of proteins and facilitate cutting into strips if necessary. The NC was washed in PBS-T 0.2% three times for 10 min on an orbital shaker. Free-sites on the blot were blocked with 5% dried skimmed-milk powder in PBS-T 0.2%. (blocking buffer) shaking for 1 h at room temperature. The blot was washed and incubated with primary antibody or biotinylated primary antibody diluted in blocking buffer and left shaking overnight at 4°C. Following washing, the blot was incubated for 2 h at room temperature with conjugated second antibody or streptavidin-HRP diluted in blocking buffer. The blots were washed followed by a further two washes in just PBS. Development of the blots was done using 0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, 0.3 mg/ml nitro blue tetrazolium in 100 mM Tris with 5 mM MgCl₂, pH 9.5 (Sigma Fast BCIP/NBT, B-5655) as an insoluble substrate with the alkaline phosphatase conjugate. Detection of peroxidase conjugate was with 0.6 mg/ml 3,3’-diaminobenzidine in 50 mM Tris pH 7.6 with 0.03% H₂O₂ or the enhanced chemiluminescence system (ECL, Amersham International, UK) (section 2.8.5).

2.8.5 ECL detection
ECL is based on the HRP/H₂O₂ catalysed oxidation of luminol. Following oxidation, luminol enters an excited state which emits light as it decays back down to ground state.
Chemical enhancers such as phenols, increase the light output over 1000-fold and its duration. The emitted light signal can be detected using blue-light sensitive X-ray film. Briefly, at the detection step, reagents 1 and 2 were mixed in equal volumes using 0.125 ml/cm² of membrane and incubated for 1 min. Excess reagent was drained off the blots which were then wrapped up in cling-film, and exposed to Kodak X-OMAT-AR film for various period of time (15 seconds to several minutes).

2.8.6 Cleveland digests of β1,4-GalTase

A similar procedure to that previously described was used (Berger, 1986). Purified human milk β1,4-GalTase was separated on a maxi 1.5 mm thick, 10% SDS-PAGE under reducing conditions as described above (section 2.8.3). The β1,4-GalTase was added to an equal volume of 2x concentrated Laemmli buffer and boiled for 3 min. Slots were each loaded with 10 μg or 25 μg of β1,4-GalTase along with one lane of rainbow markers 14.3-220 kDa (Amersham International, RPN.756) and run for 4.5 h at 20 mAmps. The gel was removed and stained in 0.1% Coomassie blue, 10% acetic acid for 10 min, then destained in 50% methanol, 10% acetic acid for no more than 30 min. Bands were then excised (0.5 cm wide) using a scalpel blade and equilibrated in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA (buffer 1) for 30 min. Excess buffer was removed from the slices and the gel bands were stored at -20°C until needed (usually 24-48 h later).

Samples were then separated on a maxi 1.5 mm thick 15% SDS-PAGE gel which had a stacking gel of about 5-6 cm. Pre-electrophoresis the day before use with the upper reservoir running buffer containing 1 mM reduced glutathione was carried out for 1 h at 20 mAmps. Reduced glutathione acts as a scavenger to eliminate reactive by-products of acrylamide polymerization. The next day fresh running buffer containing 1 mM of thioglycolic acid, in place of glutathione as it is less likely to interfere with sequence analysis, was added to the upper reservoir tank. Ten μg of soluble human milk β1,4-GalTase was loaded with 10 μl of buffer 1 containing 10% glycerol (buffer 3). Excised gel bands containing the 10 μg of β1,4-GalTase were inserted into slots with 10 μl of 20% glycerol (buffer 2). The excised gel bands containing 25 μg of β1,4-GalTase were loaded with 10 μl of buffer 2 plus 10 μl of buffer 3 containing V8 protease at 0.5 mg/ml. Control slots with only 10 μl buffer 3 containing 0.5 mg/ml V8 protease were also run as well as one lane of rainbow markers 2.35-46 kDa (Amersham International RPN.755). With the water cooling system switched on, the gel was run for 1 h at 30 mAmps or until the dye front was approximately 0.5 cm from entering the separating gel, stopped for 30 min for digestion to proceed, then continued to run again for 3 h at 35 mAmps. The gel was blotted as described below (section 2.8.7).
2.8.7 Transfer of proteins onto PVDF membranes for sequencing

All buffers used in this procedure were pre-cooled to 4°C, powder-free gloves worn throughout and everything kept very clean to avoid contamination of proteins for sequencing. Following SDS-PAGE as described above (section 2.8.6), the gel was equilibrated for two 10 min incubations in 25 mM Tris, 40 mM glycine, 20% methanol, pH 9.4 (cathode buffer). The polyvinylidene fluoride (PVDF) membrane used was Immobilon-PSQ (Millipore, Hertfordshire, UK), an extremely hydrophobic membrane that allows for high protein binding and is suitable for direct protein sequencing. The PVDF membrane was pre-soaked in 100% methanol for a few seconds, rinsed in ddH2O for 2 minutes, then equilibrated in anode buffer II (25 mM Tris, 20% methanol, pH 10.4) for 5-10 minutes before use in transfer. Two thick filter pads were soaked in anode buffer I, one in anode buffer II and three in the cathode buffer. The sandwich was made starting from the anode with the two filter pads in anode buffer I (0.3 M Tris, 20% methanol, pH 10.4), one pad in anode buffer II, PVDF membrane, gel and the three filter pads in the cathode buffer. Transfer was for 2 h at 1.2 mAmps/cm² of membrane. After transfer the PVDF membrane was rinsed briefly in ddH2O, stained briefly with Ponceau-S, cut into strips if required, the blots were either probed as described above (section 2.8.4) or stained for protein with Coomassie blue. Prior to staining, the blots were rinsed in 0.002% DTT then stained with 0.2% Coomassie Blue R-250 in 5:4:1 methanol:ddH2O: acetic acid for up to 5 min. Destaining was done in the same solvent with several changes, the final destain was in 90% methanol, 10% acetic acid to clear the background. The membrane was rinsed again in 0.002% DTT and soaked for 20 min in 0.002% DTT. Membrane were placed onto filter paper and allowed to air-dry. Bands of interest were excised using new scalpel blades, put into sterile Eppendorfs for protein sequencing to be carried out by Dr. Joe Gray at Protein Sequencing Service, University of Newcastle Upon Tyne, UK.

2.9 Molecular biology techniques

2.9.1 E. coli cultures

E. coli cells were grown at 37°C with orbital shaking at 180 rpm (Gallenkamp Orbital incubator) in LB or 2X TY (section 2.1.7). Cultures always originated from a single well-defined colony plated out on agar (LB or 2X TY agar) Petri plates, before inoculation of small (3-5 ml) cultures and expansion into large 1 L cultures grown overnight at 37°C rotating at 180 rpm. Selective medium contained ampicillin (Sigma A-9518) used at 100 μg/ml for LB agar plates and included in LB cultures of transformed E. coli cells.
2.9.2 Preparation of competent *E. coli* cells

*E. coli* strain JS5, (based on MC1061 strain, Strr, recA1/F':::Tn10 (Tet') proAB, lacIq, lacZΔM15, Bio-Rad) were used for transformations of DNA. An inoculation of JS5 cells into 5 ml LB was left rotating at 180 rpm overnight at 37°C. The following morning, 400 μl of the culture was added to 100 ml of LB containing salt supplements (2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4). Cultures were left growing for about 2 h at 37°C 180 rpm until the absorbance at 600 nm was between 0.4-0.5. The cultures were split into two 50 ml Falcon tubes and kept on ice for 15 min, then centrifuged at 3000 g for 15 min at 4°C. Supernatants were discarded and each cell pellet resuspended in 1 ml of ice-cold buffer 1 (16 mM MES, 100 mM RbCl, 10 mM CaCl2, 50 mM MnCl2, pH 5.8), pooled, and made up to 40 ml with ice-cold buffer 1. Following a 15 min incubation on ice, cells were centrifuged at 3000 g for 15 min at 4°C. The supernatant was discarded and the cell pellet resuspended in 2 ml of ice-cold buffer 2 (10 mM Pipes, 10 mM RbCl, 75 mM CaCl2, 15% glycerol (v/v), pH 6.5), incubated on ice for 15 min, and 200 μl aliquots transferred to 1.5 ml Eppendorf tubes on ice. The cells were either transformed immediately or stored at -70°C.

2.9.3 Transformation of competent *E. coli* cells

Frozen competent cells were defrosted for 5 min on ice. About 10 ng of DNA (1 ng for transformation control) was added to the cells, mixed by swirling the pipette tip and incubated for 30 min on ice (1 h if competent cells were made the same day) before being heat-shocked for 2 min in a water-bath at 42°C. Cells were put back on ice for 5 min, then 800 μl of LB added and incubated for at least 40 min at 37°C. Fifty μl of the DNA/E. coli suspension was plated onto selective agar. The remaining cells were spun down in a microfuge for 1 min at 3000 g. The excess supernatant was discarded, and the cell pellet resuspended and plated out. Plates were wrapped in cling-film, incubated upside-down, overnight at 37°C. The following morning, single colonies were counted, and transformation efficiency of the cells calculated (should be between 10^5-10^8 cells transformed (cfu)/μg DNA). Plates were stored for several weeks at 4°C.

2.9.4 DNA mini-preps

Purification of DNA was carried out using Wizard Minipreps (Promega, Madison, WI, USA) and following the manufacturer's protocol. Selective LB agar plates were divided into four and streaked out from separate 3 ml overnight cultures, then incubated upside-down, overnight at 37°C. The rest of the cultures were centrifuged at 3000 g for 15 min at 4°C. Supernatants were discarded and the cell pellet resuspended in 200 μl ice-cold resuspension buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A) and transferred to 1.5 ml Eppendorf tubes at room temperature for 5 min. Two hundred μl of cell lysis solution (0.2 M NaOH, 1% SDS) was added per tube, mixed gently by
inversion until the lysate cleared and put on ice for 5 min. Two hundred μl of ice-cold neutralization solution (1.32 M potassium acetate, pH 4.8) was added and mixed gently by inversion. Lysates were centrifuged at 18 200 g for 10 min at room temperature, and the clear supernatant carefully removed and transferred to a new 1.5 ml Eppendorf tube. One ml of thoroughly mixed Wizard Miniprep DNA purification resin was added to the supernatant and mixed by inversion of the tubes. The DNA/resin slurry was gently passed through a minicolumn which binds the DNA. Minicolumns were then washed with 2 ml of column wash buffer (83 mM NaCl, 8.3 mM Tris-HCl, pH 7.5, 2.08 mM EDTA, 58% ethanol). The minicolumn was detached from its barrel and transferred to an Eppendorf, centrifuged at 18 200 g for 2 min at room temperature to dry the resin. After transferring the minicolumn to a new Eppendorf, 50 μl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added and left for 1 min at room temperature. The DNA was eluted by centrifugation of the minicolumn at 18 200 g for 20 min at room temperature. Plasmid DNA was stored at 4°C or -20°C.

2.9.5 DNA maxi-preps

Five ml cultures were set up and left shaking at 180 rpm at 37°C for 8 h. These cultures were transferred to 1L selective broth and cultured overnight at 180 rpm at 37°C. The following morning, approximately 5 ml was poured off from each culture and aliquots frozen down in cryotubes by adding an equal volume of plasmid cultures to 2X concentrated Hoegness solution (26.5 mM K2HPO4, 2.6 mM KH2PO4, 4 mM sodium citrate dihydrate, 2 mM MgSO4, 7H2O and 8.8% (v/v) glycerol) and stored at -70°C. The 1 L cultures were poured into large autoclaved buckets and centrifuged at 3000 g for 30 min at 4°C. For each preparation supernatants were discarded and the pellets resuspended in 40 ml MAXI buffer (50 mM glucose, 25 mM Tris-HCl pH 7.5, 10 mM EDTA). Eighty ml of lysis solution was added, swirled to clarify the lysates, and 40 ml of 3 M potassium acetate added (section 2.9.4). The solution was centrifuged at 3000 g for 20 min at 4°C. The supernatant was filtered through nylon into a clean 1L centrifuge bucket and 100 ml of isopropanol was added, then centrifuged at 3000 g for 30 min at 4°C. Supernatant was discarded and the pellet rinsed with 2 ml 70% ethanol and drained off. The pellet was then resuspended with 2 ml TE and transferred to a 12 ml polyallomer tube (Beckman, 331372). An equal volume of 5 M LiCl was added, tubes covered with Nescofilm and mixed by inversion. The tubes were centrifuged at 18 200 g in a Beckman L7 ultracentrifuge using an SW40-Ti swing-out rotor for 10 min at 4°C. The supernatant was collected into a new tube and an equal volume of isopropanol was added and mixed by inversion to precipitate the DNA. Following a centrifugation at 18 200 g for 10 min at 4°C, the supernatant was poured off to waste and the pellet rinsed with 1 ml 70% ethanol and drained off. The pellet was
resuspended in 300 μl of TE and transferred to a 1.5 ml Eppendorf containing 20 μg/ml RNase A. A further 200 μl of TE was used to rinse round the Beckman tube and added to the Eppendorf. The pellet was resuspended on a shaker for 30 min at room temperature. PEG precipitation of the DNA was done by adding an equal volume of 13% PEG-8000 in 1.6 M NaCl, mixed well by inversion, then centrifuged at 18 200 g for 10 min at room temperature. The pellet was washed with 500 μl of 70% ethanol and drained off, followed by brief evaporation. Four hundred μl of TE was added and the pellet resuspended on a shaker for 5-10 min at room temperature. A further 200 μl of TE was added before phenol/ chloroform extractions were carried out in a fume hood. An equal volume of TE-saturated phenol/ chloroform/ isoamyl alcohol (25:24:1) was added, tubes mixed by inversion, then centrifuged at 18 200 g for 3 min at room temperature. The upper aqueous supernatant was transferred into a clean Eppendorf, and the phenol chloroform extraction repeated until the interface became clear. The supernatant was taken and added to an equal volume of water-saturated chloroform, mixed by inversion and centrifuged for 3 min at 18 200 g at room temperature. The supernatant was taken into a fresh Eppendorf, precipitated with 1:10 of the volume using 3 M sodium acetate and 2.5 volumes of 100% ethanol and mixed by inversion. Tubes were centrifuged at 18 200 g for 6 min at 4°C. The pellet was washed with 70% ethanol, drained and evaporated, then resuspended in 600 μl TE. Using a 1:100 dilution of the DNA, absorbances were measured at 230 nm (phenol), 260 nm (DNA), and 280 nm (protein).

2.9.6 Gel electrophoresis of DNA
Analysis of DNA quality and restriction digests was performed via agarose gel electrophoresis. An agarose gel (Seakem, FMC Bioproducts #50003; 0.75% or 1 %, depending upon the size to be separated) was made up in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3), including 1 μg/ml of ethidium bromide just prior to casting. Twenty μl of samples and DNA markers (typically about 1 μg of lambda DNA digested with Hind III and EcoR I) were loaded with 5 μl of loading dye (20% Ficoll, 0.5% Orange G). The gel was run in 1x TBE buffer at 5 V/cm, visualized using a UV transilluminator and photographed using Polaroid film type 667. Low melting-point agarose gels (Sigma A-9414) in 1x TAE (40 mM Tris, 1 mM EDTA, adjusted to pH 8.0 with glacial acetic acid) were used for elution of DNA.

2.9.7 DNA restriction digests
Restriction enzymes were used in appropriate incubation buffers as supplied by the manufacturer's, supplemented with BSA at a final concentration of 100 μg/ml where necessary. Typically, 5 units of enzyme was used per μg of DNA digest, made up to 20
µl with ddH₂O and incubated overnight at 37°C. For test digests about 1 µg of DNA was used, whereas 5-10 µg of DNA was used in the preparation of cloning fragments/vectors.

2.9.8 Ligation of plasmid vector and insert DNA
The antisense vector was constructed by digesting β1,4-GalTase cDNA out of Bluescript KS using BamH I and Hind III restriction enzymes and subcloning into the Hind III/BamH I sites of the cloning vector pcDNA3, thereby orientating β1,4-GalTase in a 3' to 5' direction. The sense construct involved cleavage of the β1,4-GalTase cDNA insert from Bluescript KS with BamH I and Xho I and subcloning into pcDNA3 using the BamH I/Xho I cloning sites, resulting in the β1,4-GalTase cDNA in a 5' to 3' direction (Figure 6.6).

Following digestion of the fragments and vectors, the DNA was run on a 1% low melting-point agarose TAE gel (section 2.9.6) (Figure 6.7). The appropriate bands were excised, transferred to an Eppendorf and melted at 70°C. The DNA was cleaned up using Promega Magic PCR minicolumns. Magic PCR Prep DNA Purification Resin (Promega) was mixed and 1 ml added to the melted gel slice, invert several times over 20 seconds. The resin/DNA mixture was pipetted into a syringe and passed through a Magic minicolumn. After washing the minicolumn with 2 ml of wash buffer (80% isopropanol) the column was detached and the resin dried by centrifuging at 18 200 g for 20 seconds. The minicolumn was transferred to a new Eppendorf and 50 µl of TE added and left for 1 min. The DNA was eluted by centrifugation at 18 200 g for 20 seconds and stored at -20°C. A small fraction (3 µl) was run on a TBE gel (section 2.9.6) to check the amount of DNA present.

A molar excess of insert: vector is required for ligation, which is usually achieved using equal amounts of DNA from each (unless the insert is very large). The ligation reaction consisted of 100 ng of cloning vector, 100 ng of cloning fragment, 1 µl (400 units) of T4 ligase (New England Biolabs, Hitchin, Hertfordshire, UK), 2.5 µl of 10X ligase buffer (500 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP, 250 µg/ml BSA) made up to a final volume of 25 µl with ddH₂O. For each cloning vector, a negative control (no insert or ligase in the ligase reaction, to test for any undigested vector) and positive control (no insert in the ligase reaction, to test for any self-ligation of vector) were included. Incubations were overnight at 16°C.

After ligation, E. coli were transformed with the plasmids (section 2.9.3), clones grown on selective medium and checked for inserts following mini-preps (section 2.9.4) and restriction digests. The orientation of the inserts were checked by differential digests and terminal sequencing (section 2.9.9) of the inserts in the plasmid.
2.9.9 DNA sequencing

DNA sequences were determined using the dideoxy chain-termination DNA sequencing method (Sanger et al., 1977). This method involves the synthesis of a particular sequence of a single-stranded DNA by a DNA polymerase, following annealing of a specific oligonucleotide primer. The sequence reaction occurs in two steps. The first is the labelling step, incorporating the four deoxynucleoside triphosphates, including radioactively labelled dATP. In the second step, the chain-termination occurs following the incorporation of a 2', 3'-dideoxy analogue (ddNTP). Inclusion of this analogue stops any further chain elongation as it lacks the 3'-hydroxy terminus required for the next phosphodiester bond. Four separate reactions, each with a different ddNTP give the complete sequence information. The reaction was carried out using the Sequenase v 2.0 DNA Sequencing Kit according to the manufacturer's instructions (United States Biochemical, Ohio, USA). Double-stranded DNA (3-5 μg) was alkaline-denatured with 2 μl of 2M NaOH in a final volume of 10 μl ddH2O. The DNA was heated to 65°C for 5 min and then allowed to cool to room temperature for 10 min. Five pmols of primer (5’GTGGAGGTCTATATAAGCAG3’ which bound to sequences in the CMV promoter or 5’TAATACGACTCACTATAGGG3’ which bound to sequences in the T7 promoter) was added along with 3 μl of 3 M sodium acetate in a final volume of 30 μl ddH2O. The DNA was precipitated by adding 75 μl of absolute ethanol and incubating in a dry-ice/ ethanol bath for 20 min. After centrifugation at 18 200 g for 10 min at room temperature, the pellet was washed with 70% ethanol and resuspended in 7.5 μl of ddH2O. The labelling reaction was started by the addition of 2 μl of 5x concentrated Sequenase buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl2, 250 mM NaCl), 1 μl of 100 mM DTT, 2 μl of labelling mix dGTP diluted 1:5 (1.5 μM of each dGTP, dCTP and dTTP), 0.5 μl of [α-35S]-dATP (specific activity of 1000 Ci/ mmol, DuPont, Dreieich, Germany) and 2 μl (3.7 units) of 1:7 diluted Sequenase v 2.0 T7 DNA polymerase, and incubated for 5 min at room temperature. The chain termination step was started by the addition of 3.5 μl of the labelled sequencing mix to each of four tubes, pre-warmed to 37°C, containing 2.5 μl of termination mix (8 μM of dideoxy and 80 μM of four deoxynucleotides) of each of the four dideoxynucleotides, and incubated for a further 5 min at 37°C. The reaction was stopped by the addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were used immediately or stored up to 48 h at -20°C.

Sequence reactions were run on 6% polyacrylamide Sequa-gels (National Diagnostics, Atlanta, Georgia, USA) in 1x TBE. The gels were polymerized the day before use and pre-electrophoresized at 55 Watts for 2 h on the day of analysis. Reaction samples were heated at 80°C for 2 min just prior to loading 4 μl per well. After running, the gels were fixed in 10% acetic acid, 10% methanol then dried under vacuum at 80°C for 15 min. Gels were then exposed to Fuji RX X-ray film for approximately 48 h.
2.10 Cell culture

2.10.1 Cell culture of COS-7 cell lines

COS-7 cells were grown in 20 ml of complete medium (section 2.1.5), with 0.5 x 10^6 cells per 80 cm^2 flask, kept flat, in a 37°C incubator with 5% CO_2. Cells were split, using EDTA (section 2.10.2) when confluent, usually every 3 days. Cell viability was invariably greater than 90%.

2.10.2 Transient transfection using DEAE-dextran

COS-7 cells were transiently transfected using the method of Aruffo et al. (1994) with modifications. The concentrations of dextran and chloroquine, as well as the incubation time had to be optimized for the cell line being used. Also, the transfection time had to be slightly adjusted according to the number of times the cells had been passaged. The longer the transfection incubation time, the better the transfection efficiency though fewer cells survived as the chloroquine and dextran mixture was cytotoxic.

The day before transfection, cells from nearly confluent flasks (usually taken at two days after splitting) were counted and re-seeded at 1.5 x 10^6 cells per flask. The following day transfection medium was prepared: 6 ml per flask of 10 % NuSerum (decomplemented, Beckon Dickinson, MA, USA), 250 μg/ml dextran (average molecular weight 500 000, stock solution at 100 mg/ml), 200 μM chloroquine (stock solution at 200 mM) and 5 μg/ml supercoiled DNA (stock solution at 1 mg/ml, section 2.9.5) made up in DMEM. Medium was removed from flasks containing cells to be transfected. The cell layer was washed with 5 ml of sterile PBS (autoclaved, Sigma P-4417) at room temperature, then the transfection mixture was added to each flask and incubated for 2 h at 37°C with 5% CO_2. Transfection mixture was removed and the cells were washed with PBS. Cells were then shocked with 3-5 ml of 10% DMSO in PBS for 2 min at room temperature. The DMSO was removed and the cell layer washed with PBS. Twenty ml of fresh complete medium was then added to each flask and the cells incubated overnight at 37°C with 5% CO_2. The next day the medium was removed and the cell layer washed twice with 3-5 ml of 0.02% EDTA in PBS solution at 37°C. One ml of 0.25% trypsin-0.02% EDTA was added to the monolayer of cells and left for 15 min at 37°C. Cells were then harvested by adding 3-4 ml of DMEM at 37°C and sloughing up and down with a 3 ml sterile Pasteur pipette. Cells from the same transfections were pooled and centrifuged at 700 g for 5 min at room temperature. Complete medium was added and the cells were resuspended back into the same number of flasks. Twenty-four hours later the medium was removed and the cells washed with 5 ml 0.02% EDTA in PBS at 37°C. Another 3 ml of 0.02% EDTA in PBS
solution was added and the cells were incubated for 30 min at 37°C before harvesting for analysis.

2.10.3 Cell culture of B cell lines

EBV-transformed B cell lines were grown at 0.5 x 10^6 cells/ml in complete medium (section 2.1.5) in a 37°C incubator with 5% CO_2. They were split every 2-3 days and re-cultured at 0.5 x 10^6 cells/ml using fresh medium and new tissue culture flasks. The cell viability was usually greater than 90%.

2.10.4 Cell culture transfection using Lipofectin

Cell cultures were set up with freshly isolated PBMC or EBV-transformed B cell lines at 2.5 x 10^6 cells/ml of complete medium (with gamma globulin-free FCS) for different incubation times (12-96 h) and with various concentrations of S-oligos (final 0.01-10 μM). Where indicated repeated addition of S-oligos during the cultures period was performed. The protocol was later modified to increase the amount of DNA entering the cell by using liposomes.

Lipofectin reagent (Life Technologies #18292-011) is a 1:1 (w/w) mixture of cationic lipid N-[1-(2,3-diolcyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) in water. Prior mixing of the Lipofectin and the negatively-charged DNA resulted in lipid-DNA complexes. These complexes facilitate the delivery of DNA into the cell more easily, presumably by a combined process of hydrophobic diffusion and electrostatic attraction, and allow subsequent expression of the imported DNA. The manufacturer's instructions for transfection with Lipofectin were only used as a guideline, since the transfection conditions had to be optimized by titration of the lipid:DNA ratio as well as the incubation and harvest times for each cell type.

Briefly, cells were washed with reduced serum culture medium (Opti-MEM, Life Technologies) without any anti-bacterial agents (which would be cytotoxic in the presence of Lipofectin). Into 35 mm Nunc tissue culture dishes, 2.5 x 10^6 cells/ml were seeded in a total volume of 800 μl. The prior mixing of lipid-DNA in Opti-MEM was done for 15 min at room temperature then 200 μl added to each well and gently mixed. Dishes were incubated for at least 6 h in a 37°C incubator gassed with 5% CO_2.

Complete culture medium (with 10% FCS, section 2.1.5) was then added to give a final cell density of 5 x 10^5 cells/ml and incubated at 37°C in 5% CO_2 until harvested. Cell supernatants were stored at -20°C and the cells washed and lysed for β1,4-GalTase activity as described in section 2.2. Where DNA vectors were used, the S-oligos were substituted with the plasmids containing the inserts and selected (section 2.10.6).
2.10.5 Labelling of antisense oligonucleotide with fluorescein

Oligonucleotide in RPMI was incubated with 5-iodoacetamidofluorescein (5-IAF, Fluka 57674) in 50 mM BIC buffer, pH 9.6, at a 10:1 ratio (w/w). The reaction continued in the dark for 4 h with continuous shaking at room temperature. Unincorporated 5-IAF was separated from the conjugated oligonucleotide by gel filtration using a G-25 NAP-5 column (Pharmacia Biotech). The column was equilibrated with 15 ml of sterile distilled H$_2$O. Sample volume of approximately 115 µl, was allowed to enter the gel bead and then an additional 385 µl of H$_2$O added. Once all the sample had entered the gel, the sample was eluted with 1 ml H$_2$O and collected into an Eppendorf. The eluted sample was concentrated using a speed-vac and then stored at -20°C.

2.10.6 Stable transfections of EBV-B cells using Lipofectin

EBV-transformed B cell lines were grown in complete medium with 10% FBS (minus any gamma globulins). Prior to transfection the cells were washed free of antibiotics using HBSS, then resuspended at 4 x 10$^6$ cells/ml in complete medium (less antibiotics). Cells were seeded out in 500 µl volumes into 24 well tissue culture plates. Three µg of PEG-precipitated maxi-DNA (at 1 mg/ml) was added to 100 µl of RPMI and gently mixed with Lipofectin (10 µl in 100 µl of RPMI) at room temperature for 15 min. The DNA-Lipofectin complexes were added to the cells and incubated at 37°C in 5% CO$_2$ for 8 h. The cells were then pelleted and resuspended in complete medium at 0.5 x 10$^6$ cells/ml and re-cultured. Two days later the cells were resuspended at 0.5 x 10$^6$ cells/ml in fresh complete medium with G418 (Life Technologies #11811-023). Cells transfected with pcDNA3 plasmids (section 2.10.4) were selected using Geneticin G418, as pcDNA3 expressed the neomycin$^R$ gene. Initially, G418 stocks were titrated against the untransfected B cell lines in order to determined the minimal effective lethal dose of the drug. G418 was dissolved in 0.1 M HEPES, pH 7.4, at 100 mg/ml, sterile filtered and stored in aliquots at -20°C. Transfectants were split every three days and resuspended at 0.5 x 10$^6$ cells/ml in fresh complete medium with G418. Supernatants were collected steriley in aliquots and stored at -20°C for analysis of the secreted IgG galactosylation status. When sufficient cell numbers were available, aliquots were stored in liquid nitrogen (section 2.10.8), or lysed for β1,4-GalTase activity and assayed for β1,4-GalTase protein. In order to improve cell viability (>85%), cells were separated on Lymphoprep (section 2.4.1). This occurred once at the end of selection just prior to freezing aliquots of the transfectants, and once at the end of cell culture time point, day 29 in figures 6.13 and 6.17.

2.10.7 Cell counts and viability

A small volume of cell suspension was diluted with an equal volume of 0.4 % trypan blue solution (Sigma T-8154) and an aliquot added to an improved Neubauer ruling
haemocytometer for counting. Viable cells excluded the dye and appeared bright using a phase-contrast microscope. Doubling time was calculated using the following formula. Doubling time = \( \Delta t/P \), where \( \Delta t \) was the period during cell culture and \( P \) was the number of times the cell population doubled during the period from \( t = 0 \) to time \( t \) calculated from the formula; \( P = \log C_t/C_0 \) divided by \( \log 2 \), where \( C_t \) was the cell concentration at time \( t \) and \( C_0 \) at time \( t_0 \).

### 2.10.8 Freezing cells

Following a wash in RPMI, cells were resuspended in ice-cold freezing medium containing 90% FCS and 10% DMSO and 1 ml aliquots, containing about \( 5 \times 10^6 \) cells, were transferred into flamed cryotubes on ice. Tops were screwed down with flamed forceps and vials stored overnight in a polystyrene box at -70°C. Frozen vials were then transferred into liquid nitrogen storage tanks.

Frozen vials of cells were thawed quickly at 37°C and added to 10 ml pre-warmed fetal calf serum. The cells were then centrifuged, counted and cultured in complete medium. When the hybridomas were cloned for the second time they were cultured in serum-free medium, which consisted of 1% Nutridoma (Boehringer Mannheim) in place of the 10% fetal calf serum in complete medium.
3. Galactosyltransferase Activity in Human B Lymphocytes
3.1 Introduction

Specific oligosaccharide structures on glycoproteins reflect the sequential activity of the corresponding glycosyltransferases and glycosidases (Kornfeld and Kornfeld, 1985; Moremen, et al., 1994), some of which may be protein-specific (Baenziger, 1994). Consequently, the inherent specificity a glycosyltransferase possesses for its donor and acceptor substrates is further 'fine-tuned' by specific amino acid sequences present on the acceptor. Glycosylation may also be cell- and tissue-specific, as certain glycosyltransferases may only be present in certain cell types (Dinter and Berger, 1995b). Further, the expression of these genes may be regulated by cell- and tissue-specific promoters (Harduin^, et al., 1993). β1,4-GalTase has been shown to galactosylate a variety of oligosaccharide structures terminating in GlcNAc residues. However, the presentation of the oligosaccharide structure, in terms of branching (Blanken, et al., 1984; Paquet, et al., 1984; Narasimhan, et al., 1985; Morita, et al., 1988) and its accessibility provided by the glycoprotein acceptor (Fujii, et al., 1990; Lund, et al., 1993b), affects the efficiency of galactosylation.

It has been suggested that altered oligosaccharide structures on glycoproteins may induce a conformational change in the protein backbone with subsequent modified biological activity (Wright and Morrison, 1994). Glycosylation changes have been associated with some diseases (Parekh, et al., 1989; Turner, 1992; Isenberg and Rademacher, 1996). For example, rheumatoid arthritis (RA) has a deficit of galactose residues on its IgG N-linked oligosaccharides compared to the control age-matched population (Parekh et al., 1988b). It is possible that the abnormally glycosylated IgG is more than a diagnostic indicator and may be involved in the pathogenesis of RA (Tsuchiya, et al., 1989; Rademacher, et al., 1994; Malhotra, et al., 1995). This agalactosyl IgG 'defect' in RA is reversible, as patients who go into remission during pregnancy concurrently normalize their level of IgG galactose (Rook, et al., 1991b).

The underlying mechanism of agalactosyl IgG production is unknown. Some studies, using the radiochemical assay with ovalbumin as the acceptor substrate, have detected decreased levels of β1,4-GalTase activity in the B cells from RA patients (Axford, et al., 1987; Wilson, et al., 1993). However, another report could only detect this reduction with asialoagalacto-IgG (AsAg-IgG) as the acceptor molecule (Furukawa, et al., 1990).

Glycosyltransferase activities are usually measured by following the transfer of a radiolabelled sugar from its high-energy sugar donor to a suitable acceptor substrate. The radioactive product is then separated from the unincorporated label and counted.
These radiochemical assays are very reproducible, though they are also time-consuming and do not readily identify the linkage formed on the newly glycosylated product. With the introduction of non-radioactive ELISA-based assays, a more convenient approach for assaying glycosyltransferase activities in serum and cells is now available (Stults and Macher, 1990; Taki et al., 1990; Zatta, et al., 1991; Keshvara, et al., 1992; Taki, et al., 1994).

These assays were modified to measure the lymphocytic β1,4-GalTase activity using a neoglycoprotein as the acceptor substrate. β1,4-GalTase specifically transfers galactose from UDP-galactose to the non-reducing end of N-acetylglucosamine in a β1,4 linkage. The synthesis of the neoglycoprotein was carried out by the coupling of many GlcNAc derivative residues to the non-glycosylated protein, BSA, resulting in the acceptor GlcNAc-pITC-BSA. The availability of the reagents and ease of the conjugation provided for an attractive alternative acceptor substrate. In the ELISA-based assay, GlcNAc-pITC-BSA acceptor was coated to the well of the plate in which the GalTase reaction proceeded. The newly formed Galβ1,4GlcNAc-pITC-BSA product was then identified with biotinylated RCAI lectin. The usefulness of the GlcNAc-pITC-BSA acceptor was extended to the radiochemical assay where newly formed product could easily be precipitated from the reaction mixture.

This chapter describes the development of the ELISA-based assay for the measurement of lymphocytic β1,4-GalTase activity. Further, the GalTase activities found in B cells from patients with RA and in non-RA patients and healthy controls are compared using the acceptor substrates, GlcNAc-pITC-BSA in both the ELISA-based and radiochemical assays (Keusch, et al., 1995), and ovalbumin in the radiochemical assay.
3.2 Results

3.2.1 Characterization of the neoglycoconjugate, GlcNAc-pITC-BSA
In order to develop an ELISA based assay for the measurement of β1,4-GalTase activity, a suitable acceptor substrate had to be synthesized. One of the easiest options was to conjugate GlcNAc sugar residues to a non-glycosylated protein such as BSA. The most commonly used method is reductive amination (Gray, 1974) which though simple also destroys the sugar ring structure. In order to maintain the ring structure, a phenyl derivative of GlcNAc was used. The chemistry involved the conversion of the p-aminophenylglycoside into an isothiocyanate by treatment with thiophosgene, the resulting GlcNAc-phenylisothiocyanate was then able to form stable products with amines in slightly alkaline conditions. As these reactive sugar isothiocyanates are available commercially, the conjugation procedure was further simplified and hazard-free. Each time the GlcNAc-pITC-BSA substrate was synthesized, the number of GlcNAc residues conjugated per BSA molecule was fairly constant with approximately 22 GlcNAc occurring on average, as assessed using the benzothiazolone hydrazone method (Manzi and Varki, 1993). This contrasted with ovalbumin, the commonly used acceptor in the GalTase reaction, which has only one N-linked oligosaccharide, of various structures, per protein molecule (Wilson, 1991). The GlcNAc-pITC-BSA was found to be stable if kept lyophilized and desiccated at -20°C, and also in PBS at 4°C, though a fresh aliquot was used each month.

The GlcNAc residues on GlcNAc-pITC-BSA were specifically recognized by the BSII lectin (Figure 3.1). When GlcNAc-pITC-BSA was run alongside underivatized BSA in an SDS-PAGE an increase in molecular weight, corresponding to the GlcNAc-pITC moiety, could be observed (results not shown).

Increasing the GlcNAc-pITC-BSA concentration to 16 mg/ml (208 μM) inhibited the GalTase reaction in the radiochemical assay, possibly due to oligomerization of the neoglycoprotein. By varying the GlcNAc-pITC-BSA concentration in the presence of a constant amount of UDP-Gal (100 μM) the apparent K_m value for this neoglycoprotein substrate with the bovine milk β1,4–GalTase was found to be much lower, 20 μM (1.45 mg glycoprotein/ml, Figure 3.2), to that previously reported for ovalbumin, 1.6 mM and 0.9 mM (18.4 and 40 mg glycoprotein/ml for bovine and human milk β1,4-GalTase respectively) (Schanbacher and Ebner, 1970; Verdon and Berger, 1983), the commonly used acceptor substrate for GalTase in the radiochemical assay.
BSII lectin binding specifically to the neoglycoconjugate, GlcNAc-pITC-BSA. 50 μg/ml of GlcNAc-pITC-BSA in PBS was coated onto ELISA plates and unbound sites were blocked with underivatized BSA. The neoglycoconjugate was then probed with 2 μg/ml of biotinylated lectins in PBS-T with 0.1 mM Ca$^{2+}$ (RCAI was at 0.5 μg/ml in just PBS-T). Following incubation with streptavidin-HRP the reaction was revealed with OPD substrate in 0.1 M citrate buffer, pH 5.0 with 0.03% H$_2$O$_2$ and stopped with H$_2$SO$_4$. Absorbances were read at 490 nm and subtracted from background binding with underivatized BSA.
Figure 3.2
Apparent $K_m$ using the GlcNAc-pITC-BSA acceptor substrate was 20 $\mu$M in the GalTase radiochemical assay when using bovine milk $\beta 1,4$-GalTase. The conditions used were as stated in section 2.3.2
3.2.2 Specificity of the β1,4-GalTase reaction

The acceptor substrate, GlcNAc-pITC-BSA was coated onto ELISA plates and the specificity of the β1,4-GalTase reaction was monitored with biotinylated lectins. As the β1,4-GalTase reaction progressed during the 1 h incubation period, the formation of the Galβ1,4-GlcNAc-pITC-BSA product resulted in a decrease in the *Bandeiraea simplicifolia II* (BSII) binding to terminal GlcNAc residues and a corresponding increase in *Ricinus communis agglutinin I* (RCAI) binding to galactose residues (Figure 3.3).

**Figure 3.3**
The specificity of the β1,4-GalTase reaction monitored with biotinylated lectins: (a) BSII lectin; (b) RCAI lectin. Error bars indicate ± 1 SEM.
Figure 3.4
Inhibition of the β1,4-GalTase activity in the ELISA method using a rabbit F(ab′)2 anti-β1,4-GalTase with either bovine milk GalTase (500 pmol/100 μl/h, left) or human HB-1 lymphocytic cell lysate (right). Grey bars: no antibody; white bars: irrelevant F(ab′)2; black bars: F(ab′)2 anti-β1,4-GalTase. Error bars indicate ± 1 SEM.

The β1,4-GalTase activity was partially inhibited by a polyclonal rabbit F(ab′)2 anti-β1,4-GalTase antibody, but not with control rabbit F(ab′)2 antibody (Figure 3.4). Inhibition of this type could theoretically be an artefact due to β1,4-GalTase transferring galactose onto exposed terminal GlcNAc on the antibody if this were present on the rabbit F(ab′)2 anti-β1,4-GalTase but not on the control F(ab′)2. However, this type of competitive substrate inhibition was excluded since lectin binding studies on both rabbit F(ab′)2 antibodies revealed only terminal sialic acids and a few terminal galactose residues, but no exposed GlcNAc (results not shown).
Figure 3.5
Linearity of the β1,4-GalTase activity in the ELISA method: (a) standard bovine milk
β1,4-GalTase (1 nmol/100 μl); (b) human lymphocytic β1,4-GalTase activity in the
HB-1 B-cell line lysate.

3.2.3 Characterization of the β1,4-GalTase activity ELISA
β1,4-GalTase activity was routinely measured over a 1 h incubation period. Using
GlcNAc-pITC-BSA as the acceptor substrate, the ELISA method was linear over a 75
min incubation period with both partially purified bovine milk GalTase and with B-cell
lysates (Figure 3.5). Measurements within and between ELISA plates proved to be very
reproducible. In experiments using human B cell lysates, HB-1, randomly distributed
over 40 wells in two separate plates, intra-plate variation was 45.2± 3.3 (mean nmol/ mg
protein/ h ± sd, c.v. 7.4%) and inter-plate variation was 42.35 ± 4.03 (c.v. 9.5%).
3.2.4 Comparison of ovalbumin and GlcNAc-pITC-BSA acceptor substrates

When GlcNAc-pITC-BSA was compared with ovalbumin in both the ELISA and the radiochemical assays, a greater sensitivity for GalTase activity was achieved when using GlcNAc-pITC-BSA (Figure 3.6).

B cell lysates were not assayed using ovalbumin as an acceptor substrate in the ELISA system as the sensitivity of the assay was generally too low for the comparison between the control and the RA B cell samples.

Figure 3.6
Comparison of two different acceptor substrates: (⊙) GlcNAc-pITC-BSA and (●) ovalbumin grade VI, using standard bovine GalTase in (a) ELISA and (b) radiochemical assay.
Figure 3.7

GalTase activity in B lymphocytes from the first study of patients with RA (●, n=11) and controls (○, n=10) using the GlcNAc-pITC-BSA acceptor substrate in (a) the ELISA method and (b) the radiochemical assay. Error bars indicate ± 1 SEM. The p values show no significant difference using the unpaired Student's t-test.

3.2.5 β1,4-GalTase activity in RA B lymphocytes

Lymphocytic β1,4-GalTase activity was readily detectable using either the ELISA or the radiochemical assays, although the absolute values obtained were different between the two assays. The limit of detection of partially purified bovine milk β1,4-GalTase was found to be much lower with the radiochemical assay when compared to the ELISA. However, when using equivalent amounts of cell lysate sample, the ELISA detected greater β1,4-GalTase activities than the radiochemical assay. Using GlcNAc-pITC-BSA as an acceptor, no statistically significant difference in CD19+ B cell β1,4-GalTase activity was observed in the first study, between 11 patients with RA and 10 normal controls individuals in either the ELISA or radiochemical assay (Figure 3.7) (Keusch, et al., 1995).
Figure 3.8
IgG from peripheral blood of individuals presented in figure 3.6 was assayed for IgG G0 (section 2.6.2). First study group, RA (●, n=11) and controls (○, n=10). Values were expressed as standard deviations above or below the age-related mean for a healthy population (Sumar, et al., 1990). Error bars indicate ± 1 SEM. The p values were obtained using the unpaired Student's t-test.

It has been established that RA patients have high levels of agalactosyl IgG when compared to their age-matched controls (Parekh, et al., 1988b). At the same time of collecting blood for B cell isolation, some plasma was stored in order to measure the IgG galactosylation. The IgG G0 assays were performed as described (see section 2.6.2). Patients in the first study had age-corrected IgG G0 which was significantly (p=0.011) raised in the RA group compared to the controls (Figure 3.8).
This first study suggested that the lack of difference between the β1,4-GalTase activities in the B lymphocytes from the RA and control groups was due to the acceptor substrate, GlcNAc-pITC-BSA.

In order to test this possibility, a second study was set-up where greater amounts of B cells were collected from RA patients and controls. This enabled the measurement of β1,4-GalTase activity in the same B lymphocyte samples with acceptor substrates, GlcNAc-pITC-BSA (in both the ELISA and radiochemical assays) and ovalbumin using the radiochemical assay.

The original observations with the GlcNAc-pITC-BSA acting as acceptor substrate were confirmed in the second study, in that no statistically significant difference in B lymphocytic β1,4-GalTase activity between the RA and control groups could be detected in the ELISA or radiochemical assays (Figure 3.9). The β1,4-GalTase activities in the second study were within the range of those obtained in the first study. Whilst there was possibly some indication in the second study, that the B cell GalTase activities in the RA group were approaching a significant difference (p=0.1279) (Figure 3.9b), combining the data from the two groups gave a p value of 0.6226. Hence, there was no overall significant difference between the β1,4-GalTase activities in the B cells from RA and controls when assaying with the GlcNAc-pITC-BSA acceptor substrate in either the ELISA or the radiochemical assays.

Using the samples from the second study, B lymphocytic GalTase activities were measured using ovalbumin as the acceptor in the radiochemical assay. This resulted in the B lymphocytic GalTase activities in the RA patients being reduced to 42% of that seen in the control group (Figure 3.10).

B cell GalTase activities from the RA patients showed a mean value of 324 pmol/mg protein/h, compared to the control group value of 770 pmol/mg protein/h. There was a good correlation between the GalTase activities detected in the samples using either GlcNAc-pITC-BSA or ovalbumin substrates in the radiochemical assay (r=0.834). A reduction in galactose transferred to ovalbumin substrate compared to GlcNAc-pITC-BSA was observed for both the RA and control groups (p<0.001). Approximately 1.75 times more galactose was incorporated into the GlcNAc-pITC-BSA in the control B cells compared to the ovalbumin, with an even greater difference of more than three times seen in the RA group.

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Figure 3.9
GalTase activity in B lymphocytes from the second study group of patients with RA (●, n=10) and controls (○, n=10) using the GlcNAc-pITC-BSA acceptor substrate in (a) the ELISA method and (b) patients with RA (●, n=11) and controls (○, n=12) in the radiochemical assay. Error bars indicate ± 1 SEM. The p values show no significant difference using the unpaired Student’s t-test.
Figure 3.10
GalTase activity in B lymphocytes from the second study group of patients with RA (●, n=11) and controls (○, n=9) using ovalbumin as acceptor substrate in the radiochemical assay. Error bars indicate ± 1 SEM. The differences were significant using the unpaired Student's t-test.
IgG from peripheral blood of individuals presented in figure 3.9 was assayed for IgG G0 (section 2.6.2). RA (●, n=11) and controls (○, n=9) was assayed for IgG G0. Values were age-matched and expressed as standard deviations of the age-related population mean (Sumar, et al., 1990). Error bars indicate ± 1 SEM. The p values were obtained using the unpaired Student's t-test.

The combined levels of IgG G0 from the first and second studies were significantly higher in the RA group compared to the age-matched controls (p=0.0321). However, when the age-corrected IgG G0 were compared in those samples where reduced GalTase activity was detected (using the ovalbumin substrate), there was no difference between the RA and control groups (p=0.352) (Figure 3.11). Thus, no association was seen between B cell GalTase activities and IgG galactosylation.
Figure 3.12
GalTase activity in B lymphocytes from RA patients (●, n=11) and controls (○, n=9), obtained using ovalbumin as the acceptor substrate in the radiochemical assay, showed no correlation with age. The mean GalTase values for the RA group (red dotted-line) and the control group (blue dotted-line).

The GalTase activities did not correlate with either age or sex in either the RA patients or the control group (Figure 3.12). No association was seen with the type of treatment received by the patients, nor was there any correlation with the duration of the arthritis. The only association observed was between the reduced GalTase activity in RA B lymphocytes and the presence of rheumatoid factor (RF). Samples, from the second study, which were positive for RF had significantly lower GalTase activities than those without RF, 0.122± 0.066 SD (n=5) and 0.532 ± 0.233 (n=4) mean GalTase activity (nmol/mg protein/h) ± SD respectively (p=0.0067). Further, those B cells from RA patients which were RF negative, did not have significantly reduced GalTase activities compared to the control population (p=0.361).
3.3 Discussion

The simple and reproducible production of a neoglycoprotein, GlcNAc-pITC-BSA has been demonstrated. Approximately 22 GlcNAc residues were covalently attached to primary amines on the BSA. GlcNAc-pITC-BSA was an excellent acceptor substrate for β1,4-GalTase, giving an approximately 80-fold lower $K_m$ (20 μM) compared to that reported for ovalbumin (1.6 mM) with bovine milk β1,4-GalTase (Schanbacher and Ebner, 1970). Using this neoglycoprotein as an immobilized-acceptor, an ELISA-based assay was developed which provided a simple approach for measuring lymphocytic β1,4-GalTase activity. Furthermore, the newly formed product giving the N-acetyllactosamine structure may be detected with the biotinylated RCAI and, because RCAI binds with high affinity to Galβ1,4 GlcNAc structures (Merkle and Cummings, 1987), only β1,4-GalTase activity is measured. In the radiochemical assay, the glycosylated products required further analysis to identify the linkage formed. Hence a greater degree of specificity was achieved compared to the more conventional radiochemical assays.

The β1,4-GalTase activity was inhibited by approximately 40% using a rabbit F(ab')2 anti-β1,4-GalTase. Although this antibody was raised against bovine milk β1,4-GalTase, Western blotting and anti-β1,4-GalTase ELISA confirmed it cross-reacted with human milk β1,4-GalTase (results not shown). Thus this antibody preparation was able to inhibit both bovine and human β1,4-GalTase activities. The possibility of the β1,4-GalTase transferring galactose onto any exposed terminal GlcNAc present on the rabbit F(ab')2 antibodies was ruled out by the finding that neither the anti-β1,4-GalTase nor the control F(ab')2 antibodies exhibited exposed GlcNAc residues (results not shown).

Using the GlcNAc-pITC-BSA acceptor, the limit of detection of partially purified bovine milk β1,4-GalTase was found to be lower with the radiochemical assay (25 pmol/100 μl/h) when compared to the ELISA (75 pmol/100 μl/h). However, when using equivalent amounts of cell lysate, the ELISA detected greater GalTase activities than the radiochemical assay. The reason for this difference was unclear. One possible explanation may be due to altered acceptor substrate recognition in a fluid-phase and a solid-phase by purified β1,4-GalTase and GalTase present in crude cell extracts. The possibility that β-galactosidase activity in lymphocytic cell lysates influenced the measurement of β1,4-GalTase activity was highly unlikely as the pH optimum for the β-galactosidase in the cell lysates was found to be acidic (pH 3.5-4.5) and only expressed at low levels (results not shown). At the pH used in the β1,4-GalTase assays (pH 7.0) no β-galactosidase activity was detectable in the cell lysates.
The β1,4-GalTase activity ELISA was found to be extremely reproducible. The reagents required (GlcNAc-pITC, biotinylated lectins and β1,4-GalTase) are commercially available and the results can be simply assessed using an ELISA plate reader. The facility to identify the carbohydrate product by employing biotinylated lectins is an advantage over the radiochemical method. Theoretically, it would be possible to adapt this assay for measurement of other glycosyltransferases. N-acetyllactosamine formed from the β1,4-GalTase reaction may act as the acceptor substrate for sialyltransferase, whose product may be identified with an appropriate biotinylated lectin, e.g. *Sambucus nigra* (SNA). This ELISA was also fully capable of measuring soluble serum β1,4-GalTase activities (results not shown). The ease and inexpense of measuring β1,4-GalTase activity in this ELISA provided a useful alternative to the radiochemical assay (Keusch, et al., 1995).

The β1,4-GalTase reaction was more readily detected using the neoglycoprotein, GlcNAc-pITC-BSA, rather than ovalbumin as the acceptor substrate. Therefore, a study was set-up to measure the B cell β1,4-GalTase activities from RA patients and controls using the GlcNAc-pITC-BSA in the newly developed ELISA system. Despite an apparent difference in sensitivity between the ELISA and radiochemical assays, the ELISA method with the neoglycoprotein GlcNAc-pITC-BSA was fully capable of measuring the levels of β1,4-GalTase activity found in human lymphocytes. However, when GlcNAc-pITC-BSA was used in the radiochemical assay or in the β1,4-linkage-specific ELISA assay no statistically significant difference between the RA patients and control group was seen. The patients in this study had age-corrected IgG G0 which was significantly raised in the RA group compared to the controls (p=0.011, unpaired Student’s *t*-test). Thus, it was likely that use of the GlcNAc-pITC-BSA neoglycoprotein accounted for the similar enzyme activities observed in these two groups. In order to test this possibility, a second study was set-up, where enough RA and control B cells were collected to repeat the assays for β1,4-GalTase activity using the GlcNAc-pITC-BSA substrate in both the ELISA and radiochemical assays, and further, to test these same samples with the conventional glycoprotein acceptor, ovalbumin, using the radiochemical assay. The sensitivity of the β1,4-GalTase ELISA was insufficient to measure activities in the B cell lysates when using ovalbumin as the substrate.

Results of the second study confirmed the findings of the first, in that B cell β1,4-GalTase activities were easily detected using the GlcNAc-pITC-BSA substrate, but no differences were observed between the B cell β1,4-GalTase activities in the RA and control groups. The studies described here only observed decreased GalTase activity (by 42%) in B cells from RA patients when using ovalbumin as the acceptor. Other
groups have previously reported a similar decrease in the B cell $\beta$1,4-GalTase activity from RA patients, although different acceptor substrates and assay procedures were used (Furukawa, et al., 1990; Axford et al., 1992; Wilson, et al., 1993). Surprisingly, no $\beta$1,4-GalTase activity was detected in B cells from either the control or RA groups when ovalbumin was used as the acceptor (Furukawa, et al., 1990), in contrast to results presented in this chapter and elsewhere (Axford, et al., 1992; Wilson, et al., 1993). Furukawa et al (1990) only observed the reduction in $\beta$1,4-GalTase activity with asialoagalacto-IgG (AsAg-IgG) acceptor and not with asialo-ovine submaxillary mucin (Gal $\beta$1,3 GalNAc), AsAg-transferrin or free GlcNAc residues (Kobata, 1990). AsAg-IgG acceptor was created by treating intact normal human IgG with sialidase followed by digestion with $\beta$-galactosidase from the streptococcus strain 6646K. The sequestered oligosaccharide chains in native IgG (Sutton and Phillips, 1983) were less efficiently galactosylated by $\beta$1,4-GalTase than either denatured IgG, or isolated IgG oligosaccharides (Fujii, et al., 1990; Wilson, et al., 1993). The isolated heavy chain of porcine IgG but not the intact porcine IgG molecule was a suitable acceptor substrate for both soluble and bound $\beta$1,4-GalTase from swine mesentery lymph nodes (Rao and Mendicino, 1978). This suggested that in vivo, the galactose residues were transferred onto a partially folded IgG molecule which had its oligosaccharides easily exposed for the terminal glycosyltransferase to act upon. At present the IgG structure presented to $\beta$1,4-GalTase in the biosynthetic pathway is unresolved (Rademacher, et al., 1996a) (section 1.3.6).

GlcNAc-pITC-BSA and ovalbumin were both good acceptors for $\beta$1,4-GalTase, although it was only possible to resolve differences in the B cell GalTase activities between the RA and control groups using ovalbumin as the acceptor. The increased rate of galactose transfer in the B cells from the RA and control groups seen with GlcNAc-pITC-BSA over the ovalbumin was greater in the RA group, though no significant difference was observed between the two groups. It may be that the number and a possible clustering of GlcNAc residues in the GlcNAc-pITC-BSA provided for a more accessible and efficient glycoprotein acceptor than that of ovalbumin. Also, the aromatic phenol spacer molecule in the GlcNAc-pITC-BSA may favour interaction with the putative hydrophobic substrate binding pocket (Aoki, et al., 1990). Hydrophobic O-glycosidic groups attached to GlcNAc had up to 2000-fold lower $K_m$, though only about a two-fold increase in the $V_{\text{max}}$ compared to the non-derivatized GlcNAc sugar (Geren, et al., 1976). Thus, in order to detect the modest decrease in the B cell $\beta$1,4-GalTase from RA patients a more restrictive substrate such as ovalbumin (which is readily available) or AsAg-IgG, in terms of branching, number of GlcNAc per protein molecule and glycoprotein conformation may be required.
The complex biantennary N-linked oligosaccharide, such as those found at the Fc region in IgG have more than 30 different glycoforms (Parekh, et al., 1985). β1,4-GalTase, from a number of different sources, readily galactosylated biantennary oligosaccharides where the GlcNAc residues were exposed on both branches, acting primarily on the Manα1,3 arm with successive galactosylation on the Manα1,6 branch occurring at a much slower rate (Blanken, et al., 1984; Paquet, et al., 1984; Morita, et al., 1988). The presence of a bisecting GlcNAc molecule, did not alter the branch specificity of bovine milk or thymus β1,4-GalTase, but decreased the galactosylation of the Manα1,3 branch by 78%, probably as a result of steric hindrance (see below) (Blanken, et al., 1984; Narasimhan, et al., 1985). Purified β1,4-GalTase maintained the same substrate specificities throughout each step of its purification, suggesting that the same enzyme was responsible for the galactosylation of each branch (Morita, et al., 1988). However, in bovine IgG only the non-bisected sugar chains were preferentially galactosylated on the Manα1,3 arm, whilst the bisected structures were predominantly galactosylated on the Manα1,6 arm (Fujii, et al., 1990). Highly-ordered branch specificity has also been observed with β1,4-GalTase in the synthesis of blood-group I structures, where galactosylation of the β1,6 branched GlcNAc residue of the trisaccharide GlcNAcβ1,3(GlcNAcβ1,6)Gal preceeded that of β1,3 linked GlcNAc (Blanken et al., 1982). Hence β1,4-GalTase exhibits very high branch specificity which alters depending on the oligosaccharide acceptor, species and glycoprotein. Time course studies revealed that B cells from controls continued to galactosylate AsAg-IgG without saturation for up to 6 hours, whilst B cells from RA patients had reached saturation by 2 hours (Furukawa, et al., 1990). It would be worthwhile determining if the galactosylation efficiencies seen in the B cells between the RA and control resulted in different branch specificities on the AsAg-IgG substrate.

Substrate specificities of glycosyltransferases are likely to play a role in the microheterogeneities of the complex oligosaccharide seen in IgG. Some of these differences may be explained in terms of branch specificities and grouped into bisected and non-bisected sugar chains on IgG. However, the influence the protein structure has on the oligosaccharide substrate should also be considered. Structural studies on the sugar chains of human IgG revealed a higher frequency of galactose present on the Manα1,6 branch rather than the α1,3 branch of non-bisected biantennary complexes, which is at complete variance to the in vitro studied models (see above) (Fujii, et al., 1990). The oligosaccharides on human IgG1 differed in the ratio of galactosylated structures found compared to bovine IgG and human IgG2. IgG1 had a higher ratio of Manα1,6 arms galactosylated to Manα1,3, including the non-bisecting structures (Fujii, et al., 1990). Although native IgG1 had more galactose on the Manα1,6 arm, the Manα1,3 arm on denatured IgG1 was preferentially galactosylated (Fujii, et al., 1990).
It may be that the \textit{in vivo} state of the IgG subclass folding differed in such a way as to affect which branch was galactosylated. The production of mouse-human chimeric IgGs in mouse J558L cells, demonstrated that each human heavy chain of IgG subclasses could have a different glycosylation profile (Lund, et al., 1993b). Further, the \(\beta1.4\)-GalTase isolated from bovine milk (and other organs including liver, kidney and serum) and myeloma cells secreting IgG\(_1\) and IgG\(_2\) subclasses had similar specificities on a variety of IgG oligosaccharide substrates (Fujii, et al., 1990). This data would suggest that the protein conformation, rather than any potential IgG-specific \(\beta1.4\)-GalTase, influenced the degree of galactosylation.

A potential caveat with the identification of glycoforms of IgG isolated from the plasma is that they represent only a fraction of the glycoforms produced from the plasma cells, the remainder being involved in complex formation, attached to cell surfaces, degraded or having already been removed from the circulation. Obviously, the IgG biosynthetic route is of upmost importance in determining how IgG is presented to \(\beta1.4\)-GalTase as an acceptor substrate for its subsequent galactosylation (section 1.3.7). The x-ray crystallography structure of IgG showed that the oligosaccharides on each heavy chain were enclosed between the C\(\gamma2\) domain and formed an integral part of the Fc structure (Deisenhofer, 1981; Sutton and Phillips, 1983). It was thought that, in rabbit IgG, at least 50\% of the Man\(\alpha1,3\) arms were devoid of galactose to permit the carbohydrate pairing with the opposite sugar chain in the space available within the Fc pocket (Sutton and Phillips, 1983). However, more fully galactosylated human IgG\(_1\) and IgG\(_3\) molecules have been produced in static culture (Kumpel, et al., 1994). In addition, a human IgG\(_4\) has been reported with digalactosylated structures present within the Fc region (Jefferis et al., 1990). It may be that an alteration in protein conformation was required to accommodate such oligosaccharide structures.

A bisecting GlcNAc caused the Man\(\alpha1,6\) arm of the biantennary oligosaccharide chain to fold back towards the core carbohydrates. This bisecting GlcNAc inhibited the subsequent processing by GnT II, GnT IV, \(\alpha1,6\)-Fuc-T and mannosidase II (Schachter et al., 1983), possibly via steric hindrance of the GlcNAc\(\beta1,2\)Man\(\alpha1,3\)Man\(\beta1,4\) region of the substrate, which has been proposed as a binding site for the above four enzymes (Brisson and Carver, 1983). Further, a \(\beta1,4\) linked galactose on the Man\(\alpha1,3\) arm prevented the same four enzymes from acting (even in the absence of a bisecting GlcNAc), and also blocked the GnT III from interacting (Schachter, et al., 1983). Hence, \(\beta1,4\)-GalTase and GnT III appeared to compete for the same agalactosylated non-bisecting biantennary oligosaccharide substrate. The low activity ratio of \(\beta1,4\)-GalTase: GnT III, rather than just the \(\beta1,4\)-GalTase activity, correlated with the incidence of hypogalactosylated IgG in patients with advanced multiple myeloma.
(Nishiura, et al., 1990). However, there was no apparent increase in any particular core oligosaccharide structures of IgG from patients with RA (Parekh, et al., 1985).

It has been suggested that the defective galactosylation of IgG in RA patients is due to the activity of an IgG-specific β1,4-GalTase (Furukawa, et al., 1990). The difference in β1,4-GalTase activity in the B cells from RA patients was attributed to an increased $K_m$ for the UDP-galactose when the AsAg-IgG acceptor substrate was used (Furukawa, et al., 1990). There was no difference in the $K_m$ for the AsAg-IgG acceptor substrate between the B cells from the RA and control groups. The $\beta$1,4-GalTase reaction occurs as an ordered mechanism with the reactants participating in the order: $\text{Mn}^{2+}, \text{UDP-galactose}, \text{GlcNAc}$ (Morrison and Ebner, 1971a; Khatra et al., 1974). In addition, the substrate binding sites are thought to be in very close proximity (Aoki, et al., 1990). Thus, it is unclear how an increased $K_m$ for UDP-galactose was observed without a change in the binding of the acceptor AsAg-IgG. There has been no compelling evidence that such an IgG-specific enzyme exists as $\beta$1,4-GalTase from different species and sources were similar by a) their ability to be regulated by the modifier protein, b) their substrate specificities and c) the ratios of specific activities which remained constant throughout the purification of $\beta$1,4-GalTase (Morita, et al., 1988; Fujii, et al., 1990). However, with the recent emergence of several different functional $\beta$1,4-GalTase genes (see below) their relevance to IgG galactosylation in health and disease states warrants investigation.

Furukawa et al (1990) also found that α-lactalbumin was able to inhibit more than twice the $\beta$1,4-GalTase activity from B cells when using AsAg-transferrin compared to the AsAg-IgG substrate (Furukawa, et al., 1990). The way in which α-lactalbumin modifies the catalytic activity of GalTase depends not only on the identity of the carbohydrate acceptor but also on the acceptor concentration in relation to its $K_m$ value (Brew, et al., 1968; Klee and Klee, 1970) as α-lactalbumin has unexpectedly been shown to increase $\beta$1,4-GalTase activity towards AsAg-biantennary sugar chains (Morita, et al., 1988). The enhanced efficiency of $\beta$1,4-GalTase utilizing UDP-GalNAc donor substrate following interaction with α-lactalbumin represents a possible regulation of $\beta$1,4-GalTase activity (Do, et al., 1995). Whether there are other modifier proteins present in cells capable of inducing $\beta$1,4-GalTase to utilise other donor substrates remains to be determined. B cell GalTase activity towards ovalbumin was inhibited by α-lactalbumin (Wilson, et al., 1993), a property also seen in bovine milk $\beta$1,4-GalTase which could also galactosylate bovine and human IgG (Fujii, et al., 1990; Wilson, et al., 1993). Cell lysates from a human B cell acute lymphoblastic leukaemic cell line (BALL-1), which cannot produce IgG, could galactosylate AsAg-IgG though at approximately 50% the level of that observed in the B cells from RA patients. The
kinetics for the β1,4-GalTase from the BALL-1 cell line were the same as those for B cells from controls (Furukawa, et al., 1990). Human liver homogenates were also able to galactosylate AsAg-IgG at similar rates to the BALL-1 cell line (Furukawa, et al., 1990). Thus, β1,4-GalTase capable of galactosylating IgG were isolated from a number of different sources and species. Whether there are some β1,4-GalTase which galactosylate IgG preferentially over other substrates needs further study. May be substrate competition experiments with B cell β1,4-GalTase would help resolve the question of enzyme specificities.

There may be structural differences between the RA and control B cell β1,4-GalTase enzymes resulting in altered folding of the binding site for UDP-galactose or for other interactions including oligomerization. Trypsinization of bovine colostrum β1,4-GalTase (51 kDa) results in a catalytically active form of the enzyme (42 kDa), usually found in milk. This lower molecular weight form of β1,4-GalTase is more symmetrical in shape and exhibits a more reversible binding of UDP-galactose to the enzyme—Mn2+ complex (Powell and Brew, 1974). If such a process occurred in B lymphocytes from RA patients then the proteases would have to be very restrictive, otherwise more general defects in B cells from RA patients would be expected to occur. Protein-engineered mutants of human β1,4-GalTase which lack Cys129 (or the equivalent Cys134 in bovine β1,4-GalTase) are thought to disrupt a disulphide bond (Cys129-Cys245) necessary for catalytic activity (Wang, et al., 1994; Boeggeman, et al., 1995). These mutants were still capable of binding GlcNAc and UDP, though with a reduced affinity for the UDP (Boeggeman, et al., 1995). Another mutant human β1,4-GalTase, Cys340Ser, exhibited enzymatic activity though had a much higher K_m for UDP-galactose (Wang, et al., 1994). At present it is unclear how β1,4-GalTase interacts with its acceptor substrates (section 1.3.4).

There have been various studies suggesting that the Golgi membrane-bound form of β1,4-GalTase self-associates (Navaratnam, et al., 1988; Bendiak, et al., 1993; Fleischer, et al., 1993), possibly via some interaction with cytoskeletal components (Yamaguchi and Fukuda, 1995). Whilst the β1,4-GalTase dimerization may play a role in Golgi retention, it has also been shown that purified soluble human β1,4-GalTase dimerizes in solution (Malissard, et al., 1996) and it has been proposed that this is its preferred structure in the Golgi for activity (Fleischer, et al., 1993). Perhaps modifications in the RA β1,4-GalTase protein contribute to a less stable dimer formation. Also if less β1,4-GalTase enzyme were present in the B cells from RA patients, there would be less dimerization leading to a decrease in the affinity which would have an effect on β1,4-GalTase activity. Further site-directed mutagenesis studies and the eagerly awaited
crystal structure of β1,4-GalTase should clarify the specific interactions involved in the β1,4-GalTase reaction.

There is now gathering evidence for the existence of several β1,4-GalTase genes. Two β1,4-GalTase from porcine trachea, one of which was α-lactalbumin independent, have been described (Sheares and Carlson, 1984). The α-lactalbumin binding domain is conserved in evolution as avian β1,4-GalTase has high homology between its functional domains and those of mammalian β1,4-GalTase (Hathaway, et al., 1991; Shaper, et al., 1995). Moreover, two functional avian β1,4-GalTase genes have been identified and expressed (Shaper, et al., 1995). Homologies based on the functional domains in β1,4-GalTase have been found in enzymes other than the known β1,4-GalTase (Bakker, et al., 1994). This increases the likelihood of the existence of a larger GalTase gene family. Very recent data from β1,4-GalTase knock-out mice, revealed residual β1,4-GalTase activity still existed, despite the absence of β1,4-GalTase mRNA following analysis with exon 1 as a probe (Asano, et al., 1997; Lu, et al., 1997). Further, glycoproteins were isolated from these β1,4-GalTase knock-outs which expressed some Galβ1,4GlcNAc sugar structures (Asano, et al., 1997; Lu, et al., 1997). Another recent study identified and expressed a human cDNA homologous to β1,4-GalTase gene (Sato, et al., 1997). The deduced amino acid sequence exhibited 35% homology to the β1,4-GalTase gene, including four conserved cysteines, two of which were predicted to be involved in a disulphide bridge essential for catalytic activity (Sato, et al., 1997). This newly identified β1,4-GalTase enzyme utilized GlcNAc-thio-para-nitrophenol as an acceptor but in contrast to bovine milk β1,4-GalTase activity it was unable to efficiently utilize AsAg-transferrin as an acceptor. Northern blot analysis revealed high expression of a single 4.7 kb mRNA transcript in a variety of tissues including heart, brain, testis and bone marrow, but very low levels in the thymus, lymph node and leukocytes (Sato, et al., 1997). A mutant CHO cell line, Lec20, which is deficient in β1,4-GalTase activity, is still able to synthesis fully galactosylated glycoproteins (Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, USA, personal communication).

Although originally no difference between GalTase activity in (age-matched) T lymphocytes from RA and controls was observed (Axford, et al., 1987; Furukawa, et al., 1990), a later study found a significant decrease in GalTase activity, using ovalbumin as the acceptor, though not as great as that seen in the B cells from RA patients (Axford, et al., 1992). Animal models of arthritis also have decreased lymphocytic GalTase activity. Splenic T and B cells from Lewis rats with adjuvant arthritis have reduced GalTase activity (Frenkel and Cohen, 1989), as do the B cells from the peripheral blood, but not the spleen, of MRL lpr/lpr mice (Axford, et al., 1994b). Reduced GalTase
activities described in this chapter and by others (Wilson, et al., 1993) have been
detected in peripheral blood CD19+ B cells implying that the defect is more likely to be
one which affects the majority of the B cells and is not one which is clone restrictive.
Further, only a very small percentage of the peripheral blood B cells are plasma B cells,
and fewer still are secreting IgG. Abnormalities of immunoglobulin glycosylation in
RA patients are restricted to IgG (Field et al., 1994a), suggesting the galactosylation
defect may be due more to the IgG substrate than to the β1,4-GalTase protein.

β1,4-GalTase activity measured in isolated B cells may be different from that seen in
their local in vivo environment. Isolated B cells do not receive the same signals from
cell-cell interaction as from soluble factors of surrounding cells. An increase in β1,4-
GalTase activities can be seen in the PBMC population following short-term culture
compared to those freshly isolated (see chapter 5). This may be due to stimulatory
effects of the culture medium and/or the culture dishes, or indeed the removal of a
modifier molecule normally present in vivo. Such a regulator, perhaps even present in
the extracellular fluid, may exist in the local B cell environment of RA patients. This
inhibitor(s) in the RA B cells would have to interact with the ovalbumin and/or the
ovalbumin binding site on β1,4-GalTase, but not affect GlcNAc-pITC-BSA or its
interaction with β1,4-GalTase. Rheumatoid factor may be a likely inhibitor for
interaction with the AsAg-IgG acceptor. However, mixing B cell homogenates from
RA patients and controls, and also using microsomal fractions (thought to be free from
rheumatoid factor) showed no evidence of such inhibition (Furukawa, et al., 1990).
β1,4-GalTase activity has also been shown to be regulated by phosphorylation. A 58
kDa β1,4-GalTase associated serine/threonine protein kinase (p58GTA) can
phosphorylate β1,4-GalTase and specifically cause an increase in the β1,4-GalTase
activity (Bunnell, et al., 1990a). However, no allelic variant of this p58GTA gene
(Delves et al., 1990; Delves et al., 1992) or the β1,4-GalTase gene has been associated
with RA (Axford and Alavi, 1994a; Jeddi, et al., 1996). There maybe different gene
regulatory factors present in the B cells from RA patients compared to those of controls.

Certain drugs which RA patients are treated with may affect β1,4-GalTase reactivities
to different acceptors. Some suggestion has been made for sulphasalazine drugs
maintaining GalTase activities and reducing IgG G0 in patients with RA (Axford, et al.,
1992), however, neither the GalTase activities or the IgG G0 levels presented in this
chapter and elsewhere (Furukawa, et al., 1990; Bodman, et al., 1992) correlated with
drug treatment. Although IgG galactosylation varied with age, no such relationship
with GalTase was observed (Figure 3.12) (Furukawa, et al., 1990). Furthermore,
patients with low GalTase activity did not necessarily have the lowest amount of
galactose present on their IgG. However, there was a strong correlation, in this small
study, between the presence of RF and reduced GalTase activities in RA B cells (p=0.0067). Moreover, B cells from RA patients without any RF had normal GalTase activity levels. Another group observed a correlation between low GalTase activity and murine MRL hybridomas producing IgG RF (Axford, et al., 1994b). Whether there is a direct relationship between B cell GalTase activity, RF production and immune complex formation requires further investigation.

Agalactosyl IgG is thought to be a pre-secretory event as RA B cells in culture secreted more IgG G0 than the control group (Bodman, et al., 1992). There has been no evidence of increased β-galactosidase activity in the serum of RA patients, in fact it was found to be significantly decreased (Mullinax, et al., 1976). Further, no cellular β-galactosidase activity could be detected at the pH the β1,4-GalTase activity assay was carried out at, ruling out an artefact basis for the reduced β1,4-GalTase activity in RA B lymphocytes. Thus, the regulation of agalactosyl IgG is unclear and there may well be other factors which play a role in the galactosylation of IgG such as the oxidative environment found in areas of inflammation in RA patients; effect of cytokines; and different assembly pathways for IgG.

In future studies, the choice of acceptor substrate for measuring β1,4-GalTase activity must be carefully considered. Fundamental questions regarding the reduced β1,4-GalTase activity seen in the RA B cells need to be addressed, in order to ascertain if the cause is one of a defective enzyme production and/or of protein function. The question of quantitative differences between the β1,4-GalTase protein levels in B cells from RA patients and controls is addressed in chapter 4. Whether the observed reduction in β1,4-GalTase activity would be sufficient for a reduced level of galactose on IgG is explored in chapter 5.
4. Production and Characterization of Monoclonal Antibodies to \( \beta 1,4 \)-Galactosyltransferase
4.1 Introduction

At present there are no commercially available monoclonal antibodies (mAbs) to any glycosyltransferases, although a number against β1,4-GalTase have been described in the literature including those against rat (Kawano et al., 1994), bovine (Ulrich, et al., 1986) human (Berger, et al., 1986) β1,4-GalTase and to a human β1,4-GalTase thought to be associated with ovarian tumours (Uejima, et al., 1992). Readily available and well characterized anti-β1,4-GalTase mAbs would be useful in studying cell localization, function and structure of β1,4-GalTase.

In 1975 Köhler and Milstein fused plasmacytoma cells with normal plasma cells, creating hybrid cells, or hybridomas, that secreted both myeloma and antibody immunoglobulin and were immortalised. Screening of the culture supernatants with specific antigens, identified groups of hybridomas which were then cloned to produce an immortal cell line synthesising a homogeneous antibody population of known specificity i.e. monoclonal antibodies. The production of anti-human β1,4-GalTase mAbs, using this hybridoma technology, and their characterization will be described in this chapter.

These mAbs were then used in several applications including the quantification of β1,4-GalTase protein. One possible explanation for the difference in B cell β1,4-GalTase activities between RA and control groups when using ovalbumin as an acceptor could be a quantitative difference, i.e. there is less β1,4-GalTase protein present in the B cells from patients with RA. In order to address this question, a method for quantifying β1,4-GalTase protein was developed. Initial attempts were based upon the binding properties of α-lactalbumin for β1,4-GalTase, but such assays proved to be insensitive. The development of an ELISA based assay was possible following the production and characterization of anti-β1,4-GalTase mAbs. Using mAbs of different binding specificity for β1,4-GalTase, a two-site ELISA binding assay was established. The β1,4-GalTase quantification assay described in this chapter was used to measure β1,4-GalTase protein from B cell lysates.
4.2 Results

4.2.1 Immunization of mice with human milk β1,4-GalTase

Three Balb/c mice were immunized with human milk β1,4-GalTase in Ribi adjuvant (two s.c. injections of 20, 10 or 5 μg, into mice 1, 2 and 3 respectively each with a final booster of 15 μg i.v.), and the two highest responders were sacrificed and spleen cells isolated. All mice gave high antibody titres with mouse 1 giving a significant signal down to 1:10^5 dilution (Figure 4.1).

![Graph showing antibody titres](image)

**Figure 4.1**

Two Balb/c mice immunized with human milk β1,4-GalTase were assayed 10 days after the second injection for antibody titres using a direct binding ELISA (section 2.7.6) (anti-β1,4-GalTase response in mouse 1 (●) and mouse 2 (○) and non-specific binding to blocking protein, 1% Marvel, in mouse 1 (■) and mouse 2 (□). Readings were obtained after 40 min development. After 2 h development the absorbances at a serum dilution of 1:62 500 were 1.676 (mouse 1) and 0.742 (mouse 2). Error bars indicate one standard error of the mean. Pre-immune serum from each mouse gave absorbances below 0.07 at 1:500 dilution.
4.2.2 Hybridomas

Spleen cells from mouse 1 were fused with mouse plasmacytoma cell line JK.Ag 8.653. The resulting hybridomas were selected in HAT medium and supernatants tested for anti-human β1,4-GalTase antibody, using a direct binding ELISA. Positive hybridomas gave absorbances greater than 0.4, which was five times above the background levels. The fusion resulted in only 5% of hybridomas which produced antibodies specific for human β1,4-GalTase (25 out of 470 hybridomas tested). The positive cell lines were re-screened a week later using a radiolabelled anti-mouse Ig (section 2.7.5) and aliquots of cell lines which showed strong reactivity (72% of the hybridomas) were frozen down. The five most strongly positive hybridomas (greater than 6.5 times above background levels) were then expanded. These hybridomas were cloned by limiting dilution and at each stage hybridoma supernatants tested for reactivity to human milk β1,4-GalTase.

4.2.3 Isotyping of anti-β1,4-GalTase mAbs

After the first round of cloning by limiting dilution the majority of the hybridomas still contained mixtures of antibodies and further limiting dilution was necessary before B6, E7, G4 and H11 were cloned. A previously selected hybridoma, B8, was omitted as the reactivity to β1,4-GalTase was weak, possibly suggesting the existence of a spontaneous non-secreting variant. All the cloned hybridomas secreted mAbs of the IgG1 isotype (Figure 4.2) with kappa light chains (Figure 4.3). The B6b, E7a, G4b and H11b clones were expanded over several weeks and aliquots frozen down. The clones were grown up in serum-free medium (section 2.1.5) in triple decker flasks up to stationary phase. Very similar levels of 1B6, 1E7, 5G4 and 1H11 IgG1 secretion were observed (mean 12.3 μg/ml IgG ± 0.48, n=4) which yielded more than 2 mg of total mAb from each flask. The mAbs were purified on separate protein-G columns, quantified and stored in aliquots at -20°C.
(a) Anti-human β1,4-GalTase mAbs and (b) total isotypes present from different hybridoma cell lines after the second cloning by limiting dilution. IgG1 (■), IgG2a (□), IgG2b (■), IgG3 (■), IgM (□□), IgA (□□). Error bars represent one SEM. Non-specific binding to blocking protein, 1% Marvel (---). (section 2.7.7).
4.2.4 Specificity of anti-β1,4-GalTase mAbs

Some contaminating proteins such as IgG and α-lactalbumin have been found in low amounts in commercially available β1,4-GalTase which could elicit an antigenic response when used as a source of immunogen (Ulrich, et al., 1986). These mAbs only reacted against β1,4-GalTase, and there was no recognition to other proteins even at the longer development time (Figure 4.4b). Human β1,4-GalTase was strongly recognized by all mAbs. 1H11 mAb also recognized bovine β1,4-GalTase to a similar extent as human β1,4-GalTase (Figure 4.4a). The longer development time showed that the reaction against human β1,4-GalTase was saturated, and that all mAbs recognized the bovine β1,4-GalTase though to different extents (Figure 4.4b). Overnight development was considered valid as the non-specific reaction observed with an irrelevant isotype control mAb remained at background levels.
Figure 4.4
Specificity of anti-β1,4-GalTase mAbs (section 2.7.8) after (a) 1 h and (b) a 16 h development. Proteins coated at 5 μg/ml: human milk β1,4-GalTase (■); bovine milk β1,4-GalTase (□); human α-lactalbumin (■); bovine α-lactalbumin (□); human IgG (□); BSA (□). One standard error of the mean is indicated.
All the mAbs were able to detect denatured β1,4-GalTase from both human and bovine milk following SDS-PAGE and transfer to nitrocellulose membranes (Figure 4.5). The reactivity of 1H11 mAb with human and bovine milk β1,4-GalTase was very similar whereas the other mAbs reacted more intensely with the human milk β1,4-GalTase (a broad band between 47-54 kDa). Further, 1H11 mAb recognized two major bands of 42 kDa and 48 kDa and a minor band of 28 kDa, in bovine milk β1,4-GalTase, whereas the other mAbs only detected a single band. 1E7 and 5G4 mAbs bound to a 48 kDa band, and 1B6 mAb bound to a 50 kDa band in bovine milk β1,4-GalTase (Figure 4.5).

As the carbohydrate structures on β1,4-GalTase are able to elicit an immune response (Childs, et al., 1986), the specificity (protein or carbohydrate) of the anti-β1,4-GalTase mAbs was investigated in Western blots, using a recombinant soluble form of human β1,4-GalTase expressed in E. coli (provided by Dr. Hubert Appert) (Aoki, et al., 1990). This enzyme was provided in an unpurified form from the periplasmic fraction of E. coli (Figure 4.6a) and had to be concentrated 10-fold before detection. All the anti-β1,4-GalTase mAbs could detect this non-glycosylated form of β1,4-GalTase as two major bands of 36 kDa and 34 kDa which was in agreement to the size of the expressed protein (Figure 4.6b) (Aoki, et al., 1990). The 34 kDa band was probably derived from the 36 kDa band through proteolysis (Aoki, et al., 1990). An excess of E. coli lysate control (>12-fold, pIN-III) was loaded onto the SDS-PAGE gel and transferred, but no background reaction was detected (Figure 4.6b). 1E7 mAb detected the major 48 kDa band in bovine milk β1,4-GalTase, but using the more sensitive ECL detection system, a minor band of 46 kDa was also apparent (Figure 4.6b, compare to Figure 4.5, lane 7).

A purified recombinant human β1,4-GalTase from S. cerevisiae (rhGalTase, provided by Prof. Eric Berger) (Malissard, et al., 1996) was analysed by SDS-PAGE and revealed considerable heterogeneity due to the hyperglycosylation at the single N-glycosylation site. The heavily glycosylated band was more apparent after silver staining than with Coomassie blue (Figure 4.7a, lanes 3 and 7). Cleavage of the N-glycans from rhGalTase with Endo H resulted in a gel shift from a high molecular diffuse band (of approximately 100-200 kDa) to a major band at 51 kDa and two bands at 43 and 42 kDa (Figure 4.7a, lanes 7 and 8). The anti-β1,4-GalTase mAbs reacted equally well with the rhGalTase before and after treatment with Endo H as shown by Western blotting (Figure 4.7b), confirming that the carbohydrate structures were not involved in the epitopes of these mAbs. Another anti-β1,4-GalTase mAb, 2/36/118 (provided by Prof. Eric Berger), only detect the deglycosylated rhGalTase, suggesting that the large N-glycans were causing steric hindrance to this epitope (Figure 4.7b). A number of low molecular weight bands (around 30 kDa and 14.3 kDa) in all the β1,4-GalTase preparations (Figure 4.7a, lanes 2-4) were detected with 1H11 mAb (Figure 4.7b).
Figure 4.5

Western blot detection (section 2.8.4) of human milk β1,4-GalTase (lanes 2-5) and bovine milk β1,4-GalTase (lanes 6-9) using anti-β1,4-GalTase mAbs: 1B6 (lanes 2 and 6); 1E7 (lanes 3 and 7); 5G4 (lanes 4 and 8) and; 1H11 (lanes 5 and 9). Molecular weight markers are indicated in kDa.
Figure 4.6
(a) Coomassie blue stain of 10% SDS-PAGE gel under reducing conditions, as used for transfer shown in (b) ECL detection of a Western blot probed with 1E7 mAb.
Bovine milk β1,4-GalTase (11 µg, lane 1); periplasmic fraction of *E. coli* expressing soluble β1,4-GalTase in pIN-GT (50 µg, lane 2); periplasmic fraction of *E. coli* containing pIN-III vector alone (607 µg, lane 3). Molecular weight markers are indicated in kDa (lane 4).
Figure 4.7
(a) 10% SDS-PAGE gel under reducing conditions was stained with silver (lanes 1-4) or Coomassie blue (lanes 5-8). Molecular weight markers in kDa (lanes 1 and 5); human milk β1,4-GalTase (lanes 2 and 6); rhGalTase from *S. cerevisiae* with (lanes 4 and 8) and without Endo H treatment (lanes 3 and 7). (b) ECL detection of a Western blot probed with anti-β1,4-GalTase mAbs. Human milk β1,4-GalTase (1 μg, lane 1); rhGalTase from *S. cerevisiae* with (1 μg, lane 3) and without Endo H treatment (1 μg, lane 2).
Figure 4.8
Fixed and permeabilized B cells were stained with 1E7 mAb and rabbit F(ab')₂ anti-mouse IgG-FITC. Cells were mounted onto glass slides and photographed under uv light.

The anti-β1,4-GalTase mAbs could also detect β1,4-GalTase present within the Golgi of fixed and permeabilized B cells (Figures 4.8 and 5.16). The staining intensity achieved with 1H11 mAb was weak, compared to that observed with 1B6, 1E7 and 5G4 mAbs, possibly indicating a less accessible epitope and/or poor binding affinity to β1,4-GalTase in situ. The overexpression of β1,4-GalTase resulted in some β1,4-GalTase localized at the cell surface of COS-7 cells (Figures 5.12a and b), though not in B cells (Figure 5.15b).
Preliminary experiments on the effect of anti-β1,4-GalTase mAbs on β1,4-GalTase activity were performed with 1E7 mAb. The mAbs were checked for exposed GlcNAc residues which could theoretically act as a competitive substrate for β1,4-GalTase in the activity assay. Only 1H11 mAb had high levels of GlcNAc, whilst all the mAbs had exposed sialic acid and galactose residues to varying extents, with very low levels detected in the isotype negative control (results not shown). No inhibitory action on β1,4-GalTase activity was found in the presence of 1E7 mAb when compared to the isotype control, which unexpectedly showed about 25% inhibition at the low levels of β1,4-GalTase (Figure 4.9). At low β1,4-GalTase activity, 1E7 mAb appeared to enhance the β1,4-GalTase activity (Figure 4.9).
A fraction of the protein-G purified mAbs were conjugated to biotin and their reactivity to β1,4-GalTase assessed (Figure 4.10). All the anti-β1,4-GalTase mAbs reacted well against human milk β1,4-GalTase though only 1H11 mAb maintained strong cross-reactivity to bovine β1,4-GalTase in this ELISA system.

4.2.5 Epitope Mapping of β1,4-GalTase

The biotinylated mAbs were used in combination with unlabelled mAbs in a direct binding competition ELISA against human milk β1,4-GalTase, and also bovine β1,4-GalTase in the case of biotinylated 1H11 mAb, to determine if distinct epitope binding regions existed (Figure 4.11).
Figure 4.11
A competition ELISA of biotinylated anti-β1,4-GalTase mAbs binding to human milk β1,4-GalTase in the presence of unlabelled anti-β1,4-GalTase mAbs 1B6 (■); 1E7 (□), 5G4 (□) and; 1H11 (□) (section 2.7.13). 1H11* was assayed against bovine milk β1,4-GalTase. 100% binding for each biotinylated mAb was obtained in the absence of other mAbs. Error bars of one standard of the mean are shown.

A decrease in the absorbance at 490 nm of greater than 10%, when incubated in the presence of unlabelled mAbs, was interpreted as competitive inhibition. The epitope(s) that 1B6, 1E7 and 5G4 mAbs were binding to on human milk β1,4-GalTase were very closely associated, as indicated by their strong inhibition of binding (>95%). Biotinylated 1H11 mAb on the other hand appeared to be interacting with a distinct epitope, as less than 10% inhibition of binding was observed in the presence of the other mAbs with either human or bovine β1,4-GalTase (Figure 4.11). Notwithstanding the binding affinity of the mAbs, this represented approximately 250-fold more IgG molecules in competition for β1,4-GalTase than biotinylated 1H11 mAb. The binding of biotinylated 1B6, 1E7 and 5G4 mAbs to β1,4-GalTase was inhibited by about 20-25% in the presence of unlabelled 1H11 mAb.
Crude preparations of β1,4-GalTase mutant proteins expressed in E. coli (provided by Dr. Hubert Appert) (Zu, et al., 1995) were used in a direct binding ELISA to determine important epitope regions on β1,4-GalTase used by the anti-β1,4-GalTase mAbs (Figure 4.12). The expression levels of the mutant β1,4-GalTase in E. coli appeared to be quite low and varied between the mutants as an overnight ELISA development was required for detection. The anti-β1,4-GalTase mAbs were raised against the soluble form of human milk β1,4-GalTase (amino acids 41-400). 1H11 mAb binding was apparently unaffected by the different β1,4-GalTase deletion mutants, indicating that the first 115 amino acids in β1,4-GalTase were not involved in its epitope. The TSSS
mutant which involved a loss of negative charge from EDDD to TSSS a few amino acids downstream from the proposed UDP-galactose binding site (Aoki, et al., 1990), appeared to show a slight decrease in binding with 1H11 mAb, though this could be due to different amounts of the mutant β1,4-GalTase preparations coated onto the ELISA plates. The other mAbs all showed decreased binding to the β1,4-GalTase mutants to various extents, suggesting some loss of epitope determinants. No cross-reaction to bovine β1,4-GalTase was seen with 2/36/118 mAb.

To further define epitope regions, Cleveland digests of purified human milk β1,4-GalTase were performed, transferred onto PVDF membranes and probed with the mAbs. It was possible to generate consistent band patterns of β1,4-GalTase following digestion with V8 protease (Figure 4.13a). Moreover, differential binding patterns of the anti-β1,4-GalTase mAbs to these β1,4-GalTase fragments was observed (Figure 4.13b). Unfortunately, attempts at N-terminal protein sequencing of the relevant excised bands were unsuccessful.
Figure 4.13
Cleveland digests of human milk β1,4-GalTase with V8 protease (section 2.8.6).
(a) Coomassie blue stained SDS-PAGE gel. (b) Western blot probed with 1H11 mAb. (c) Western blots probed with mAbs 2/36/118, 1E7, 1H11 and IgG1 isotype control. Molecular weight markers in kDa (lane 1); 10 μg soluble β1,4-GalTase (lane 2); 10 μg β1,4-GalTase excised gel band (lane 3); 25 μg β1,4-GalTase excised gel band with 10 μl V8 protease at 0.5 mg/ml (lane 4); 10 μl V8 protease at 0.5 mg/ml (lane 5).
4.2.6 Kinetics of anti-β1,4-GalTase mAbs binding to β1,4-GalTase

These experiments were performed using surface plasmon resonance (SPR, in collaboration with George Panayotou at the Ludwig Institute, London). This approach monitored the association (k\text{ass}) and dissociation (k\text{diss}) rate constants of immobilized mAb with soluble β1,4-GalTase over real time (see section 2.7.14) (Figure 4.14). Different types of purified β1,4-GalTase protein were used in the SPR measurements and the kinetic data calculated using the BIAcore software (Pharmacia) (Table 4.1). When human milk β1,4-GalTase or rhGalTase (Endo H-treated) were used, the binding did not reach equilibrium and the k\text{diss} was much slower than that observed for the other proteins. The k\text{ass} data could not be fitted for the rhGalTase (Endo H-treated) binding curves and the k\text{diss} data fitted best, though poorly, with human milk β1,4-GalTase and rhGalTase (Endo H-treated) using a two-site binding model where the majority (>85%) of the sites had slow dissociation rate constants (in the 10^{-4} \text{s}^{-1} range).

The anti-β1,4-GalTase mAbs were all of a moderately high affinity (binding constant, K\text{B}, approximately 10^8 \text{M}^{-1}) when interacting with human milk β1,4-GalTase. The rhGalTase preparations and bovine milk β1,4-GalTase all resulted in lower affinities with all the mAbs when compared to human milk β1,4-GalTase. These lower binding constants were mainly attributable to much higher k\text{diss} rates than changes in the k\text{ass}, such as the estimated 80-fold increase in k\text{diss} calculated for 1E7 and 5G4 mAbs with the rhGalTase. 1E7 and 5G4 mAbs had k\text{ass} and k\text{diss} rate constants which were very similar to each other when tested against all the different types of β1,4-GalTase. In fact the K\text{B} values for the mAbs were always in a similar range for each of the β1,4-GalTase proteins assayed. However, the K\text{B} ranking of 1H11 mAb among the other mAbs increased when measured with bovine milk β1,4-GalTase and the N-deglycosylated rhGalTase.
Table 4.1
Summary of anti-\(\beta_1,4\)-GalTase mAbs association (\(k_{\text{ass}}\), M\(^{-1}\) s\(^{-1}\)) and dissociation (\(k_{\text{diss}}\), s\(^{-1}\)) rates with different \(\beta_1,4\)-GalTase preparations. The binding constant (\(K_B\), M\(^{-1}\)) indicates the relative affinity of the mAbs. Human milk \(\beta_1,4\)-GalTase (hGalTase), recombinant human \(\beta_1,4\)-GalTase expressed in \(S.\ cer\)e\(v\)i\(s\)i\(a\)e\(e\) (rhGalTase), rhGalTase treated with Endo H (rhGalTase\(^a\)), rhGalTase that has been mutated to remove the N-glycosylation site, N-deglycosylated rhGalTase, (rhGalTase\(^b\)) and bovine milk \(\beta_1,4\)-GalTase (bGalTase). nf, data could not be fitted.

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Figure 4.14
An overlay of four separate sensorgrams showing immobilized anti-β1,4-GalTase mAbs interacting with soluble rhGalTase. The rhGalTase was injected (between the two arrows) and associated with the mAb, then as the rhGalTase injection was replaced with just buffer (downward arrow) the dissociation rates of mAb-rhGalTase complexes were measured.
Figure 4.15
Human milk β1,4-GalTase (15 ng/ml) was measured in the quantification assay (section 2.8.1). Biotinylated anti-β1,4-GalTase mAbs 1B6 (■, 2.5 μg/ml), 1H11 (□, 2.5 μg/ml) or a combination of 1B6 and 1H11 (■, each at 1.25 μg/ml) were bound onto streptavidin-coated ELISA plates. The captured β1,4-GalTase protein was detected with an affinity-purified rabbit anti-β1,4-GalTase protein antibody. Non-specific binding with an irrelevant biotinylated isotype control mAb (2.5 μg/ml, dotted line). Error bars show one standard error of the mean.

4.2.7 Quantification of β1,4-GalTase protein
The basic quantification of β1,4-GalTase protein was achieved using a two-site ELISA. A capture mAb, 1B6, was coated onto one half of an ELISA plate, with an irrelevant isotype control mAb, MOPC-21 IgG1, coated on the other half. Following the addition of β1,4-GalTase standards and samples, biotinylated 1H11 mAb was added and the assay developed using a streptavidin-HRP conjugate. Modifications were made to the assay to accommodate smaller assay volumes and increase the sensitivity. Instead of coating the mAbs directly onto the plastic ELISA plates, biotinylated mAbs were added to streptavidin coated plates. This was to provide for a stronger binding of the mAbs within the ELISA plate, with the aim of increasing the reproducibility and sensitivity of the assay. Further, synergistic binding to β1,4-
GalTase was observed when biotinylated anti-β1,4-GalTase mAbs, 1B6 and 1H11, were jointly bound to the streptavidin-coated ELISA plates (Figure 4.15). The captured β1,4-GalTase could then be detected with a rabbit anti-β1,4-GalTase antibody. Various efforts were made to enhance the detection signal, including the use of a streptavidin-peroxidase polymer, however, the maximal signal: noise ratio was achieved using a directly conjugated goat anti-rabbit IgG-peroxidase antibody. Different coating buffers, antibody concentrations and incubation times were also optimized for the quantification of β1,4-GalTase protein ELISA. The assay proved to be sensitive down to 1 ng/ml of β1,4-GalTase protein (50 pg per well) and very reproducible (Figure 4.16).
Figure 4.17
Quantification of β1,4-GalTase protein in B cell lysates from the second study (section 2.1.1) of RA patients (n=12, black circles) and controls (n=12, white circles). B cell lysates from 2B6 (white triangles) and JAC-10 (black triangles) cell lines were assayed for β1,4-GalTase protein at three time points over a one month period in culture. Error bars indicate one standard error of the mean.

The interplate variation was between 1.3-6.1% cv using β1,4-GalTase protein concentrations between 0-60 ng/ml, with an average cv of 3.11% ± 1.81.

This ELISA was able to quantify β1,4-GalTase protein from various cell lysates and also secreted soluble forms found in cell culture supernatants (Figures 4.17, 5.11 and 5.17). The basis for the decreased β1,4-GalTase activities found in B cells from RA patients is unknown, although it has been suggested that a reduced affinity for UDP-galactose may be a contributing factor (Furukawa, et al., 1990). B cell lysates from the same samples used in the second study of RA patients and controls (see section 2.1.1 and chapter 3) were assayed for β1,4-GalTase protein using the quantification ELISA (section 2.8.1, Figure 4.17). The line of best-fit was determined for the standard curve using linear regression and the regression equation used to calculate β1,4-GalTase protein in the samples (InStat, GraphPad Software, San Diego, CA, USA). Very low
levels of B cell β1,4-GalTase protein were detected in the samples (mean 2.32 ± 0.98 sd and; mean 1.47 ± 0.78 sd ng/ml in 12 controls and 12 RA patients respectively), due to limited amounts of material available. These β1,4-GalTase protein values were finally expressed over the total protein in the B cell lysates assayed (Figure 4.17). No statistically significant difference was observed between the amounts of B cell β1,4-GalTase protein from the control and RA patients. Neither was there any correlation between the expression levels of β1,4-GalTase protein and β1,4-GalTase activity in either the control or RA groups. EBV-transformed B cell lines, 2B6 and JAC-10, had significantly higher levels of β1,4-GalTase protein than the peripheral blood B cells from the controls and RA patients (p<0.0006 and p<0.015 for the 2B6 and JAC-10 cell lines respectively). When investigating B cells transfected with the human β1,4-GalTase cDNA, a 2.5:1 ratio of cellular β1,4-GalTase protein:β1,4-GalTase activity was observed (see chapter 5). These transfectants also secreted a similar ratio of β1,4-GalTase protein (3.0 ± 0.79 and 0.6 ± 0.35 ng β1,4-GalTase protein/10^6 viable cells/day for the sense and antisense transfected B cells respectively).
4.3 Discussion

Four anti-β1,4-GalTase mAbs have been produced and extensively characterized enabling their subsequent use in a number of applications including ELISA, Western blotting, SPR and immunofluorescence.

5% of anti-β1,4-GalTase specific hybridomas resulted from the fusion. Similar results have been reported elsewhere (Berger, et al., 1986). All four anti-β1,4-GalTase mAbs reacted with human and also cross-reacted to bovine milk β1,4-GalTase. In the direct binding ELISA the signal obtained against β1,4-GalTase from both species was very similar in the case of 1H11 mAb, although the other mAbs reacted preferentially with human milk β1,4-GalTase. The apparent loss of signal against bovine milk β1,4-GalTase with all the biotinylated mAbs except 1H11 mAb was most probably due to different assay development times as a result of alternative detection systems used (alkaline phosphatase or peroxidase, compare Figures 4.4 and 4.10). The Western blot clearly showed 1H11 mAb binding to proteins of approximately 28 kDa and 42 kDa, as well as to a similar sized protein (48 kDa) as 1E7 and 5G4 mAbs when using purified bovine milk β1,4-GalTase (Figure 4.5). Only 1B6 mAb detected a separate single band of approximately 50 kDa in the bovine milk β1,4-GalTase. If this 50 kDa polypeptide is processed to yield 48 kDa, 42 kDa, and other smaller molecular fragments, then it is unclear why the mAbs which bound to these lower molecular weight proteins did not also bind to the 50 kDa protein. It may be, though unlikely, that the epitopes for 1E7, 5G4 and 1H11 mAbs only become apparent following proteolysis of the high molecular protein. Alternatively, another β1,4-GalTase gene encoded the β1,4-GalTase protein which only 1B6 mAb recognized. It is unclear if the β1,4-GalTase found in milk is encoded by a single gene. The milk β1,4-GalTase enzymes were purified from pooled milk samples, thus increasing the likelihood of some heterogeneity. Western blots of purified human milk β1,4-GalTase showed very similar binding patterns of the anti-β1,4-GalTase mAbs to proteins of 47-54 kDa, although 1H11 mAb also bound a number of lower molecular weight proteins (Figure 4.5). The lower molecular weight bands were more apparent using ECL detection in the Western blots (14.3 kDa and 29 kDa, Figure 4.7b, lane 1), and could also be seen in the protein gel of highly purified β1,4-GalTase when stained with silver (Figure 4.7a, lane 1), suggesting degradation products.

The anti-β1,4-GalTase mAbs were found to be protein-specific as assessed by their reactivity to recombinant human β1,4-GalTase expressed in E. coli. Periodate oxidation of β1,4-GalTase did not result in any significant changes in anti-β1,4-GalTase mAbs binding (results not shown). This was further confirmed when the large N-glycans on rhGalTase, expressed in S. cerevisiae, were shown to have no effect on the binding of
the anti-β1,4-GalTase mAbs, with the exception of 2/36/118 mAb. The single N-linked glycosylation site in β1,4-GalTase is located in the stem region, the most heterogeneous region in β1,4-GalTase between species. 2/36/118 mAb did not cross-react with bovine β1,4-GalTase, which has two N-linked glycosylation sites, and was precluded from binding to heavily glycosylated rhGalTase, would suggest the oligosaccharides were masking the epitope, possibly located within the stem region (see below).

A slightly higher molecular weight species was observed in the human milk β1,4-GalTase sample (53 kDa) compared to the Endo H treated rhGalTase (51 kDa), which was also apparent in the immunoblots. This was probably due to the four extra amino acids in the N-terminal sequence of the human milk β1,4-GalTase compared to rhGalTase (starting from positions 41 and 45 respectively, see Figure 1.3), and possibly extra glycosylation and other post-translational modifications. The heterogeneity of the purified rhGalTase protein was partially due to its extensive N-linked glycosylation, in common with other glycoproteins when expressed in *S. cerevisiae*. Charge heterogeneity has previously been shown to involve both N- and O-linked glycosylation on rhGalTase (Malissard, et al., 1996). Further, silver stained protein gels revealed a larger degree of heterogeneity in these purified rhGalTase preparations, notably in lower molecular weight protein bands (mostly at 14.3, 17 and 29-33 kDa, Figure 4.8a, lanes 3 and 4). Most of these protein bands were detected in the immunoblot with IH11 mAb following ECL (Figure 4.8b, lanes 2 and 3). Some of these immunoreactive proteins were also common to the purified human milk β1,4-GalTase lane suggesting low molecular weight degradation products of β1,4-GalTase, possibly due to the fact that these proteins had undergone two freeze-thaw cycles (Figure 4.8b, lane 1).

The crystal structure of β1,4-GalTase or indeed any mammalian glycosyltransferase is unknown. However, the predicted secondary structure for all Golgi glycosyltransferases cloned to date indicate a type II membrane-bound protein with a short cytosolic amino terminal tail, anchored in the membrane by a stretch of approximately 20 hydrophobic amino acids, followed by a long stem region containing site(s) for N-linked glycosylation and terminating in the large globular catalytic domain (Paulson and Colley, 1989). The areas of highest homology (>90%) in β1,4-GalTase between the species lies within the functional domains. It would be reasonable to predict a similar overall structure between human and bovine β1,4-GalTase. Differences in sequence, even by one amino acid, may have an influence on antibody binding whilst the overall structure would probably be maintained. Hence, to a certain extent it is possible to identify amino acids which are important in determining immunodominant epitopes using mAbs.
None of the other mAbs were able to inhibit 1H11 mAb binding, indicating that the epitope recognized by 1H11 mAb on human and bovine β1,4-GalTase was segregated from those recognized by 1B6, 1E7 and 5G4 mAbs which all competed with each other for binding. The 20-25% inhibition exhibited by 1H11 mAb on the other mAbs binding to β1,4-GalTase may have been due to a slight steric hindrance or a conformational change in the epitopes for 1B6, 1E7 and 5G4 mAbs following 1H11 mAb interaction. Further, these epitopes were more likely to be continuous rather than discontinuous epitopes as β1,4-GalTase which had been reduced, SDS-treated and boiled then separated by SDS-PAGE and transferred to nitrocellulose was still recognized by all the anti-β1,4-GalTase mAbs. However, limit renaturation of protein following electrophoresis onto nitrocellulose may still be possible. The anti-β1,4-GalTase mAbs were raised against the soluble human milk β1,4-GalTase and hence were unlikely to react to any sequences in the amino terminal up to Leu^1 cleavage site, unless a repetitive epitope existed. The anti-β1,4-GalTase mAbs bound to the E. coli β1,4-GalTase deletion mutants in ELISA, though binding was apparently diminished especially with the rhGalTase. However, as the β1,4-GalTase mutant proteins were present in low amount in an unpurified form it was not possible to quantify any loss in binding. When the small amount of sample containing rhGalTase was concentrated (>10x) and assessed by Western blotting, the anti-β1,4-GalTase mAb binding could be detected using ECL. The fact that 1H11 mAb always reacted strongly with the β1,4-GalTase mutants in immunoblotting and ELISA suggested that sufficient β1,4-GalTase protein was available for detection. Qualitatively, it would appear that the binding of 1H11 mAb was unaffected by the deletion of the first 115 amino acids in β1,4-GalTase, whereas 2/36/118 and 5G4 mAbs were greatly affected by all the mutants. 1E7 mAb exhibited a similar binding to 5G4 with the β1,4-GalTase mutants except with TSSS (rhGalTase which included a four point amino acid substitution in the catalytic domain at residues 315-318), where 1E7 gave a stronger signal. These results suggest that the epitope for 2/36/118, 5G4 and 1E7 were within amino acids 41-115 of β1,4-GalTase, with the 1E7 mAb epitope biased towards the carboxyl end of this domain. 1B6 mAb had a slightly stronger signal than the other mAbs (excluding 1H11 mAb) to rhGalTase and was less affected by the rhGalTase mutant. Taken together with the competition ELISA results, 1B6 mAb appeared to be binding to an epitope partially overlapping 5G4 and 1E7 mAbs which extended further downstream than amino acid 116. Thus the data suggested that 5G4, 1E7 and 1B6 mAbs were binding to non-identical, though overlapping epitopes, whilst 1H11 mAb bound to a distally distinct epitope in an area that has homology with bovine milk β1,4-GalTase. It was unclear what the N-terminal amino acids were in the bovine milk β1,4-GalTase preparation as the sample was not sufficiently pure. Two cleavage sites in bovine milk β1,4-GalTase at amino acids 79 and 96 have been reported in the literature, however as more
molecular weight species have been found there are likely to be other sites. One potential site might be akin to the transmembrane cleavage site used in human milk β1,4-GalTase, where homologous sequences exist. Although these *E. coli* β1,4-GalTase mutants were all enzymatically active, indicating correct overall folding, there may be slight distortions in the protein structure (see the rhGalTase SPR data), or clipping of the protein (Aoki, et al., 1990) which could deleteriously affect some epitopes. Attempts were made to identify β1,4-GalTase fragments containing relevant epitopes using Cleveland digests in conjunction with immunoblotting. As the human milk β1,4-GalTase had been processed by V8 protease, the amino terminal end should have been suitable for sequencing. The lack of N-terminal sequence signals from the excised protein bands was probably due to insufficient protein. The anti-β1,4-GalTase mAbs binding profiles observed with the digested β1,4-GalTase further supported distinct epitopes for 2/36/118, 1E7 and 1H11 mAbs (Figure 13b). Another approach to identify epitope binding domains may be to use overlapping peptides particularly of the sequences found in the stem region.

The binding of anti-β1,4-GalTase mAbs to fixed and permeabilized B cells resulted in characteristic Golgi staining, the subcellular location of β1,4-GalTase, indicating that the epitopes on β1,4-GalTase were readily accessible in situ. However, the epitope recognized by 1H11 mAb may have been less accessible as weaker Golgi staining was observed. The epitope may either be hidden by the folding of β1,4-GalTase (possibly dimer formation) or other proteins in the Golgi location are interacting with β1,4-GalTase and masking the pertinent epitope. As 1H11 mAb bound soluble bovine milk β1,4-GalTase to a similar degree as soluble human milk β1,4-GalTase, the epitope would be predicted to lie in a homologous region. It would therefore be interesting to assess 1H11 mAb binding to β1,4-GalTase in bovine B cells to establish if the epitope is efficiently presented and thus provide some further information on how β1,4-GalTase is folded in vivo in different species. Qualitative and quantitative measurements of β1,4-GalTase are possible with these epitope-specific monoclonals which, with the likelihood of β1,4-GalTase crystals in the near future, will also prove useful in structural studies.

1E7 mAb was found to enhance β1,4-GalTase activity at low concentrations of β1,4-GalTase. The epitope mapping of β1,4-GalTase would suggest that 1E7 is binding in the stem region of β1,4-GalTase which may stabilize the orientation of β1,4-GalTase and enhance its catalytic activity. No β1,4-GalTase inhibition was seen with 1E7 mAb when compared to the isotype control, further supporting a lack of interaction with the...
predicted UDP-galactose, GlcNAc or Mn^{2+} binding sites, present in the carboxyl end of β1,4-GalTase (Aoki, et al., 1990), regions of homology between species. The reason for the observed inhibition with the isotype control in the β1,4-GalTase activity assay was unclear as this mAb did not recognize β1,4-GalTase neither were there significant amounts of exposed GlcNAc residues on this mAb which could potentially act as a competitive substrate. It remains to be determined if this isotype control was interacting with another component of the β1,4-GalTase activity assay, such as the acceptor substrate. One report showed a variable effect of anti-GalTase mAbs (including the 2/36/118 mAb) on β1,4-GalTase activity over time (Berger, et al., 1986). After two days incubation there was a decrease in activity, whereas a 9 day incubation led to an increased β1,4-GalTase activity. The assay system was fairly crude in that hybridoma supernatants were used, containing variable amounts of mAbs, and other glycoproteins, including β1,4-GalTase (which had been heat-inactivated). Hence, in this assay the mAbs could have been binding to the β1,4-GalTase in the hybridoma medium, and the active β1,4-GalTase could have been binding to exposed GlcNAc residues on the other glycoproteins present as well as any present on the mAbs, consequently these results were difficult to interpret. Other mAbs against bovine milk β1,4-GalTase did not inhibit β1,4-GalTase activity (Ulrich, et al., 1986). Approximately 40% inhibition of bovine milk β1,4-GalTase activity was observed in the presence of a polyclonal anti-β1,4-GalTase (Figure 3.4). It is unlikely that the antibodies in the polyclonal preparation are directly interacting with the active site, which is thought to be a hydrophobic pocket, thus unlikely to be surface exposed and hence a poor antigenic site. Rather, the interaction at several epitopes regions is probably having a cumulative effect, possibly though a conformational change, resulting in the inhibition of β1,4-GalTase activity.

It may be interesting to investigate further the differences in the anti-β1,4-GalTase mAbs glycosylation. If these mAbs also cross-reacted to murine β1,4-GalTase then they may be interacting with β1,4-GalTase as they transit through the Golgi of the hybridomas during their synthesis. Such an interaction may influence the galactosylation of anti-β1,4-GalTase mAbs.

The highest affinity binding constants, measured by SPR, for all the anti-β1,4-GalTase mAbs were observed with human milk β1,4-GalTase (10^8 M^{-1}) followed by bovine milk β1,4-GalTase (10^7 M^{-1}) and then the rhGalTase (with or without N-glycosylation, 10^6 M^{-1}). In some instances, similar K_{B} were observed between the anti-β1,4-GalTase mAbs, such as the binding of 5G4 and 1H11 to rhGalTase, yet there were 5-fold differences in their K_{ass} and k_{diss} values. The change in K_{B} among the mAbs were due primarily to increased k_{diss} rates. Similar characteristics between the rhGalTase protein and human milk β1,4-GalTase have been reported (Malissard, et al., 1996). However,
small differences between the β1,4-GalTase enzymes were observed, such as the increase in $K_m$ for GlcNAc of 2.5-3-fold seen with N-deglycosylated rhGalTase and rhGalTase respectively and a 50% decrease in the $K_m$ for glucose (in the presence of 1 mg/ml α-lactalbumin) suggesting that subtle changes in the folding of β1,4-GalTase were slightly affecting the acceptor binding domains. Such differences along with a modest shift in circular dichroic spectra in N-deglycosylated β1,4-GalTase compared to the human milk β1,4-GalTase (Malissard, et al., 1996) may have influenced the high $k_{diss}$ observed with the anti-β1,4-GalTase mAbs. Similar $K_B$ for the anti-β1,4-GalTase mAbs were seen with both rhGalTase and the N-deglycosylated rhGalTase which further supports the lack of involvement of N-glycans in the interaction of the mAbs. It was perhaps significant that the $K_B$ obtained with bovine milk β1,4-GalTase was greater than that with the rhGalTase proteins. Whilst glycosylation of β1,4-GalTase has been shown to be unnecessary for β1,4-GalTase activity *per se*, the correct glycosylation of β1,4-GalTase may be involved in subtle structural orientation which permits optimal enzymatic activity.

The binding data would not always mathematically fit to a simple one or two site binding equation. No $k_{ass}$ data could be fitted for the Endo H-treated rhGalTase experiments. There were several possible explanations, though without further biochemical analysis, the real reason(s) will remain unclear. It may be that the preparations should have been further purified following digestion with Endo H in order to prevent any potential interaction between the released oligosaccharides and the β1,4-GalTase protein. One possibility was that the β1,4-GalTase protein was self-associating and dissociating into aggregates. The soluble forms of rhGalTase and N-deglycosylated β1,4-GalTase have been suggested to exist as dimers in solution (Malissard, et al., 1996). Hence, the possibility of larger β1,4-GalTase aggregates existing and resulting in epitopes being hidden or exposed as the complexes form or dissociate may be envisaged. The response signal obtained by the mAb binding a single epitope on β1,4-GalTase may be overestimated if the β1,4-GalTase protein had self-associated into a large aggregate. Further, distortions in the dissociation rates may result from large aggregates of β1,4-GalTase forming, where a type of 'rolling' of epitopes in the aggregates may be conceived in this fluidic system. Some of the epitopes in the aggregate would be accessible whilst others were inaccessible, but all the time the aggregate would still be bound to the mAb resulting in a prolonged response signal. Another possibility is that the folding of the enzyme is such that the epitopes are not completely accessible to the mAbs.

The similar $K_B$ with human and bovine milk β1,4-GalTase for 1H11 mAb, was reflected in the fact that this mAb could clearly detect these two proteins to similar extents in ELISA and immunoblotting. However, it is not correct to compare $K_B$
obtained in an SPR set-up with other assay systems. It should be borne in mind that the β1,4-GalTase in the SPR experiments was a purified soluble form. In a direct binding ELISA, β1,4-GalTase is coated directly onto the ELISA plate, which can result in a significant amount of protein being denatured and which may therefore affect the orientation of some epitopes.

A sensitive and reproducible ELISA system was developed for the quantification of β1,4-GalTase protein from cell lysates and soluble secreted β1,4-GalTase. This assay used 1B6 and 1H11 mAbs which bound to non-competitive epitopes on β1,4-GalTase. Enhanced sensitivity was achieved with both biotinylated 1B6 and 1H11 mAbs linked to streptavidin-coated ELISA plates, probably due to a decrease in the dissociation rates. Others have shown that orientation of mAbs using protein-G or anti-Ig antibodies enhanced sensitivity of a similar assay specific for hCG, although in this instance streptavidin capture of biotinylated synergistic antibody pairs was not advantageous (Klonisch et al., 1996). The other advantage in having two anti-β1,4-GalTase mAbs of different epitope specificities may be to capture and quantify β1,4-GalTase protein which might only express one of these epitopes, possibly as a result of processing. Even though there was no correlation between total amount of protein and β1,4-GalTase protein, it was estimated that future studies would require approximately 3 x 10^5 cells per assay well for the detection of β1,4-GalTase protein in B cells. On average only 12 μg of total protein was assayed per well of the ELISA from the controls and RA patients, compared to 26 μg of total protein used from the B cell transfectants. Further improvements in the assay sensitivity, short of using higher affinity mAbs, might be achieved using chemiluminescence detection.

The low levels of β1,4-GalTase protein detected in the B cells from controls and RA patients should be regarded as preliminary data, as the values obtained were at the limit of sensitivity of the assay. This data suggests that the reduction in β1,4-GalTase activity observed in RA B cells was not due to a reduced amount of β1,4-GalTase protein within these cells. This would imply that the β1,4-GalTase present in the RA B cells was qualitatively different from that in the control B cells. It was unlikely that the quantification ELISA could distinguish between active and inactive β1,4-GalTase or different post-translational modifications, which may partially explain the lack of correlation between β1,4-GalTase protein and activity levels in either the RA or control groups. The relationship between β1,4-GalTase protein expression levels and activity is discussed further in chapter 5.
5. Regulation of Cellular $\beta_{1,4}$-Galactosyltransferase Activity using Antisense and Sense Nucleic Acids
5.1 Introduction

β1,4-GalTase is regarded as a housekeeping gene since it is involved in the biosynthesis of glycans passing through the Golgi in almost all eukaryotic cells. However, variable expression levels of β1,4-GalTase are found depending upon the cell type, and the cell cycle, differentiation and developmental stage (Lopez, et al., 1989; Pouncey, et al., 1991; Roth et al., 1991; Shaper, et al., 1994; Kudo and Narimatsu, 1995; Cai, et al., 1996; Rajput, et al., 1996). Further, active forms of β1,4-GalTase have been described not only in the Golgi, but also on the plasma membrane and as a secreted soluble form in various extracellular fluids (Strous, 1986; Shur, 1991). It is unlikely that β1,4-GalTase galactosylates substrates outside the Golgi in vivo, due to insufficient concentrations of UDP-galactose available, but it could act as a lectin. Correct compartmentalization of β1,4-GalTase, UDP-galactose, appropriate cations and acceptor substrates within the Golgi are all necessary though not always sufficient for effective β1,4 galactosylation. There are a number of factors which could potentially influence the galactosylation process such as the conformation of the acceptor substrate (Narasimhan, et al., 1985), substrate secretion rates, β1,4-GalTase modifier protein (α-lactalbumin), post-translational modifications on β1,4-GalTase, as well as the expression levels of donor substrate and β1,4-GalTase. Experiments described in this chapter investigated the different levels of B cell β1,4-GalTase expression and its relationship to IgG galactosylation.

IgG has a conserved N-linked glycosylation consensus site in the Cy2 region at Asn\textsuperscript{297} occupied by complex type oligosaccharides. Even a mAb derived from a single B cell clone can possess about 30 different glycoforms due to the presence of differences in terminal sugars, bisecting GlcNAc and branching fucose residues. The ratios of the different glycoforms in IgG remain constant for an individual, though well documented changes were observed with age (Parekh, et al., 1988b). Low IgG galactosylation was apparent in babies and young children, with maximal levels of galactose seen at around 30 years old, thereafter there was a steady reduction in the amount of galactose found on IgG with age, which resulted in an increased proportion of IgG glycoforms terminating in GlcNAc. An increased frequency of hypogalactosylated IgG (increased IgG G0 glycoforms) have been reported in a restricted number of diseases including RA, juvenile chronic arthritis, Crohn's disease and tuberculosis (Parekh, et al., 1989). Apart from being a useful aid as a diagnostic marker (Young, et al., 1991), IgG G0 has been shown to have significantly reduced binding to C1q and to FcγRI (Tsuchiya, et al., 1989). Moreover, the IgG G0 glycoform has been implicated in the pathogenesis of RA (Rademacher, et al., 1994). The mechanism of IgG G0 production is unknown. Whilst a reduction in β1,4-GalTase activity has been noted in the B cells of RA patients using
certain acceptor substrates (see chapter 3), it is unclear whether this reduction is the 
cause of the hypogalactosylated IgG seen in these patients. A recent report suggested 
that the β1,4-GalTase activity levels expressed in different B cell lines did not reflect 
the galactosylation profiles on the secreted IgG (Kumpel, et al., 1994).

Experiments were designed to establish different β1,4-GalTase activity levels within the 
same B cell line and investigate their subsequent IgG galactosylation states. As no 
specific inhibitors of β1,4-GalTase were available, an antisense approach was used to 
specifically regulate the amount of B cell β1,4-GalTase expressed. Oligonucleotides 
(oligos) which are complementary to the mRNA of interest are known as antimessenger 
or antisense. The effect of adding antisense to mRNA has resulted in the inhibition of 
discrete gene expression. Oligodeoxynucleotides with phosphorothioate linkages (S-
oligos), which are more resistant to nucleases, have been shown to specifically 
hybridize to their target mRNA forming DNA-RNA duplexes. Specific cleavage of the 
RNA portion of these complexes by RNase H nuclease prevented translation of the 
sense transcripts and consequently suppressed the expression of that particular gene 
(Wagner et al., 1993). However, the complete mechanism(s) involved in antisense 
remain to be fully elucidated but may be multiple including transcriptional control, 
inhibition of translation and inhibition of splicing (Leonetti et al., 1993). Studies 
described in this chapter also inhibited β1,4-GalTase expression by the transfection of 
veectors expressing the β1,4-GalTase cDNA in the antisense orientation. In this 
approach, the presence of antisense β1,4-GalTase RNA would have hybridized with the 
steady-state level of target sense β1,4-GalTase transcripts thus blocking their translation 
(Melton, 1985). In order to establish B cells with different expression levels of β1,4-
GalTase, other cells were transfected with the β1,4-GalTase cDNA in the sense 
orientation.
5.2 Results

5.2.1 β1,4-GalTase antisense S-oligos in PBMC cultures

Initial experiments aimed at altering cellular β1,4-GalTase activity levels were performed using antisense S-oligos. These S-oligos consisted of short sequences (15 and 21 mers) complementary to regions within exon 1 of the human β1,4-GalTase gene. Specifically, those bases encoding the initiation start sites and the transmembrane spanning domain were targeted. As a control, S-oligos containing the respective sense sequence of bases for the target area were included. Isolated human PBMCs from healthy individuals were cultured in the presence of these antisense S-oligos and assayed for β1,4-GalTase activity. Despite varying different culture conditions no significant decrease in β1,4-GalTase activity was observed in the PBMC cultures (Figure 5.1).

![Figure 5.1](image_url)

Isolated PBMC (2.5 x 10^6 cells/ml) were cultured for 48 h in the presence of various concentrations of antisense (red bars) or control, sense (green bars), 15 mer S-oligos directed against the coding region for the transmembrane domain (section 2.10.4). β1,4-GalTase activity was assayed from these cell lysates. Results are the mean of two independent cultures each assayed in duplicate, with one standard error of the mean.
5.2.2 Cellular β1,4-GalTase activity during cell culture

Short-term culture of isolated PBMC was shown to stimulate β1,4-GalTase activity (Figure 5.2). This effect was observed in PBMC from different individuals as well as in PBMC populations which had undergone monocyte depletion by adherence to tissue culture plastic flasks (results not shown). As a stable, basal β1,4-GalTase activity was required for further studies, an established B cell line, JAC-10, was tested (Figure 5.2). The β1,4-GalTase activities of peripheral B cells may be affected following transformation in some cases (Wilson, et al., 1993) but not others (Furukawa, et al., 1990). The β1,4-GalTase activities measured in the JAC-10 EBV-transformed B cell fell within the range obtained from normal peripheral blood B cells.

![Figure 5.2](image_url)

**Figure 5.2**

Isolated PBMC (1 x 10^6 cells/ml, black circles) and B cell line, JAC-10, (0.5 x 10^6 cells/ml, white circles) were cultured for the times indicated and assayed for β1,4-GalTase activity following cell culture (1 x 10^6 cells/ml). Results are the mean of two independent cultures each assayed in duplicate, with one standard error of the mean.
Figure 5.3
15 x 10^6 JAC-10 cells were cultured at various densities and harvested 48 h later. 2 x 10^6 JAC-10 cells were lysed and aliquots assayed for β1,4-GalTase activity. Error bars indicate one standard error of the mean.

The β1,4-GalTase activity observed in this B cell line was much more constant than those achieved with the PBMC cultures. There was a gradual decrease in β1,4-GalTase activity and β1,4-GalTase protein (results not shown) in the B cell line following continuous cell culture over several months, though relatively stable levels were maintained even at different cell densities (Figure 5.3).

5.2.3 Transfection of β1,4-GalTase antisense S-oligos in B cell cultures
Liposomes were used to improve the uptake and thus the intracellular concentration of S-oligos, prior to cell culture (Bennett et al., 1992; Wagner, 1994). Lipofectin which is a cationic mixture of DOTPMA and DOPE lipids was mixed with the negatively-charged DNA to form lipid/DNA complexes with an overall positive charge. These complexes were then transfected into cells, which occurs via electrostatic forces and lipid diffusion, through the lipid bilayer of the cell plasma membrane.
EBV-transformed B cell lines, 2B6 and JAC-10 (2 x 10^6 cells/ml), were transfected with 15 mer S-oligos using Lipofectin. Antisense (red bars), sense (green bars) or no S-oligos (blue bars) were transfected into B cell lines for 6 h, cultured a further 48 h then cells were assayed for β1,4-GalTase activity. Error bars indicate one standard error of the mean.

However, B cell lines transfected with the sense and antisense S-oligos using Lipofectin, showed no statistical differences in their β1,4-GalTase activities (Figure 5.4). There was a non-specific reduction in β1,4-GalTase activity in the JAC-10 B cells cultured in the presence of S-oligos compared to no S-oligos, which was not due to cytotoxicity as all cultures were more than 95% viable.

The delivery of the S-oligos into cells was checked by transfection of antisense S-oligo-fluorescein (5-IAF, see section 2.10.5) into the JAC-10 B cell line. These cells were examined under a uv microscope and the majority showed bright staining, indicating that the S-oligos were effectively taken up by the B cells (see Figure 5.5).
Figure 5.5
JAC-10 B cells were transfected with antisense S-oligo 5-IAF using Lipofectin and photographed under UV light, 6 h post-transfection.

Figure 5.6
2.5 x 10⁶ cells/ml JAC-10 B cells were transfected with 8 μM antisense (red bars) S-oligo 5-IAF or without S-oligo 5-IAF (blue bars) for the times indicated and cultured for a further 48 h. Cells were lysed and assayed for β1,4-GalTase activity. Error bars indicate one standard error of the mean. * denotes a significant statistical difference of p<0.05 as determined by a Student's t-test.
Whilst the S-oligos appeared to be endocytosed by the transfected B cells, the inhibitory effect they had on the β1,4-GalTase activity was minimal (Figure 5.6). Specific hybridization of the antisense S-oligo to the sense S-oligo and to total mRNA isolated from JAC-10 cells was verified by dot blot analysis using 5' end labelling of the S-oligos as the probes (results not shown). Due to a number of other potential problems associated with antisense S-oligos such as their effective targeting and intracellular concentration as well as their expense, a vector approach was used.

5.2.4 β1,4-GalTase antisense/sense vector constructs
Vector constructs containing the full-length human β1,4-GalTase cDNA in the sense and antisense orientation were made (Figure 5.7), transfected into cells, which were later assayed for β1,4-GalTase activity. Clone CT7-J20 (kindly given by Dr. Michiko Fukuda, La Jolla, CA, USA) containing the full-length human β1,4-GalTase cDNA in Bluescript KS was digested with Hind III/BamH I and with BamH I/Xho I to generate the cloning antisense and sense fragments respectively (Figure 5.7 and Figure 5.8a, lanes 5 and 6). These fragments (approximately 1.3 kb) were subcloned into the appropriately digested eukaryotic expression vector pcDNA3 (Figure 5.8a, lanes 10 and 11), under the control of the human intermediate-early cytomegalovirus (CMV) promoter. Constructs were checked for correct ligation by double digests to excise the β1,4-GalTase insert (Figure 5.8a, lanes 15 and 22). The antisense and sense orientation on the β1,4-GalTase inserts was checked by differential nuclease restriction digestions using Xba I/Sac II (Figure 5.7 and Figure 5.8a, lanes 17 and 23). This double digest only cleaved a very small fragment of 105 bases from ASpcDNA3 which was not visible on the gel (Figure 5.8a, lane 17) but a visible larger fragment of 1.3 kb from the SpcDNA3 vector was obtained (Figure 5.8a, lane 23) indicating correct orientation. The orientation of β1,4-GalTase inserts in the ASpcDNA3 (antisense) and SpcDNA3 (sense) constructs was verified by DNA sequencing the 5' end of the inserts (Figure 5.8b).

5.2.5 Transfection of β1,4-GalTase antisense/sense vectors into COS-7 cells
Initially these constructs were transfected, using DEAE-dextran, into COS-7 cells (African green monkey kidney cells) to test their function in a transient high-expression system. The COS-7 cell lines have an origin-defective SV40 virus integrated in their chromosomal DNA, which produces large T antigen but no virus particles. When a plasmid with the SV40 origin is transfected into COS-7 cells the large T antigen causes the plasmids to replicate to a high copy number (10^3-10^4/cell). Hence any cDNA under the control of an efficient promoter in the transfected plasmid will be highly expressed.
Figure 5.7
Clone CT7-J20 contained the full-length cDNA for human β1,4-GalTase (blue) at the EcoR I site in Bluescript KS. The β1,4-GalTase cDNA was excised out of Bluescript (coloured inserts) and subcloned into eukaryotic expression vector, pcDNA3, in the sense (BamH I and Xho I digests, SpcDNA3) and the antisense (Hind III and BamH I digests, ASpcDNA3) directions. Endonuclease restriction sites on the cloning fragment are schematically indicated. Other restriction sites (writing perpendicular to figure) are part of the polylinker sequence of pcDNA3 on the respective cloning vectors. Inserts were under the expression of the CMV promoter. The vector could be selected in ampicillin or neomycin medium.
Figure 5.8
(a) Analysis of Maxi-prep DNA via agarose electrophoresis. Lambda DNA Hind III/EcoR I (lanes 1, 7, 12, 18 and 24) molecular weight markers in kb. Undigested, supercoiled CT7-J20 (containing GalTase cDNA) in Bluescript KS (lane 2), pcDNA3 (lane 8), antisense GalTase vector ASpcDNA3 (lane 13) and sense GalTase vector SpcDNA3 (lane 19). Single digest with EcoR I on CT7-J20 (lane 4). Single digest with BamH I to linearize CT7-J20 (lane 3), pcDNA3 (lane 9), ASpcDNA3 (lane 14), SpcDNA3 (lane 20). Double digests with Hind III/BamH I on CT7-J20 (lane 5), pcDNA3 (lane 10), ASpcDNA3 (lane 15), and SpcDNA3 (lane 21). Double digests with BamH I/Xho I on CT7-J20 (lane 6), pcDNA3 (lane 11), ASpcDNA3 (lane 16) and SpcDNA3 (lane 22). Double digests with Xba I/Sac II on ASpcDNA3 (lane 17) and SpcDNA3 (lane 23). Excised GalTase insert (~1.3 kb) seen in lanes 4-6, 15, and 21-23.
Figure 5.8

(b) DNA sequencing of the 5' end of the GalTase inserts in the (i) ASpcDNA3 and (ii) SpcDNA3 constructs. Identical sequences are seen in the bracketed area which represents part of the T7 promoter sequence in the parent plasmid pcDNA3. Sequences above this region represent the GalTase inserts.
Figure 5.9

(a) Eukaryotic expression vector, pCR3lacZ, encoding β-galactosidase (black circles) and pcDNA3 without any insert (white circles) were transiently transfected into COS-7 cells. The cells were harvested at the times indicated after transfection and stained in the presence of X-gal reagent and the number of positively transfected cells (stained blue) were counted the next day (section 2.5).

(b) Light microscopy of positively transfected pCR3lacZ COS-7 cells and stained with X-gal.
COS-7 cells were transfected with SpcDNA3 (sense, green bars), ASpcDNA3 (antisense, red bars) and pcDNA3 (control without an insert, blue bars) (section 2.10.2). Cells were harvested 48 h later, and assayed for transient expression of GalTase activity using the radiochemical assay in the presence of either GlcNAc-pITC-BSA (solid bars) or ovalbumin (hatched bars) acceptor substrates (section 2.3.2).

The transfection conditions were established using the pCR3/LacZ vector which contained the bacterial gene lacZ, β-galactosidase, under the control of the CMV promoter. Positively transfected cells appeared blue after incubation with X-Gal reagent, with maximum expression levels detected 48 h post-transfection (Figure 5.9). Transfection efficiencies between 20-50% were observed. Cells transfected with pcDNA3 (no insert) did not result in any blue staining.

The sense (SpcDNA3), and antisense (ASpcDNA3) constructs and pcDNA3 (no insert) were transfected into COS-7 cells and assayed for GalTase activity (Figure 5.10). There was more than a 10-fold increase in GalTase activity in COS-7 cells transfected with the SpcDNA3 constructs compared to those with the control (pcDNA3) or ASpcDNA3 constructs.
Figure 5.11
Transient expression of β1,4-GalTase protein (cellular, solid bars; secreted, hatched bars) was assayed (section 2.8.1) in COS-7 cells 48 h post-transfection with SpcDNA3 (sense, green bars), ASpcDNA3 (antisense, red bars) and pcDNA3 (control without an insert, blue bars) (section 2.10.2).

Both GlcNAc-pITC-BSA and ovalbumin proved to be very good acceptors for the SpcDNA3 transfected cells, though a slightly higher incorporation of galactose was achieved with the former acceptor substrate. Similar low endogenous GalTase activity was seen in both the pcDNA3 and ASpcDNA3 COS-7 transfectants.

The high levels of GalTase activity expression observed in COS-7 cells transfected with SpcDNA3 was reflected qualitatively in the β1,4-GalTase protein detected from these cells. Quantitatively, approximately 8-fold more β1,4-GalTase protein compared to GalTase activity was seen in the SpcDNA3 transfected COS-7 cells. There was also a much higher level of β1,4-GalTase protein secreted into the cell culture medium by COS-7 cells transfected with SpcDNA3 compared to the ASpcDNA3 and pcDNA3 (Figure 5.11). In fact, very little endogenous β1,4-GalTase protein could be detected in the COS-7 cell lysates or supernatants using the anti-β1,4-GalTase mAbs.
COS-7 transfectants were also analysed for cell surface expression of β1,4-GalTase using flow cytometry (Figure 5.12b). Due to the nature of the transfection procedure and subsequent high protein expression, there is a certain amount of cell death occurring. At the time of harvesting the cells post-transfection, cell viability was always greater than 85%. Further damage and stress to the cells can also occur during the staining and washing procedure for flow cytometry analysis. Therefore, transfected cells were also labelled with propidium iodide (PI) dye to assess the integrity of the plasma membrane during cell surface analysis (Figure 5.12b). A construct used for cell surface expression of human chorionic gonadotropin (kindly given by Alison Jackson, UCL) was tested alongside the GalTase constructs (Figure 5.12b). Low (10-15%, depending upon transfection efficiencies), but significant levels of β1,4-GalTase were observed at the cell surface in SpcDNA3 transfected COS-7 cells, which was maximal 48 h post-transfection. No cell surface expression of β1,4-GalTase was detected in untransfected, pcDNA3 or ASpcDNA3 transfected COS-7 cells indicating that the high levels of GalTase expression resulted in novel surface expression. All the surface β1,4-GalTase expression observed was present on intact viable cells as assessed by exclusion of the PI dye (Figure 5.12b) and confocal microscopy (Figure 5.12a).

Figure 5.12
(a) Confocal microscopy of SpcDNA3 transfected COS-7 cells stained for cell surface expression of β1,4-GalTase using anti-β1,4-GalTase mAb, 1E7, followed by rabbit F(ab')2 anti-mouse IgG-FITC.
Figure 5.12

(b) Flow cytometry showing i) surface expression of β1,4-GalTase in SpcDNA3 (green) but not ASpcDNA3 (red) or pcDNA3 (black) transfected COS-7 cells using anti-β1,4-GalTase mAb, 1E7, followed by rabbit F(ab′)2 anti-mouse IgG-FITC. Dead cells were gated-out based on forward and side scatter profiles. Cells with non-intact cell membranes in ii) SpcDNA3 transfectants or iv) hCG transfectants were stained positive with PI. iii) A positive control for cell surface expression was a construct expressing the fusion molecule hCG linked at its carboxyl terminus to the transmembrane domain of MHC class I molecule, H2-D^b. This fusion protein was also expressed using pcDNA3.
5.2.6 Transfection of β1,4-GalTase antisense/sense vectors into B cells

The sense (SpcDNA3) and antisense (ASpcDNA3) β1,4-GalTase constructs as well as control (pcDNA3 without an insert), were transfected into the B cell line, JAC-10, using Lipofectin. These vectors had the neomycin resistance gene, enabling for selection of the transfected B cells with G418 over several weeks. Prior to transfections, the JAC-10 B cell line had been cultured in the presence of G418 to determine the lowest concentration of drug which caused total cell death. These titrations were repeated for different batches of G418 and different B cell lines (Figure 5.13). Following transfection, it took six weeks of cell culture in G418 selective medium to generate sufficient transfectants to freeze down several aliquots of cells. Transfectants were continuously cultured in the presence of G418 and resuspended in fresh medium every three days at 0.5 x 10⁶ cells/ml.

Figure 5.13

Cell cultures of three different B cell lines, BRAD-3 (black circles), JAC-10 (crosses) and 2B6 (white circles) in the presence of various concentrations of G418 over several days. Cell viability were assessed by exclusion of trypan blue dye (section 2.10.7).
The selection and subsequent culture of B cells transfected with pcDNA3 was not as successful as those performed with the SpcDNA3 and ASpcDNA3 vectors. The reason for this was unlikely to be a reflection on the plasmid, but simply a less efficient transfection resulting in fewer copies of plasmid into the cells. The transfection efficiencies were not directly measured, though during the first week of selection 10-15% more transfected cells survived compared to untransfected cells.

The β1,4-GalTase activities were measured at several time points during the continuous culture of the B cell transfectants (Figure 5.14). A maximum 40% increase in β1,4-GalTase activity was observed in the SpcDNA3 transfectants, with a corresponding 40% decrease detected in the ASpcDNA3 transfectants, when compared to the control pcDNA3 transfectants. The differences in β1,4-GalTase activities between the SpcDNA3 and ASpcDNA3 B cell transfectants became less during the course of cell culture, with a trend towards the levels observed in the control pcDNA3 B cell transfectants (Figure 5.14). Compared to the high levels of β1,4-GalTase expression seen in the COS-7 transfectants, a relatively modest 2.5-fold difference in β1,4-GalTase activity was detected between the SpcDNA3 and ASpcDNA3 B cell transfectants. When the B cell transfectants were stained for cell surface expression of β1,4-GalTase none was observed via flow cytometry (Figure 5.15a). Intracellular β1,4-GalTase staining following a fixative and permeabilization step, resulted in higher levels of detection in the SpcDNA3 compared to the ASpcDNA3 B cell transfectants (Figure 5.15b). This intracellular β1,4-GalTase expression in the B cell transfectants was localized to the Golgi apparatus using confocal microscopy (Figure 5.16). Moreover, a higher level of B cell β1,4-GalTase expression resulted in a greater number of Galβ1,4GlcNAc structures, as determined by staining of fixed and permeabilized transfectants with biotinylated RCA1 lectin (Figure 5.15c).
Figure 5.14
B cell transfectants were thawed from frozen, re-cultured for the times indicated, then assayed for β1,4-GalTase activity (section 2.3.1). Sense GalTase cDNA (SpcDNA3, green bars), antisense GalTase cDNA (ASpcDNA3, red bars), or control plasmid (pcDNA3 without an insert, blue bars, assayed at three time points between the days indicated). Error bars of one standard error of the mean are shown.
Figure 5.15
Flow cytometry analysis of (a) intact and (b) fixed and permeabilized B cell transfectants, SpcDNA3 (green) and ASpcDNA3 (red) stained with anti-β1,4-GalTase mAb, 1E7 and rabbit F(ab')2 anti-mouse IgG-FITC. (c) Fixed and permeabilized B cell transfectants were analysed for Galβ1,4GlcNAc structures by staining with biotinylated RCA1 and streptavidin-FITC. Negative control (black) in (a) and (b) was an irrelevant IgG1 mAb MOPC-21, whilst in (c) cells were only incubated with streptavidin-FITC. There was no difference between the transfectants with the negative controls.
Figure 5.16
Confocal microscopy of fixed and permeabilized B cell transfectants, (a) SpcDNA3 and (b) ASpcDNA3 stained with anti-β1,4-GalTase antibody mAb, 1E7, followed by rabbit F(ab′)2 anti-mouse IgG-FITC (section 2.7.15).
Figure 5.17

β1,4-GalTase protein levels were quantified in B cell transfectants, SpcDNA3 (sense, green) and ASpcDNA3 (antisense, red) using a sandwich ELISA (section 2.). *Cellular β1,4-GalTase (ng GalTase/mg total protein, filled bars) and secreted β1,4-GalTase (ng GalTase/10⁶ viable cells, hatched bars). The average of eight different time points are shown with one standard error of the mean.

The increased expression of β1,4-GalTase in the B cell SpcDNA3 transfectants was quantified using the sandwich ELISA described in chapter 4. Approximately 5-fold more cellular β1,4-GalTase protein was detected in SpcDNA3 compared to the ASpcDNA3 B cell transfectants (Figure 5.17). Secreted β1,4-GalTase protein was assayed in supernatants collected after three days secretion. Despite a 5-fold increased secretion of β1,4-GalTase protein from the SpcDNA3 compared to the ASpcDNA3 transfectants, the ratio of secreted: cellular β1,4-GalTase protein was similar in both the SpcDNA3 and ASpcDNA3 transfected B cells (about 1:3.3). This ratio and level of β1,4-GalTase protein expression remained relatively constant during cell culture (Figure 5.17).
It was interesting to note that the 5-fold increase in the level of β1,4-GalTase protein only translated into a maximum 2.5-fold increase in β1,4-GalTase activity when comparing the SpcDNA3 and ASpcDNA3 B cell transfectants. Further, when the β1,4-GalTase activity was calculated as a fraction of the amount of β1,4-GalTase protein present, the estimated specific activity of the ASpcDNA3 transfectants was higher than that of the SpcDNA3 transfectants (7.5 ± 1.52 and; 2.4 ± 0.29 nmol/ng β1,4-GalTase protein/h ± standard deviations respectively measured at four time points, Figure 5.18). However, it is perhaps inappropriate to represent the β1,4-GalTase activity per amount of β1,4-GalTase protein present as the activity measurement were not assayed using purified β1,4-GalTase.

B cell transfectants were cultured in medium lacking gamma globulins and the de novo secreted IgG collected and stored frozen in aliquots. The IgG concentration in all samples was determined, adjusted to 2 µg/ml and analysed for oligosaccharide structures terminating in galactose. When culture supernatants were stored at 4°C, a decrease in the binding of IgG to the lectins (especially BSII) was observed compared to freshly thawed samples (results not shown). Therefore all measurements on IgG galactosylation were performed on freshly thawed aliquots, assayed at the same time in duplicate plates. The initial ELLA simply measured the binding ratio of BSII lectin (which recognized exposed GlcNAc residues) to RCAI lectin (which bound to exposed galactose) on equivalent amounts of isolated IgG. These studies showed an increased in RCAI binding to IgG recovered from SpcDNA3 compared to ASpcDNA3 B cell transfectants. The ELLA showed good correlation with known amounts of IgG G0 (kindly supplied by the Department of Biochemistry, Oxford University, UK using the hydrazinolysis technique) (Sumar, et al., 1990), but was not able to distinguish between the different types of galactosylated structures and so measured a mixture of G0, G1 and G2 types. In order to quantify the lectin binding, a number of IgG G0 standards were included in the ELLA. The variation in lectin binding to IgG secreted from the B cells cultures was expressed as IgG G0.

At all time points there was a higher frequency of IgG G0 (IgG lacking galactose) secreted from B cells transfected with the antisense β1,4-GalTase cDNA, ASpcDNA3 vector when compared to those B cells transfected with sense β1,4-GalTase cDNA, SpcDNA3 (Figure 5.18). Concordantly, the levels of β1,4-GalTase activity and β1,4-GalTase protein were always significantly less in the ASpcDNA3 transfected B cells than in those transfected with SpcDNA3 (Figure 5.18). The gradual increase in β1,4-GalTase activity detected in the ASpcDNA3 transfectants over time was accompanied by an increase in IgG galactosylation (decrease in the IgG G0 structures) (Figure 5.18). However, very similar β1,4-GalTase activities detected in the SpcDNA3 B cell
transfectants resulted in more than 30% difference in galactosylated IgG (compare culture times at day 7 with day 19, and day 16 with day 29, Figure 5.18). Despite variations in the β1,4-GalTase activity seen over culture time, most notably with the ASpcDNA3 B cell transfectants, hardly any change in the β1,4-GalTase protein was observed.
Figure 5.18

EBV-B cell transfectants, SpcDNA3 (green) and ASpcDNA3 (red) were thawed from frozen, re-cultured for the times indicated, then assayed for secreted IgG galactosylation (IgG G0, filled bars), β1,4-GalTase activity (nmol/mg protein/h, horizontal striped bars) and cellular β1,4-GalTase protein (ng β1,4-GalTase/mg total protein, vertical striped bars). Student's t-test significance values *p<0.05, **p<0.01 and ***p<0.001.
5.3 Discussion

These results have described a number of experiments where the level of cellular β1,4-GalTase activity has been specifically altered and the subsequent consequences on glycoprotein galactosylation investigated. Antisense was used as a means of inhibiting β1,4-GalTase expression, initially attempted with short sequences of S-oligos. Later experiments used a eukaryotic expression vector containing the full-length β1,4-GalTase cDNA in either the sense or antisense orientation to alter β1,4-GalTase expression levels.

Preliminary experiments regarding the regulation of β1,4-GalTase activity used PBMC from healthy individuals, as donor material was readily available and further studies using PBMC from RA patients were planned. Unfortunately, the inhibition of β1,4-GalTase activity in PBMC cultures using antisense S-oligos was unsuccessful. It was found that the short-term cultures of isolated PBMC (with or without monocyte depletion) resulted in the activation of β1,4-GalTase activity. Hence, any inhibitory effect of the S-oligos was likely to have been masked by the increased β1,4-GalTase activity. The activation of β1,4-GalTase in the PBMC cultures was not investigated further, but was unlikely to be due to exogenous β1,4-GalTase from the fetal calf serum in the culture medium as the serum had been heat-inactivated and the stimulation of β1,4-GalTase activity increased over several days in cell culture. As a specific control of cellular β1,4-GalTase activity was required, alternatives to the PBMC cultures were explored.

An EBV-transformed B cell line, JAC-10, gave constant levels of β1,4-GalTase activity over sufficient periods of culture time to enable experiments to be performed. Similar levels of β1,4-GalTase inhibition were observed in B cells transfected with sense or antisense S-oligos using Lipofectin. A number of non-specific effects with antisense have been reported (Wagner, 1994; Gura, 1995). It was unclear if sense S-oligos were the appropriate controls to use in antisense experiments as they have also been reported to act as competitors for regulatory proteins involved in gene expression (Leonetti, et al., 1993). An alternative control may have been a scrambled sequence of similar base composition to the antisense S-oligo. Fluorescein labelled antisense S-oligos confirmed their cellular uptake, though the hydrophobic fluorescein moiety may have enhanced the process. Hence the actual uptake and subsequent apparent inhibition by the S-oligos may have been overestimated. The lack of significant inhibition of β1,4-GalTase activity using the antisense S-oligos opened up a number of potential trouble-shooting areas including the effective intracellular localization of the internalized S-oligos at sufficient and stable concentrations, and the efficiency of recruiting RNase H for
hydrolysis of the RNA strand of the DNA-RNA duplex. Problems in using antisense were compounded by the lack of criteria with regards to mechanism of action, target sequence, type of analogue, length of oligo etc. for effective inhibition of gene expression (Zon and Stec, 1991), though some advances have been achieved more recently (Fakler et al., 1994).

Given the difficulties associated with antisense S-oligos in gene inhibition, as well as the expense, a different practical approach was embarked upon. The complete human β1,4-GalTase cDNA sequence was subcloned into a eukaryotic expression vector in the antisense and sense orientations, which were then either transiently or stably expressed in mammalian cell lines.

The β1,4-GalTase sense (SpcDNA3) and antisense (ASpcDNA3) constructs were initially tested in a high expression system using the COS-7 cell line. COS-7 transfection efficiencies, measured using the reporter gene lacZ, varied between 20-50%. The cell passage number was partially responsible for the variability. SpcDNA3 transfected COS-7 cells transiently expressed more than 10-fold the GalTase activity seen in the control pcDNA3 transfectants using either GlcNAc-pITC-BSA or ovalbumin as acceptors. Both these glycoproteins were excellent substrates for the transfected and endogenous COS-7 GalTase (ASpcDNA3 and pcDNA3 transfectants), though slightly higher levels of galactose incorporation were observed with GlcNAc-pITC-BSA. ASpcDNA3 had no effect on the GalTase expression in the transfected COS-7 cells when compared to the endogenous levels. No inhibition of COS-7 GalTase activity was found when cells were transfected with antisense bovine β1,4-GalTase cDNA (Masibay and Qasba, 1989). The homologies between the African green monkey kidney β1,4-GalTase and human β1,4-GalTase would be predicted to be relatively high considering the known homologies of human β1,4-GalTase with β1,4-GalTase from more distantly related species. However, it maybe that for the effective antisense inhibition, near complete complementarity was required with the target sequence (Nyce and Metzger, 1997) and/or a longer incubation period was required to influence the de novo synthesis of the COS-7 GalTase. Extremely high levels of cellular β1,4-GalTase protein were also measured in the SpcDNA3 transfected COS-7 cells. The barely detectable levels of β1,4-GalTase protein found in the pcDNA3 and ASpcDNA3 transfectants may have been due to the low amounts of protein present in these cells but possibly also to low cross-reactivity of the mAb anti-human β1,4-GalTase antibodies. These mAbs stained about 30% of COS-7 cells which had been fixed and permeabilized, however this recognition may have been further reduced in the β1,4-GalTase protein quantification ELISA where cell lysates were assayed and disruption of β1,4-GalTase epitopes could occur. The ratio of cellular: secreted β1,4-GalTase protein was more than 8:1 in the SpcDNA3 transfected COS-7 cells. The normal cellular distribution of β1,4-GalTase
protein was altered in the SpcDNA3 cells, as exemplified by cell surface staining. Experiments were not carried out to address the question of preferential localization of different β1,4-GalTase isoforms. The broad cell surface expression was probably a reflection on the vector copy number within the transfected cell. The maximal cell surface expression coincided with maximal β1,4-GalTase expression at 48 h. It was apparent that very high expression of full-length β1,4-GalTase (long form) could result in its novel cell surface expression, in agreement with some (Dinter and Berger, 1995a) but not other groups (Nilsson, et al., 1991; Russo, et al., 1992) who claimed that the normal cellular distribution of β1,4-GalTase occurred in high and low level expression systems. The disparity between these two observations may be due to differences in the methodologies, such as the amount and type of DNA transfected, length of β1,4-GalTase cDNA insert Lopez, et al., 1989, transfection conditions, expression vectors used and cell surface analysis.

The stable expression of different levels of β1,4-GalTase activity in the same B cell line was demonstrated. It was estimated that between 10-15% of B cells were positively transfected with the sense, antisense and control plasmids using Lipofectin, which were selected for in G418 medium over six weeks and aliquots frozen down. Although the estimated transfection efficiencies were similar for each vector, it is probable that unequal amounts of DNA were taken up by the transfected cells as judged by the different expression levels of cells analysed by flow cytometry and the fact that the pcDNA3 (control) transfected cells were fewer in number and did not survive as well or as long as the SpcDNA3 and ASpcDNA3 transfectants. Following the re-culture (medium always supplemented with G418) of selected transfectants, there was a steady reduction in plasmid expression over time. At the final time point in culture (day 29 in Figures 5.14 and 5.18), the viability of the transfectants fell to 75%, hence a Lymphoprep density gradient step was included prior to cell analysis. This data was consistent with a loss of plasmid from the transfectants during cell division. For the maintenance of high expressing transfectants in culture it has been recommended to reclone transfectants after several weeks in culture (Dr. Hirotaka Yamamoto, Chicago Institute of Neurosurgery, IL, USA, personal communication). Further, it may be advantageous to use vectors with the EBV origin of replication and the nuclear antigen, EBNA-1, which allow high-copy episomal replication in EBV-transformed mammalian cell lines.

The maximum expression levels of β1,4-GalTase activity in B cells transfected with SpcDNA3 and ASpcDNA3 were shown to be 40% higher and lower respectively compared to the pcDNA3 transfectants. This modest change in β1,4-GalTase expression was examined for any effect it had on β1,4-GalTase cell localization as well
as in situ galactosylation. The stable and moderate expression of β1,4-GalTase in the B cell SpcDNA3 transfectants showed a similar Golgi staining pattern to that observed in ASpcDNA3 transfectants and untransfected B cells. Further, the flow cytometry analysis of the transfectants did not reveal any cell surface staining. This was in contrast to the COS-7 SpcDNA3 transfectants transiently overexpressing β1,4-GalTase, which resulted in significant cell surface expression. It was possible that variation between cell types (Tang, et al., 1995), the expression levels (Teasdale, et al., 1994) and different post-translational modifications to β1,4-GalTase, influenced the distribution of the expressed protein. The increased β1,4-GalTase expression observed in the B cells transfected with SpcDNA3 had a similar ratio of cell associated (Golgi): secreted β1,4-GalTase protein as the ASpcDNA3 transfectants of 3.3:1 respectively. Thus a moderate increase in β1,4-GalTase expression did not appear to disrupt the normal subcellular distribution of β1,4-GalTase. This was further supported by the significantly enhanced number of Galβ1,4GlcNAc structures synthesized by sense transfected B cells, indicating correct Golgi localization and functional activity of β1,4-GalTase within these B cells.

A striking difference was observed between the expression levels of β1,4-GalTase activity and β1,4-GalTase protein. There was eight-fold more β1,4-GalTase protein than activity expressed in the SpcDNA3 transfected COS-7 cells compared to the ASpcDNA3 or pcDNA3 transfectants. This may have been slightly over-estimated due to the low cross-reactivity of the mAb anti-GalTase antibodies to the endogenous COS-7 β1,4-GalTase protein present in the ASpcDNA3 or pcDNA3 transfectants. Highly overexpressed proteins can result in their misfolding, inactivation and their retention in the endoplasmic reticulum (ER) (Russo, et al., 1992; Teasdale, et al., 1992), which may account for a portion of the β1,4-GalTase protein in the SpcDNA3 transfected COS-7 cells. However, these explanations were unlikely to account for approximately twice as much β1,4-GalTase protein as β1,4-GalTase activity expressed in the SpcDNA3 transfected B cells compared with the ASpcDNA3 transfectants. There was no evidence of the expressed β1,4-GalTase being retained in the endoplasmic reticulum of the B cells. If a portion of the overexpressed β1,4-GalTase protein was inactivated by degradation, the epitopes for two anti-β1,4-GalTase mAbs which captured the β1,4-GalTase from the cell lysates in the β1,4-GalTase quantification ELISA remained intact. Further, if processing occurred it would have taken place in the Golgi as this was the only location in which β1,4-GalTase was detected using the anti-β1,4-GalTase mAbs. Quiescent NIH 3T3 cells stimulated with serum exhibited a similar 2-fold increase in β1,4-GalTase protein over activity (Masibay, et al., 1991).
A small fraction of cellular β1,4-GalTase was constantly secreted out of the cell, for as yet undetermined reasons. There is a cleavage site in the β1,4-GalTase stem region which may be used to release either a specific lectin out of the cell and/or regulate the amount of β1,4-GalTase associated in the Golgi as a means of controlling galactosylation and Golgi localization. The presence of catalytically active β1,4-GalTase has been described in extracellular secretions including milk, serum, amniotic fluids, and cell culture supernatants (Strous, 1986; D’Agostaro, et al., 1989). The ratio of cell (Golgi) associated to secreted β1,4-GalTase remained constant irrespective of the β1,4-GalTase expression level in the B cell transfectants. At day 29 (Figure 5.18), ASpcDNA3 transfectants expressed 80% of the β1,4-GalTase activity of SpcDNA3 transfectants, yet there was more than a 4.5-fold increase in β1,4-GalTase protein detected in the SpcDNA3 transfectants. This suggested that a large proportion of the β1,4-GalTase protein was catalytically inactive.

One possible explanation might be that the excess inactive β1,4-GalTase protein was involved in effective stabilization of active β1,4-GalTase and/or acting as a specific receptor in the retention of glycoconjugates awaiting galactosylation. β1,4-GalTase along with a number of other Golgi proteins have been described as dimers Bendiak, et al., 1993; Fleischer, et al., 1993). Recently a ST6Gal I dimer which accounted for a third of the total enzyme in the Golgi of HeLa cells was found to be catalytically inactive, but could still bind its acceptor substrate (Ma and Colley, 1996). If in the Golgi of control B cells, β1,4-GalTase consisted of a mixture of monomers, dimers and larger complexes, then the increased or decreased expression of β1,4-GalTase may be influencing the structural and subsequent functional aspects of the enzyme differently. The excess β1,4-GalTase protein would be inactive possibly through aggregate formation and/or post-translational modifications which may be reversible, allowing for further regulation of β1,4-GalTase activity.

An additional process may account for the large amount of inactive β1,4-GalTase protein in the SpcDNA3 transfected B cells. Two naturally expressed forms of ST6Gal I in the liver encoded by two different RNA which differed by a single base have been reported (Ma, et al., 1997). The translational products either had a Tyr123 or a Cys123 and exhibited alternative catalytic and processing properties. As both types of ST6Gal I were encoded by a single gene, a post-transcriptional modification was thought to have occurred, possibly RNA editing. The predominant form of ST6Gal I Tyr123 was catalytically more active, and underwent higher turnover and secretion from the cell compared to the ST6Gal I Cys123 (Ma, et al., 1997). It would be interesting to investigate if β1,4-GalTase also exhibits such discrete isoforms.

A reduction in β1,4-GalTase activity in B cells below a critical amount (approximately 25 nmol/mg protein/h) resulted in secretion of IgG with more than half these antibodies
lacking galactose. The reduced amount of β1,4-GalTase protein seen in the ASpcDNA3 transfected B cells disrupted the efficient functioning of the endogenous β1,4-GalTase to such an extent as to affect its activity and galactosylation of substrates. However, the very slight increase in β1,4-GalTase protein, observed in the later time points of the ASpcDNA3 B cell transfectants (days 19 and 29, Figure 5.18) caused a marked rise in the β1,4-GalTase activity. The β1,4-GalTase from the ASpcDNA3 transfectants was reduced in quantity compared to that present in the SpcDNA3 transfectants, though was estimated to be of higher enzymatic quality (of potentially greater specific activity by comparing the β1,4-GalTase activity assayed in cell lysates over the actual amount of β1,4-GalTase protein present in the lysates), possibly as a result of its particular structural form in the Golgi.

The fact that SpcDNA3 and ASpcDNA3 B cell transfectants did not simply result in an up and down regulation of β1,4-GalTase expression may have been due to several different reasons. The effect the transfections had on β1,4-GalTase expression had to take into account the endogenous B cell expression levels which was likely to express both long and short forms of β1,4-GalTase. Differential regulation of the long and short β1,4-GalTase forms has been shown to vary in a number of cell types. Both these isoforms are found in the Golgi although it still remains controversial as to which form, if any, is preferentially localized to the cell surface (Lopez, et al., 1991; Russo, et al., 1992; Masibay, et al., 1993; Youakim, et al., 1994a). No distinction between the two protein forms could be made in the present studies, however only Golgi localized β1,4-GalTase was observed in transfected and untransfected B cells. It was unclear what if any feedback signals to the B cell transcription of β1,4-GalTase occurred following transfection with β1,4-GalTase sense and antisense vectors. Assuming that the ASpcDNA3 expression acted primarily by inhibiting the housekeeping levels of B cell β1,4-GalTase mRNA translation, then a suppression of both the long and short endogenous β1,4-GalTase was observed. It has previously been demonstrated that expression of the full length β1,4-GalTase cDNA resulted in the translation of a single protein (long) and that no leaky translation from the second internal initiation site occurred (Russo, et al., 1990). Hence, overexpression of the long β1,4-GalTase form in the SpcDNA3 transfectants would perturb the endogenous B cell expression ratio between the long and short of B1,4-GalTase protein. The two forms of ST6 Gal I which had a single amino acid substitution appeared to have different turnover rates and cell locations (Ma, et al., 1997). If such a situation existed with regards to β1,4-GalTase, then a further level of β1,4 galactosylation regulation would be operating.

Differences in β1,4-GalTase expression and cell localization have been observed in various cell types dependent upon their state of differentiation and/or proliferation.
Increased Golgi but not cell surface β1,4-GalTase activity, probably mediated through cAMP, was observed during the induced differentiation of PC12 pheochromocytoma cells (Roth, et al., 1991). Differentiation of F9 embryonal carcinoma cells with retinoic acid also specifically induced Golgi β1,4-GalTase activity (Lopez, et al., 1989; Roth, et al., 1991). When both retinoic acid and dibutyryl cAMP were used, a synergistic effect on β1,4-GalTase expression was detected (Amos et al., 1990; Roth, et al., 1991). During the differentiation of F9 cells, a 6.5-fold increase in β1,4-GalTase mRNA transcript compared to the 21-fold increase in β1,4-GalTase activity was observed (Kudo and Narimatsu, 1995). Both β1,4-GalTase mRNA transcripts, long (4.1 kb) and short (3.9 kb), increased at the same ratio 1:2.5-3.8 during the course of F9 differentiation. Their accumulation was due to mRNA stabilization (Kudo and Narimatsu, 1995).

Treatment of rat parotid gland acinar cells with isoproterenol caused cell proliferation and stimulated cell surface β1,4-GalTase 40-fold, whilst the Golgi β1,4-GalTase activity was unaltered (Marchase, et al., 1988). Quiescent HeLa cells re-entered their cell cycle following stimulation with serum, and equally expressed, during G1, increased levels in both the 4.1 kb and 3.9 kb β1,4-GalTase mRNA transcripts (Pouncey, et al., 1991). Early β1,4-GalTase mRNA expression (separate transcripts were not probed for) was also observed in NIH 3T3 cells post serum stimulation (Masibay, et al., 1991). Increased β1,4-GalTase activities were detected both in the Golgi and the cell surface, with relatively more expression on the cell surface (Masibay, et al., 1991). The peak expression of β1,4-GalTase mRNA (early G1) did not parallel that of β1,4-GalTase protein which peaked 8-10 h post serum stimulation and remained stable until cell division (Masibay, et al., 1991). When F9 cells were stimulated with serum, maximal expression of the long β1,4-GalTase mRNA occurred in early G1 while the short β1,4-GalTase mRNA was expressed during late G1 and through the remainder of the cell cycle (Maillet and Shur, 1994).

F9 cells induced to differentiate into endoderm-like cells, had increased β1,4-GalTase activity with concomitant increased glycosylation and glycoprotein biosynthesis (Lopez, et al., 1989; Amos, et al., 1990). Large changes in cellular glycosylation have been observed in cells that have undergone transformation. Oncogenic transformation of NIH 3T3 cells activated a number of glycosyltransferases causing an increased synthesis of glycoprotein structures, particularly polylactosaminoglycan chains, sialylation, and branching of glycans (Easton, et al., 1991). The effect of specifically increasing β1,4-GalTase activity by overexpression in the SpcDNA3 B cell transfectants, resulted in a significant increase in the total number of Galβ1,4GlcNAc structures produced by these cells, as determined by increased RCAI binding.
Moreover, 10-20% more galactosylation on IgG secreted from the SpcDNA3 B cell transfectants was detected compared to the ASpcDNA3 transfectants at the equivalent time points. However, overexpression of β1,4-GalTase in F9 cells, via gene transfection, had no apparent effect on the biosynthesis of lysosomal-associated membrane glycoprotein-1 (LAMP-1) (Youakim and Shur, 1993). Other factors, including the β1,4-GalTase expression levels, glycoprotein substrates, nucleotide sugar levels and cell types are probably all influential in determining glycosylation. As F9 cells normally express cell surface as well as Golgi β1,4-GalTase, the elevated cell surface expression may have influenced the cellular glycosylation through cell-cell interaction or through growth control.

The changes observed in β1,4-GalTase expression during the cell cycle suggest that GalTase may play a role(s) in cell events and signalling as well as its characteristic role in galactosylation within the Golgi. Expression of a serine/threonine protein kinase, p58GTA, has led to specific stimulation of β1,4-GalTase activity (Bunnell, et al., 1990a). The p58GTA kinase was renamed PITSLREβ1 (one of 10 isoforms) and belongs to the p34cdc2 gene family (Xiang et al., 1994). Increased expression of PITSLREβ1 in CHO cells led to inhibition of normal cell cycle (Bunnell, et al., 1990b; Bunnell, et al., 1990c) and was involved in apoptosis (Lahti, et al., 1995). Swiss 3T3 cells that stably overexpressed cytoskeletal-associated surface β1,4-GalTase had a decreased growth rate, possibly due to direct interaction with the EGF receptor (Hinton, et al., 1995). However, overexpression of cell surface β1,4-GalTase in PC12 cells increased the rate and amount of neurite outgrowth on laminin, a recognized substrate for β1,4-GalTase (Begovac et al., 1991; Huang, et al., 1995).

It may be argued that the difference in β1,4-GalTase activities between the SpcDNA3 and ASpcDNA3 B cell transfectants would have been greater if the assays had been performed in the presence of a more restrictive acceptor than GlcNAc-pITC-BSA. A small variation in β1,4-GalTase activity may therefore have had a disproportionately large effect on IgG galactosylation due to the inaccessibility of the oligosaccharides within Cγ2 of IgG. However, it appeared that factors other than the β1,4-GalTase activity alone had a more significant effect on the IgG galactosylation. A 40% increase in IgG galactosylation (decrease in IgG G0) was observed, with only a 1.1-1.3 fold-increase in the corresponding β1,4-GalTase activities (see days 16 and 19 in Figure 5.18). In fact, in the SpcDNA3 B cell transfectants, the increased IgG galactosylation led to the complete absence of IgG G0 (hypergalactosylated) until the end of culture. It was unclear what caused this large increase in IgG galactosylation. B cells cultured at different cell densities resulted in a more fully galactosylated IgG from the low density cultures (Kumpel, et al., 1994). Whilst B cell activities between different B cell lines differed, their IgG galactosylated states were similar (Kumpel, et al., 1994). It would
have been interesting to have compared the β1,4-GalTase activities in the same B cell line cultured at the two different densities. It may be that B cell differences in the cell division, IgG assembly, secretion etc. also influenced the galactosylation of IgG. Early time points in culture saw a similar cell doubling time for both the SpcDNA3 and ASpcDNA3 transfectants of approximately 66 h. However, a decrease in the doubling time for both SpcDNA3 and ASpcDNA3 to 33 and 41 h respectively, coincided with the increased IgG galactosylation. There was no apparent reason for this increase in cell proliferation, and it was unlikely to be due to the altered β1,4-GalTase expression levels as both types of transfectants were similarly affected at the same time point. Following this surge in cell division, the doubling time increased to around 78 h for the later time points. Thiol compounds have been shown to alter the doubling time and rate of IgG secretion of B cell hybridomas (Mattingly et al., 1992). The amount of IgG secreted from the SpcDNA3 and ASpcDNA3 B cell transfectants was also seen to fluctuate during cell culture. At the early time points in culture where IgG was not fully galactosylated, ASpcDNA3 cultures secreted a higher, but not statistically significant, amount of IgG compared to the SpcDNA3 B cell transfectants (3.03 ± 0.76 and 1.66 ± 0.32 μg IgG/10^6 cells respectively). The lowest levels of IgG secretion from both ASpcDNA3 and SpcDNA3 B cell transfectants (0.73 ± 0.18 and 1.2 ± 0.87 μg IgG/10^6 cells respectively) coincided with the production of more fully galactosylated IgG. This correlation may be coincidental and further experiments would be required to investigate this relationship. A number of glycoproteins have been identified whose subpopulations, based on their reactivity to concanavalin A, differed in their secretion rates (Pous et al., 1992). Studies aimed at prolonging the transit time of glycoconjugates through the Golgi, by decreasing cell culture temperature to 20°C, led to increased glycosylation of LAMP-1 and -2 (Wang et al., 1991). A murine hybridoma which had more than a three-fold increase in antibody secretion than another hybridoma, from the same parental cell line, also showed higher glycosyltransferase activities including 12-fold more GalTase (Cole et al., 1993). Bisecting GlcNAc structures altered the expression of cell surface but not secretory glycoproteins (Sultan et al., 1997). Tunicamycin treated murine hybridoma and lymphoma cells failed to express membrane bound IgM or IgA, but secretion of IgA, but not IgM, occurred in lymphoma cells in the presence of tunicamycin (Sitia et al., 1984). However, others have shown that IgG and IgM secretion from rat hybridoma cells could occur efficiently when cultured in the presence of swainsonine or deoxynojirimycin which blocked glucosidase and mannosidase activities respectively (Hashim and Cushley, 1988). Also two isoforms of IgE which differed by a six amino acid carboxy terminus on the heavy chain and in their glycosylation were both equally secreted by B cells (Batista et al., 1996). The lack of tailpiece glycosylation on IgA1 resulted in higher polymer (greater
than dimers) forms, and affected a number of effector functions, but the secretion rate was unaltered (Chuang and Morrison, 1997).

The specific rates of cell growth, and of heavy (H) and light (L) chain assembly were important criteria in the rate of antibody secretion (Bibila and Flickinger, 1992). Further, the IgG assembly pathway may be influential in the presentation of its oligosaccharide substrate to the terminally acting glycosyltransferases. It has been suggested that the formation of intra- and inter-disulphide bond formation in IgG could occur in the ER and Golgi depending upon the tissue, species or IgG subclass (Rademacher, et al., 1995). However, one report implied that the thiol-mediated retention of unpolymerized immunoglobulins did not occur in or beyond the Golgi (Valetti and Sitia, 1994). Two main pathways in IgG assembly, at least for murine IgG, have been demonstrated: pathway 1, where IgG1, IgG2a and IgG3 were assembled in the H-H2-L-H2L2 order and; pathway 2, where IgG2b (and IgM) followed the H-HL-(H2L)-H2L2 sequence. The initial formation of intermediates with disulphide-bonded heavy chains (pathway 1) resulted in low IgG galactosylation (high IgG G0) whereas IgG assembly via pathway 2 produced more fully galactosylated IgG (low IgG G0) presumably due to the degree of exposure of the oligosaccharide chain to β1,4-GalTase (Sutherland, et al., 1972). Further, it was proposed that a cell could use two or more IgG assembly pathways at any one time (Baumal, et al., 1971). Although the assembly pathways for human IgG are currently unknown, this model could potentially explain the dramatic change from low to high IgG galactosylation observed in the ASpcDNA3 and SpcDNA3 B cell transfectants (days 16 and 19 Figure 5.18) i.e. a switch from pathway 1 to pathway 2. Future studies should examine IgG glycosylation in the F(ab')2 and Fc domain separately, from a population of high and low galactosylated IgG. If both variably galactosylated IgG molecules were trafficking through the same pathway, their F(ab')2 galactosylation (unrestricted access to β1,4-GalTase, (Endo et al., 1989)) should be similar. Clearly, the site(s) and regulation of disulphide bond formation, such as through glutathione levels and protein-disulphide isomerase would be critical to the control of IgG glycosylation via this assembly model (Petersen and Dorrington, 1974; Kroning et al., 1991). Other factors, including glucocorticoids and IL-6, have been shown to influence glycosylation on a number of glycoproteins (Haffar et al., 1988; Pilkington, et al., 1996). Increased levels of serum IL-6 have been correlated with a high incidence of IgG G0 (Nakao, et al., 1991; Rook, et al., 1991a; Rademacher, et al., 1995). Interestingly, IL-6 is also an autocrine growth factor for EBV-transformed B cells and known to induce Ig production (Van Snick, 1990). Unfortunately, the levels of IL-6 were not monitored in the SpcDNA3 and ASpcDNA3 B cell cultures, thus it was unclear what effect, if any, on IgG glycosylation occurred. Women with RA often undergo remission during pregnancy with a concomitant
increase in IgG galactosylation. However, post-partum the IgG G0 glycoform increases and flare-up of RA symptoms occurs (Rook, et al., 1991b). Approximately 13% of all women first develop RA in the post-partum period (Rademacher, et al., 1996b).

Non-covalent interactions between amino acids residues and the core hexasaccharide (GlcNAc2Man3GlcNAc) have been shown to affect the synthesis of the Fcγ2 oligosaccharide chain and in vitro effector functions (Lund et al., 1995; Lund et al., 1996). A single amino acid change, Tyr296Ala, on a human chimeric IgG3 produced in CHO cells resulted in a 1.5-fold increase in agalactosylated chains (63%) compared to the wild type antibody (42%), and no sialylation (wild type contained 3.5% sialylated structures). Moreover this mutant was estimated as having significantly reduced occupancy of the glycosylation site at Asn297. Other single amino acid substitutions of contact residues with the core hexasaccharide dramatically increased the level of galactosylation and sialylation on the IgG3, but no correlation existed between the levels of galactosylation or sialylation and recognition by guinea-pig C, human C1q and human FcγRII (Lund, et al., 1996). It would be interesting to determine if such single amino acid substitutions affected the rate of IgG assembly or secretion. In contrast to these results, two murine IgG2a anti-dinitrophenyl mAbs of equivalent monosaccharide content (which predicted a non-bisected, fucosylated G1 structure) and identical sequences within the heavy chain constant region, exhibited differential reactivity to lectins, β1,4-GalTase (suggestive of altered orientation of the outer exposed GlcNAc on one of the Man α1,3 arms) and N-glycosidase F (indicative of altered inner carbohydrate core orientation) (White et al., 1997). This study suggested that the orientation of the oligosaccharide on IgG affected its ability to activate complement. The mAb with the more accessible oligosaccharides was less capable of activating complement via the classical pathway than the mAb which had its oligosaccharides sequested (White, et al., 1997). One possible explanation for the altered oligosaccharide orientation in these two mAbs, may be that the different variable regions of the two mAbs had some influence on the constant domain, including the carbohydrates, through quartenary interactions. The removal of the differentially orientated oligosaccharide chains from the two mAbs had opposite effects on C1q binding compared to their glycosylated states (White, et al., 1997)). Either the deglycosylation led to a conformational change in the polypeptide or amino acids were revealed, which increased or decreased the binding to C1q.

A subtle change in conformation, particularly around the lower hinge region of Cγ2 (residues 233-237), may be expected to alter FcγR binding (Jefferis, 1990). Further, the low affinity receptors, FcγRII and FcγRIII, rather than the high affinity FcγRI, would be the most likely affected. However, interaction between the outer sugar residues on the Man α1,6 arm and the Fc surface domain, in a human chimeric IgG3, were not required
for recognition of FcγRI and FcγRII (Jefferis et al., 1995). However, other groups have found that IgG molecules lacking of terminal galactose altered the effector functions in vitro and in vivo.

The anti-D mAbs secreted from the JAC-10 B cell line, used in studies presented in this thesis, had previously been assayed for functional activity by the group which produced this cell line (Kumpel, et al., 1994). IgG samples stored at 4°C resulted in impaired FcγRIII-mediated lysis of erythrocytes by K cells compared to freshly secreted IgG (Kumpel, et al., 1994). Reduced and differential binding of IgG to lectins was observed after storage at 4°C (results not shown), and therefore only results obtained from freshly thawed samples were presented in this thesis. Culture conditions are also known to affect protein glycosylation (Goochee and Monica, 1990; Patel, et al., 1992). Higher IgG galactosylation was obtained in another EBV-transformed B cell line, BRAD-3, by culturing under low as compared to high cell density culture conditions (Kumpel, et al., 1994). Fully galactosylated anti-D mAbs were more active in FcγRI and FcγRIII-mediated lysis of erythrocytes in ADCC assays than a less galactosylated version of the same mAb (Kumpel, et al., 1994). However, an IgG\textsubscript{1} anti-D mAb which was differentially galactosylated did not show any change in FcγRI binding neither was the rosette formation via FcγRI affected by the galactosylation status of the mAbs (Kumpel, et al., 1994). Agalactosylated IgG (completely lacking galactose) has been shown to have significantly decreased binding to Clq and FcγRI on monocytic cell line U937 (Tsuchiya, et al., 1989). Another group produced two B cell lines from the same donor which secreted differentially galactosylated IgG\textsubscript{1} anti-D mAbs. The weakly galactosylated mAb exhibited a decrease in binding to FcγRI, FcγRIII and FcγRI-mediated rosette formation compared to the more fully galactosylated mAb (Cant, et al., 1994). The decreased binding to FcγRIII by the hypogalactosylated mAbs was the most affected (Cant, et al., 1994; Kumpel, et al., 1994).

The absence of IgG G0 structures secreted from the SpcDNA3 B cell transfectants was indicative of highly galactosylated IgG (days 19 and 29, Figure 5.18). This and other reports of hypergalactosylated human IgG (Lund, et al., 1993a; Kumpel, et al., 1994) do not conform to the oligosaccharide pairing suggested by the X-ray crystallography data for rabbit IgG Fc (see section 1.3.6) (Sutton and Phillips, 1983). An approximately 20% shift in galactosylated IgG structures was observed between the SpcDNA3 and ASpcDNA3 B cell transfectants which was due to the differences in their β1,4-GalTase activities (day 7, Figure 5.18). However, the greatest change in IgG galactosylation (about 40%) and possibly the one more likely to affect IgG effector function, arose with little alteration in the B cell β1,4-GalTase activities and may be due to other contributing factors as discussed above.
Perhaps the most significant experiments in evaluating the biological effects of the IgG G0 glycoform have been performed in a murine collagen-induced arthritis model. The transfer of IgG containing autoantibodies against type II collagen into T cell-primed DBA/1 mice, was found to result in an earlier and more severe arthritis when the agalactosylated IgG glycoform was used (Rademacher, et al., 1994). Later it was shown that degalactosylated anti-mouse collagen type II mAbs were able to transfer disease (Rademacher, et al., 1996b), whereas the highly galactosylated mAbs localized to the joint but were weakly pathogenic (Holmdahl et al., 1990). It was reported that agalactosylated IgG could activate complement via mannose-binding protein (Malhotra, et al., 1995). As mannose-binding protein and agalactosylated IgG are both present in the synovial fluid, it was suggested that their interaction and subsequent activation of complement contributed to the chronic inflammation in the joints seen in RA patients (Malhotra, et al., 1995).

Other mechanisms for IgG G0 involvement in the pathogenesis of RA have been proposed. F(ab')2 glycosylation has been implicated in immune complex formation (Hymes, et al., 1979; Hay et al., 1991). Also, the increased incidence of GlcNAc residues in immune complexes from RA patients (Bond, et al., 1995) and in an animal model of arthritis, MRL-lpr/lpr mice (Bond, et al., 1990), suggested that specific sugar residues may be involved in the formation of these complexes. It has been proposed that the IgG G0 glycoform is directly involved in self-associating immune complexes in RA, via galactose present on the F(ab')2 of one IgG interacting with a lectin pocket in the Fc domain of another IgG G0 molecule (Rademacher, et al., 1988). Alternatively, the loss of galactose may have exposed novel antigenic sites to which RF could bind. A subgroup of monoclonal IgM RF have been identified which bind with a higher affinity to IgG G0 glycoforms in preference to fully galactosylated IgG (Soltys, et al., 1994; Newkirk, 1996). The slow clearance of immune complexes containing IgG G0 from the circulation and an ability to activate complement could all be contributing to the aetiology of RA. Further, if autoantibodies were present in these complexes, the site of inflammation may be localized to the autoantigen.

Experiments presented here demonstrated that higher β1,4-GalTase activities in the same B cell line resulted in higher IgG galactosylation. It would be interesting to attempt to correct the defective galactosylation in RA B cells using a similar gene transfection approach. However, there are a number of practical problems associated with the use of B cells, not least the availability of sufficient material and the high transfection efficiencies that would be required. Initial attempts at using PBMC or co-transfection of B cells with SpcDNA3 and a selective cell surface marker followed by rapid enrichment were unsuccessful. Perhaps, immortalized B cell lines from RA patients could be tested initially. The antisense/ sense approach in controlling the
expression of glycosyltransferases has recently been used elsewhere, and may prove useful in understanding their functions (Hiraiwa, et al., 1996) and how glycosylation affects the cellular processes (Yoshimura, et al., 1995; Zeng, et al., 1995).
6. General Discussion
6.1 Introduction

The work presented in this thesis has investigated B cell β1,4-GalTase expression and the ability of this enzyme in vitro and in vivo, to galactosylate glycoproteins. These studies included examining the role of β1,4-GalTase in IgG galactosylation in patients with RA. In addition, several anti-β1,4-GalTase mAbs were produced and used in the immunodetection of β1,4-GalTase in various assay systems.

6.2 β1,4-GalTase activity

An ELISA-based assay was developed to measure lymphocytic β1,4-GalTase activities. Further development of ELISA-based glycosyltransferase assays with a large array of acceptor substrates would seem feasible especially given the relatively simple synthesis of 1-N-glycyl-β-derivatives of oligosaccharides (Manger et al., 1992; Wong et al., 1994). The assay developed herein utilized a neoglycoprotein, GlcNAc-pITC-BSA, which proved to be a very efficient acceptor substrate for β1,4-GalTase. Successive glycosyltransferase reactions on acceptors in a solid-phase may prove a useful method in the development of specific oligosaccharide structures.

The modest 2.5-fold increase in β1,4-GalTase activity in the SpcDNA3 B cell transfectants, compared to the ASpcDNA3 transfectants, resulted in an increase in the number of cell-associated Galβ1,4GlcNAc structures. Thus, sufficient UDP-galactose was present in the B cell Golgi to allow for enhanced galactosylation. At present, it is unclear whether the 2-fold increase in β1,4-GalTase protein expression over β1,4-GalTase activity, observed between the SpcDNA3 and ASpcDNA3 transfectants, was an important structural requirement for β1,4-GalTase localization and/or functional activity in situ.

Increased β1,4-GalTase activity was observed in the presence of 1E7 mAb. This mAb recognized an epitope in the stem region on β1,4-GalTase which may help to stabilize the β1,4-GalTase or cause it to associate into a more active form.

6.3 The structure of β1,4-GalTase

Three out of the four of the most reactive anti-β1,4-GalTase mAbs produced had important epitope binding areas between amino acids 41-116. Hence, this stem region may contain immunodominant epitopes. The anti-β1,4-GalTase mAbs which
recognized the stem, experienced a greater decrease in $K_B$ when binding to the rhGalTase forms compared to the human milk $\beta_{1,4}$-GalTase. These epitopes were also readily accessible on $\beta_{1,4}$-GalTase localized in the Golgi. This would support the view that the stem region, due to its high proline and glycine content, may be of a flexible nature. The binding of 1H11 mAb to $\beta_{1,4}$-GalTase in situ was relatively weak compared to the other anti-$\beta_{1,4}$-GalTase mAbs, which may reflect a conformational change in $\beta_{1,4}$-GalTase when localized in the Golgi, or possibly the masking of the epitope by other proteins interacting with $\beta_{1,4}$-GalTase.

The synergistic binding of 1B6 and 1H11 mAbs to human milk $\beta_{1,4}$-GalTase indicated that either (i) the epitopes are simultaneously accessible and possibly positioned on the same face of the molecule, or (ii) the $\beta_{1,4}$-GalTase had self-associated, in which case the epitopes remain equally exposed and are not directly involved in the $\beta_{1,4}$-GalTase complex. The second possibility of purified $\beta_{1,4}$-GalTase forming aggregates could potentially be tested using pairs of mAbs which recognize overlapping epitopes. If an increase in $K_{ass}$ or decrease in $K_{diss}$ occurred with these mAbs, resulting in synergistic binding, it would indicate that the exposed epitopes were occurring on oligomers of $\beta_{1,4}$-GalTase rather than on monomeric $\beta_{1,4}$-GalTase. 1B6 and 1H11 mAbs did not have to be closely associated with each other, as the synergistic binding was observed when these mAbs were randomly captured onto ELISA plates, suggesting that the epitopes were positioned at distant regions from each other. This was further supported by the differential binding of 1H11 and 1B6 mAbs to the protein-engineered $\beta_{1,4}$-GalTase deletion mutants.

Hyperglycosylated rhGalTase proteins were expressed more efficiently in S. cerevisiae than N-deglycosylated $\beta_{1,4}$-GalTase (Malissard, et al., 1996). The overall $K_B$ of the anti-$\beta_{1,4}$-GalTase mAbs with the hyperglycosylated rhGalTase and the N-deglycosylated rhGalTase proteins were similar despite some differences seen in the $K_{ass}$ and $K_{diss}$ rates. This would suggest that the presence of N-linked carbohydrates (high-mannose structures) in rhGalTase were not affecting the overall conformation. However, the correct native oligosaccharides structures found in human and bovine milk $\beta_{1,4}$-GalTase may help in the folding of $\beta_{1,4}$-GalTase, as optimal enzyme activities and higher $K_B$ of the anti-$\beta_{1,4}$-GalTase mAbs were obtained with these proteins. The carbohydrates on $\beta_{1,4}$-GalTase may be an important factor in determining if $\beta_{1,4}$-GalTase can be crystallized for structural studies. Some glycoproteins will only crystallize following their deglycosylation, which results in a more homogeneous structure. However, the recent crystallization of TCR was only possible when expressed in a glycosylated form (Garcia et al., 1996).
6.4 The regulation of $\beta_{1,4}$-GalTase expression

B cell $\beta_{1,4}$-GalTase expression was regulated via the transfection of sense and antisense human $\beta_{1,4}$-GalTase cDNA. A maximum 40\% increase and decrease in $\beta_{1,4}$-GalTase activity in the B cell transfectants compared to the endogenous B cell $\beta_{1,4}$-GalTase activity was achieved. In theory, the inhibitory effect of antisense $\beta_{1,4}$-GalTase cDNA would have been controlled mainly at the translational level, whereas the increased expression of $\beta_{1,4}$-GalTase in the SpcDNA3 transfectants would have been regulated at the transcriptional, translational and post-translational levels.

ASpcDNA3 B cell transfectants were still expressing B cell $\beta_{1,4}$-GalTase, albeit at reduced levels, whereas the SpcDNA3 transfectants were expressing B cell $\beta_{1,4}$-GalTase and the transfected full-length $\beta_{1,4}$-GalTase protein cloned from human placenta (Masri, et al., 1988). Both types of $\beta_{1,4}$-GalTase were recognized by the anti-$\beta_{1,4}$-GalTase mAbs, and enzyme activities were readily detected using either GlcNAc-pITC-BSA or ovalbumin as acceptor substrates. No attempt was made to differentiate between the endogenous B cell $\beta_{1,4}$-GalTase expression and the stably transfected $\beta_{1,4}$-GalTase in the SpcDNA3 transfectants. Hence, the transfected B cells may have also been regulating their own $\beta_{1,4}$-GalTase expression in response to the altered levels. The stable transfections were performed in the same B cell line so as to minimize variables. B cells probably transcribed $\beta_{1,4}$-GalTase mainly from the 4.1-kb start site with lower levels from the 3.9-kb start site, in common with most somatic cell types (Harduin, et al., 1993).

The $\beta_{1,4}$-GalTase protein expressed in these B cell transfectants, and in the untransfected B cells, was found in the Golgi and also secreted into the culture medium. Although the amount of $\beta_{1,4}$-GalTase expressed in the Golgi and the culture medium varied between the SpcDNA3 and ASpcDNA3 B cell transfectants, the ratio of cell associated (Golgi): secreted soluble $\beta_{1,4}$-GalTase remained constant (3.3:1) in both the SpcDNA3 and ASpcDNA3 B cell transfectants. This indicates a specific functional role(s) for the secreted $\beta_{1,4}$-GalTase, rather than a mechanism for maintaining a constant level of $\beta_{1,4}$-GalTase within the Golgi.

6.5 The regulation of IgG galactosylation

It was possible to incorporate more galactose into IgG during its biosynthesis by utilizing B cells of higher $\beta_{1,4}$-GalTase activity. At equivalent time points in culture, the SpcDNA3 B cell transfectants consistently secreted more (up to 20\%) highly
galactosylated IgG than the ASpcDNA3 transfectants. This would imply that the levels of β1,4-GalTase were at sub-saturating levels within the Golgi, but a large proportion of IgG G0 structures were still secreted from the SpcDNA3 B cell transfectants. It may be that the IgG secreted from these B cell transfectants possessed variable region glycosylation, in which case further analysis of the F(ab')2 and Fc portions of the IgG molecule would be required to determine the location of the incorporated galactose residues.

Determining the various control points in protein glycosylation has important implications for the biotechnology industry and for some diseases. The expression system may not have the necessary complement of glycosyltransferases, or may use inappropriate glycosyltransferases resulting in an absent or novel oligosaccharide structure on the expressed glycoprotein. The consequences of these effects on the properties of the protein may be numerous, including loss or altered functional activity, antigenicity, proteolysis and altered clearance from the circulation (Varki, 1993). The affect the oligosaccharide moiety has on the biological activity of glycoproteins is often specified by the protein structure and the specific cell types expressing these glycoproteins and glycosyltransferases (Dharmesh et al., 1993). For example, the clearance rate of lutropin and hence the in vivo bioactivity is regulated by the sulphated N-linked oligosaccharides, SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα (Baenziger et al., 1992).

Fully galactosylated IgG structures were secreted from SpcDNA3 B cell transfectants which had been cultured for a longer time period, without any increase in β1,4-GalTase activity. This indicates that the level of β1,4-GalTase activity alone is not always sufficient to determine the degree of galactosylation on IgG. The rate of IgG assembly and the pathway of the de novo synthesized IgG may also be mechanisms which influence the interaction with β1,4-GalTase (section 1.3.7.5). A similar increase in IgG galactosylation was observed from the ASpcDNA3 B cell transfectants following a longer period in culture, although 10-15% IgG G0 structures were still secreted. These ASpcDNA3 B cell transfectants did show a small but steady increase in β1,4-GalTase activity throughout culture as the antisense effect was attenuated, probably through loss of plasmid copy numbers per transfectant.

It is unclear what mechanisms are acting at these later time points in culture to cause the shift of 40% IgG G0 structures to fully galactosylated IgG, and whether there are equivalent processes operating in the SpcDNA3 and ASpcDNA3 B cell transfectants. Different cell density growth conditions appeared to have had a greater impact on IgG galactosylation than the levels of B cell β1,4-GalTase activity (Kumpel, et al., 1994).
There are a number of other factors associated with cell culture which have an effect on glycosylation (Goochee and Monica, 1990; Patel, et al., 1992). Ammonium ions, which are a by-product of glutamine metabolism, decreased the level of sialic acid on IgG from murine plasma cells, without affecting the secretion rates (Thorens and Vassalli, 1986). The cell division time of HepG2 cells influenced GnT V activity which altered the final glycosylation of transferrin (Hahn and Goochee, 1992). We have also found that the increased IgG galactosylation during murine pregnancy was not correlated to any increase in splenic β1,4-GalTase mRNA or activity (Jeddi, et al., 1997). IgG galactosylation appears to be regulated at multiple levels, and these may be different depending upon the culture conditions in vitro or the local in vivo environment.

6.6 β1,4-GalTase expression in RA

The previously reported differences between the B cell β1,4-GalTase activities in RA and controls were not detectable using GlcNAc-pITC-BSA, but could be observed in the same samples when ovalbumin was used as the acceptor substrate. Thus it appears that the different B cell β1,4-GalTase activities found in patients with RA and in controls are to some extent dependent on the acceptor substrate. AsAg-IgG may also be a useful acceptor in vitro, although the oligosaccharides are buried within the Fcγ domain and it is less readily obtainable than ovalbumin. Data from RA and control B cell lysates suggest that both groups express similar levels of β1,4-GalTase protein and β1,4-GalTase mRNA (Jeddi, et al., 1996). This would indicate that a qualitative difference(s) in B cell β1,4-GalTase protein was a more probable cause for the reduced activity in RA patients, as previously argued (Furukawa, et al., 1990).

Higher serum levels of IgG G0 were found in patients with RA compared to the controls, but there was no correlation in the paired samples between reduced B cell β1,4-GalTase activity in the patients and the increased frequency of IgG G0 in the studies reported in this thesis. Peripheral blood (PB) is not a rich source for plasma cells, although B cells isolated from the PB of patients with RA can be activated in vitro to secrete high levels of IgG G0 (Bodman, et al., 1992). However, this does not necessarily mean that B cells in PB are the usual source for IgG G0 production. The major sites for spontaneous production of IgG G0 vary between animal models of arthritis, but have included PB and spleen in murine MRL lpr (Bodman, 1995).

Increased IgG G0 production has also been linked to increased levels of IL-6 (Nakao, et al., 1991; Rook, et al., 1991a). This cytokine is a B cell differentiation factor and induces the proliferation of plasma cells (Van Snick, 1990). IL-6 modulated the
activities of a number of glycosyltransferases, including β1,4-GalTase, in a human myeloma cell line (Nakao, et al., 1990). However, we have not detected changes in splenic β1,4-GalTase mRNA or activity in mice transgenic for IL-6, despite the increase in IgG G0 structures (Jeddi et al., manuscript in preparation). Another possible mechanism controlling IgG G0 production appears to operate during pregnancy, whereby a fall in IgG G0 in RA, animal models of arthritis and in patients with Crohn's disease is concomitant with a remission of the disease (Rook, et al., 1991b; Thompson, et al., 1992; Pilkington, et al., 1996).

The IgG G0 structures have been implicated in the disease pathogenesis of RA (Rademacher, et al., 1994). These structures may be self-associating into small complexes which are not effectively cleared by the reticuloendothelial system, and could lead to their deposition in tissues. When these IgG G0 structures are produced within the synovium and complexes form, accessory cells can release inflammatory mediators resulting in severe swelling of the joint. Further, local production of IgG G0 autoantibodies by plasma cells in the joint may direct the inflammatory reaction to the autoantigen, resulting in tissue damage and deformity. The possible failure of other mechanisms, such as the loss of negative feedback of IgG G0 molecules through ineffective FcγR interaction, may perpetuate IgG G0 production.

6.7 Future work on β1,4-GalTase

The acceptor substrate preferences, including branch specificities, of the recently described β1,4-GalTase enzymes need to be resolved and correlated with their specific cell/tissue expression (Asano, et al., 1997; Lu, et al., 1997; Sato, et al., 1997). Other evidence also supports the existence of multiple β1,4-GalTase in mammalian cells (Sheares and Carlson, 1984; Shaper, et al., 1995) (Dr. Pamela Stanley, Albert Einstein College of Medicine, New York, USA, personal communication). The donor substrate preferences of these novel β1,4-GalTases should also be assessed in the presence of α-lactalbumin (Do, et al., 1995). It is likely that several more β1,4-GalTase enzymes could be detected using sequences found within the newly cloned β1,4-GalTase, and thus expand this gene family (Bakker, et al., 1994). As both the carbohydrate and the aglycone moieties can be involved in glycosyltransferase reactions, acceptor substrates such as GlcNAc-pITC-BSA may prove useful in identifying different β1,4-GalTase activities (Baenziger, 1994; Portner, et al., 1996).

X-ray crystallography of several recombinant forms of β1,4-GalTase is currently being
attempted by several independent groups. The production of conformationally-dependent anti-β1,4-GalTase mAbs may also be of use in structural studies of β1,4-GalTase.

Small molecular inhibitors, specifically against the active site of β1,4-GalTase would be useful reagents in understanding β1,4-GalTase functions as well as those of defectively galactosylated glycoproteins.

A recent report has shown that a recombinant soluble form of α1,3-GalTase expressed in human 293 cells was comparable to the full-length enzyme in its ability to galactosylate both membrane-bound and secreted de novo synthesized glycoproteins, although its efficiency was perhaps less than the full-length enzyme with the membrane-bound glycoproteins (Cho and Cummings, 1997). The processing of β1,4-GalTase within the Golgi produces soluble β1,4-GalTases which may have a specific function, such as a GlcNAc binding lectin. Investigation of the proteases and the specific cleavage sites in β1,4-GalTase may prove worthwhile.

6.8 Future work on the defective galactosylation of IgG in RA

It would be interesting to specifically upregulate β1,4-GalTase activity in B cells from RA patients to determine if the galactosylation defect in IgG could be corrected. This may be attempted via gene transfection or possibly through the expression of non-glycosylated anti-β1,4-GalTase mAbs, which can upregulate β1,4-GalTase activity. Such approaches, if successful, may be tested in animal model of arthritis.

The use of different β1,4-GalTase transcriptional start sites in RA may lead to alternative cell localization (Youakim, et al., 1994a). Another possible explanation for the different β1,4-GalTase activities between RA and controls may be the existence of multiple β1,4-GalTase enzymes present within individual cells. Funukawa et al (1990) first proposed the existence of an IgG-specific β1,4-GalTase, which in the light of novel β1,4-GalTases being described, deserves further investigation. AsAg-transferrin was not the acceptor of choice for one of the recently cloned β1,4-GalTase (Sato, et al., 1997).

Tn syndrome, a glycosylation disorder found in some hematopoietic cells, is due to a deficiency in β1,3-GalTase, which can in some cases be a result of continual (although reversible) repression of a functional allele (Thurnher et al., 1993). No inhibitors of B
cell β1,4-GalTase activity have been found in RA (Furukawa, et al., 1990; Axford, et al., 1994b).

Although no allelic variants of the β1,4-GalTase gene have been associated with RA (Axford and Alavi, 1994a; Jeddi, et al., 1996), it is entirely possible that structural differences between the RA and control B cell β1,4-GalTase proteins exist. The highly purified human milk β1,4-GalTase protein has many different isoforms (Gerber, et al., 1979). Enzyme activities between the isoforms using various acceptors was considered to be similar by the authors, although an approximately 3-fold difference between the less acidic forms was observed (Gerber, et al., 1979). Some of the charge heterogeneity differences may be due to post-translational modifications, such as phosphorylation which is known to affect β1,4-GalTase activity (Strous, et al., 1987; Bunnell, et al., 1990a). The different isoforms of human milk β1,4-GalTase may also be due small structural differences as demonstrated by the generation of different peptide fragments following Cleveland digests with V8 protease and chymotrypsin (Berger, 1986). Although milk is a rich source of β1,4-GalTase, it is difficult to account for its heterogeneity as it is usually purified from pooled samples of different donors and may also have been derived from several different cell types.

Structural differences in β1,4-GalTase from patients with RA may be examined using the epitope specific anti-β1,4-GalTase mAbs in permeabilized B cells. These mAbs could also be used in combination with anti-phosphoserine mAbs to examine post-translational modifications. Whether different forms of B cell β1,4-GalTase result from cell specific proteases, in patients with RA, remains to be determined. It is however noteworthy that a single amino acid change (Tyr\(^{123}\)Cys) found in two naturally occurring types of ST6Gal I, altered their catalytic activity, processing and cellular localization (Ma, et al., 1997). A similar structural change in B cell β1,4-GalTase could explain the different activities found in patients with RA. A switch in RNA editing between the two forms could account for the reversible nature of IgG G0 observed in patients during pregnancy (Rook, et al., 1991b).

Further examination of the T cell population from patients with RA have reported a decrease in the β1,4-GalTase activity (Axford, et al., 1992), contrary to another report (Furukawa, et al., 1990). If such a reduction in β1,4-GalTase activities did exist in T cells then the galactosylation of a number of other glycoproteins would be expected to be affected. The galactosylation defect in RA patients has so far been limited to IgG (Field, et al., 1994a). This may be due to the fact that the oligosaccharides in IgG are buried within the Fc\(\gamma\) domain and therefore present a less accessible substrate to β1,4-
GalTase. The recent crystallization of the T cell receptor (TCR) has demonstrated that there is an N-linked oligosaccharide between the Cα and the Cβ domains (Garcia, et al., 1996). Therefore, it may be worthwhile examining the TCR for glycosylation abnormalities from patients with RA. Further, the B cell β1,4-GalTase activities in other diseases (apart from RA) which are associated with high incidence of IgG G0 should be assayed.

A significant association between reduced GalTase activity and the presence of RF was detected and, therefore, may be important in complex formation (Hay, et al., 1991; Axford, et al., 1994b). Examination of β1,4-GalTase in the CD5+ B cell population in RA, which is expanded and secretes RF, would be useful (Burastero, et al., 1988). Although it would be practically demanding to obtain sufficient sample material, glycosyltransferases assays may soon be sensitive enough to accommodate such small samples (Palcic, 1997).

The production, assembly and possible compartmentalization of IgG in human plasma cells needs to be investigated. An oxidative environment, such as that found in inflammatory sites in patients with RA, may cause the IgG interchain disulphide bridges to form faster or even in a different order which could influence the IgG galactosylation.

The regulation of B cell β1,4-GalTase expression can alter the galactosylation of IgG. However, IgG galactosylation may also be altered by several, probably independent, processes operating at multiple junctions. Further studies in the regulation of β1,4-GalTase expression will clarify the mechanisms involved in the biosynthesis of glycoconjugates and the function of the carbohydrate structures.
7. References


Klonisch, T., Panayotou, G., Edwards, P., Jackson, A.M., Berger, P., Delves, P.J.,
Lund, T. and Roitt, I.M. (1996). Enhancement in antigen binding by a combination of
synergy and antibody capture. *Immunology* 89: 165-171.

immunoglobulin G. *Glycobiology* 1: 5-8.


Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation

Purification and characterization of recombinant human beta 1-4 galactosyltransferase

isomerase and immunoglobulins by pokeweed mitogen in human lymphocytes.
*Hybridoma* 10: 651-657.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in

Kudo, T. and Narimatsu, H. (1995). The β1,4-galactosyltransferase gene is post-
transcriptionally regulated during differentiation of mouse F9 teratocarcinoma cells.
*Glycobiology* 5: 397-405.

of Golgi vesicular galactosyltransferase (lactose synthetase). *Eur. J. Biochem.* 195:
243-250.


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β1,4-Galactosyltransferase activity in B cells detected using a simple ELISA-based assay

Jeremy Keusch, Peter M. Lydyard, David A. Isenberg and Peter J. Delves

Departments of Immunology and 1Rheumatology Research, Arthur Stanley House, University College London Medical School, London W1P 9PG, UK

To whom correspondence should be addressed

Lymphoblastoid B cells can be used for neoglycoprotein synthesis due to the presence of a novel enzyme-linked immunosorbent assay (ELISA)-based method. This assay proved to be much simpler to use than the lengthy and expensive radiochemical assays commonly used, and has the additional advantage that it specifically identifies the enzyme mediating transfer via the Galβ1,4GlcNAc linkage. A F(ab')2 antibody against GalTase was able to specifically inhibit the reaction. Greater sensitivity for β1,4-GalTase activity was obtained using GlcNAc-pITC-BSA as an acceptor substrate rather than ovalbumin. Low levels of β-galactosidase activity were detectable in lymphocyte cell lysates at acidic pH, although such activity was not detectable at the neutral pH used in the β1,4-GalTase assay. Using this assay with the GlcNAc-pITC-BSA acceptor, similar β1,4-GalTase activities were observed in CD19+ B cells from patients with rheumatoid arthritis (RA) to those seen in normal control individuals.

Key words: ELISA/β1,4-galactosyltransferase/lymphocyte/neoglycoprotein/radiochemical

Introduction

Glycosyltransferases are a family of an estimated 100 different membrane-bound enzymes involved in the assembly of carbohydrates on glycoproteins and glycolipids. One of the best studied glycosyltransferases is β1,4-galactosyltransferase (β1,4-GalTase) which catalyses the transfer of galactose from UDP-galactose to the non-reducing terminal end of N-acetylglucosamine (GlcNAc). The GlcNAc can be the free monomer sugar or part of an oligosaccharide chain, which may be attached to a protein or lipid.

Specific oligosaccharide structures on glycoproteins reflect the sequential activity of the corresponding glycosyltransferases (Kornfeld and Kornfeld, 1985), some of which may be protein specific (Baenziger, 1994). In some disease states altered glycosylation may be seen, such as in certain malignancies where an increase in sialylation occurs on serum α1-acid glycoprotein (Moule et al., 1987). It has been suggested that altered oligosaccharide structures on glycoproteins may induce a conformational change in the protein backbone with subsequent modified biological activity (Wright and Morrison, 1994). In patients with rheumatoid arthritis (RA), serum IgG has a lower level of galactose when compared to aged-matched controls, resulting in an increased number of the oligosaccharide chains within Fcγ terminating in GlcNAc (Parekh et al., 1988). This alteration in IgG glycosylation may have an important role to play in the pathology of RA (Rademacher et al., 1994; Malhotra et al., 1995).

The agalactosyl IgG 'defect' in patients with RA is reversible, as illustrated by the fact that patients who go into remission during pregnancy concurrently normalize their level of IgG galactose (Rook et al., 1991). The cause of the reduced galactose levels on IgG in these patients is currently unknown. Whilst B cells from RA patients have been shown to exhibit decreased GalTase activities when using ovalbumin as an acceptor (Axford et al., 1987; Wilson et al., 1993), in a study by Furukawa et al. (1990) only asialoagalacto-IgG, and not other substrates, was capable of detecting this reduction. All of these studies employed radiochemical assays to measure the amount of radiolabelled galactose incorporated into the glycoprotein acceptor. These assays are very time consuming, expensive and do not readily identify the linkage on the newly formed glycosylated product. Recently, a number of non-radioactive enzyme-linked immunosorbent assay (ELISA)-based assays have been developed for the determination of glycosyltransferase activities in serum or cells (Stults and Macher, 1990; Taki et al., 1990; Zatta et al., 1991; Keshvara et al., 1992). We have modified these assays to measure lymphoblastoid β1,4-GalTase activity using a neoglycoprotein, N-acetylglucosamine-phenylisothiocyanate-bovine serum albumin (GlcNAc-pITC-BSA), as an acceptor molecule. This assay is compared to a commonly used radiochemical assay.

Results

Specificity of the β1,4-GalTase reaction

The specificity of the β1,4-GalTase reaction was monitored in the ELISA plate with biotinylated lectins. As the β1,4-GalTase reaction progresses during the 1 h incubation period, the formation of the Galβ1,4-GlcNAc-pITC-BSA product results in a decrease in the Bandeiraea simplicifolia II (BSII) binding to terminal GlcNAc residues and a corresponding increase in Ricinus communis agglutinin I (RCAI) binding to galactose residues (Figure 1). The GalTase activity was specifically inhibited by a polyclonal rabbit F(ab')2 anti-GalTase antibody, but not by control rabbit F(ab')2 antibody (Figure 2). Inhibition of this type could theoretically be an artefact due to GalTase transferring galactose onto exposed terminal GlcNAc on the antibody if this were present on the rabbit F(ab')2 anti-GalTase but not on the control F(ab')2. However, this type of competitive substrate inhibition was excluded since lectin-binding studies on both
rabbit F(ab')₂ antibodies revealed only terminal sialic acids and a few terminal galactose residues, but no exposed GlcNAc (results not shown).

**Characterization of the ELISA**

β1,4-GalTase activity was routinely measured over a 1 h incubation period. Using GlcNAc-pITC-BSA as the acceptor substrate, we found that the ELISA method was linear over a 75 min incubation period with both partially purified bovine milk GalTase and with B-cell lysates (Figure 3). Measurements within and between ELISA plates proved to be very reproducible. In experiments using HB-1 B-cell line lysates randomly distributed over 40 wells in two separate plates, intra-plate variation was 45.2 ± 3.37 (mean nmol/mg protein/h ± SD, CV 7.4%) and inter-plate variation was 42.35 ± 4.03 (CV 9.5%).

**Comparison of ovalbumin and GlcNAc-pITC-BSA acceptors**

We found that ~22 GlcNAc residues were present per BSA molecule on the acceptor substrate neoglycoprotein GlcNAc-pITC-BSA as assessed using the benzothiazolone hydrazone method (Manzi and Varki, 1993), compared to ovalbumin which has only one N-linked oligosaccharide, of various structures, per protein molecule. The GlcNAc-pITC-BSA has a much lower apparent $K_m$, 20 μM (1.45 mg glycoprotein/ml, Figure 4).
compared to that previously reported for ovalbumin, 900 µM (40 mg glycoprotein/ml) (Verdon and Berger, 1983), the commonly used acceptor substrate for GalTase in the radiochemical assay. Indeed, when GlcNAc-pITC-BSA was compared with ovalbumin in both the ELISA and the radiochemical assays, a greater sensitivity for GalTase activity was achieved when using GlcNAc-pITC-BSA (Figure 5).

\[ \beta 1,4\text{-GalTase activity in CD}19^+ \text{lymphocytes} \]

Lymphocytic GalTase activity was readily detectable using either the ELISA or the radiochemical assays, although the absolute values obtained were different between the two assays. The limit of detection of partially purified bovine milk GalTase was found to be much lower with the radiochemical assay when compared to the ELISA. However, when using equivalent amounts of cell lysate sample, the ELISA detected greater GalTase activities than the radiochemical assay. Using GlcNAc-pITC-BSA as an acceptor, no statistically significant difference in GalTase activity was observed between 11 patients with RA and 10 normal control individuals using purified CD19⁺ B-cell populations in either the ELISA (Figure 6a, \( P > 0.2 \), unpaired Student's t-test) or the radiochemical assay (Figure 6b, \( P > 0.9 \)).

\[ \text{Discussion} \]

We have described an ELISA-based assay which provides a simple approach for measuring lymphocytic \( \beta 1,4\text{-GalTase activity} \). Furthermore, the newly formed product giving the \( N\)-acetyllactosamine structure may be detected with the biotinylated RCAI and, because RCAI binds with high affinity to Gal\( \beta 1,4 \) GlcNAc structures (Merkle and Cummings, 1987), only \( \beta 1,4\text{-GalTase activity} \) is measured. Thus, a greater degree of specificity is achieved compared to the more traditional radiochemical assays.

The enzyme activity could be blocked with a rabbit \( \text{F} \text{(ab')}^2 \) against GalTase. Although this antibody was raised against bovine milk GalTase, Western blotting and anti-GalTase ELISA confirmed that it cross-reacted with human milk GalTase (results not shown). Thus, this antibody preparation was able to inhibit both bovine and human GalTase activities. The possibility of the GalTase transferring galactose onto any exposed terminal GlcNAc present on the rabbit \( \text{F} \text{(ab')}^2 \) antibodies was ruled out by the finding that neither the anti-GalTase nor the control \( \text{F} \text{(ab')}^2 \) antibodies exhibited exposed GlcNAc residues.

The limit of detection of partially purified bovine milk GalTase was found to be lower with the radiochemical assay when compared to the ELISA. However, when using equivalent amounts of cell lysate, the ELISA detected greater GalTase...
Fig. 6. RA and control β1,4-GalTase B-cell activity using (a) the ELISA method and (b) the radiochemical method. Mean ± 1 SE of the mean is indicated.

Despite an apparent difference in sensitivity between the ELISA and radiochemical assays, the ELISA method with the neoglycoprotein GlcNAc–pITC–BSA is fully capable of measuring the levels of GalTase activity found in human lymphocytes. It has been observed that, using the radiochemical assay with ovalbumin as an acceptor, B cells from patients with RA have reduced levels of GalTase activity compared to controls (Axford et al., 1987; Wilson et al., 1993). In contrast, Furukawa et al. (1990) were only able to detect reduced B-cell GalTase activity in patients with RA when they used β-galactosidase-treated IgG, but not other commonly used acceptors, in the radiochemical assay. When we used the GlcNAc–pITC–BSA in the radiochemical assay or in the β1,4-linkage-specific ELISA assay we found no statistically significant difference between the two groups. The patients with RA used in the present study had significantly decreased levels of IgG galactose compared to the levels in the control group (p < 0.002, unpaired Student’s t-test). It is thus likely that the use of the GlcNAc–pITC–BSA neoglycoprotein accounts for the similar enzyme activity observed in these two groups. This substrate possesses ~22 GlcNAc molecules per BSA molecule, leading to a much lower apparent Km value that reported for ovalbumin. The oligosaccharide structures found on ovalbumin may, therefore, not be the ideal substrate upon which to sensitively measure lymphocytic β1,4-GalTase activity.

The ELISA method we describe was found to be extremely reproducible. The reagents required (GlcNAC–pITC, biotinylated lectins) are commercially available and the results can be simply assessed using an ELISA plate reader. The facility to identify the carbohydrate product by employing biotinylated lectins is an advantage over the radiochemical method. Theoretically, it would be possible to adapt this assay to examine other substrates and to measure the activities of other glycosyltransferases. N-Acetyllactosamine formed from the β1,4-GalTase reaction may act as the acceptor substrate for sialyltransferase, whose product may be identified with an appropriate biotinylated lectin, e.g. Sambucus nigra (SNA). When other considerations such as the rapidity and relative inexpense of the assay are borne in mind, then the ELISA we have described makes an attractive alternative assay for the investigation of lymphocytic β1,4-GalTase activity.

Materials and methods

Reagents

UDP-[6-3H]galactose was obtained from Amersham International (Amersham, UK), streptavidin–horseradish peroxidase (streptavidin–HRP) from Dako (High Wycombe, UK), biotinylated RCAI, BSII and SNA lectins from Vector Laboratories (Bretton, Peterborough, UK) and human milk β1,4-GalTase from Boehringer Mannheim (Lewes, East Sussex, UK). All other reagents, including bovine milk β1,4-GalTase, were from Sigma (Poole, Dorset, UK) unless otherwise stated.

Subjects

Peripheral blood (PB) was obtained from patients with RA who fulfilled the revised criteria of the American Rheumatism Association (Arnett et al., 1987). Control PB was donated by healthy volunteers. Assays were performed using B-cell lysates from 11 patients with RA (eight female, three male; age range: 36–88 years) and 10 control subjects (nine female, one male; age range: 30–78 years).
Lymphocytic β1,4-GalTase activity ELISA

34–78, mean 57 years) and 10 controls (three female, seven male; age range 26–65, mean 41 years). It has previously been shown that enzyme activity is not age related (Furukawa et al., 1990). Serum IgG galactoside levels were measured in these patients as previously described (Bodman et al., 1994).

Rabbit β1,4-GalTase antibodies

Rabbits were injected i.m. with 150 μg bovine milk β1,4-GalTase in complete Freund’s adjuvant, repeated at two-weekly intervals in Freund’s incomplete adjuvant. Titres were tested using a direct binding ELISA with bovine milk β1,4-GalTase coated onto the plate. IgG was purified from the antiserum using a protein G column and then pepsin digested (Hudson and Hay, 1989). The F(ab′)2 fragments generated were affinity purified on a bovine milk β1,4-GalTase-Sepharose column (Lopez et al., 1985). Verification of purity and specificity of these rabbit F(ab′)2 antibodies was performed using SDS–PAGE and Western blotting, respectively.

Analysis of the terminal glycosylation states of the rabbit F(ab′)2 antibodies

One hundred microlitres of rabbit F(ab′)2 anti-GalTase or non-immune rabbit F(ab′)2 were coated onto a Maxisorp microtitre plate (Nunc, Denmark) at 10 μg/ml in phosphate-buffered saline (PBS) for 2 h at 37°C. The plate was washed three times with 0.15 M PBS (pH 7.4), 0.05% Tween 20 (PBS-T), then blocked with 200 μl of 1% BSA in PBS at 1 h at 37°C. After washing, the plate was incubated with 100 μl of either SNA–biotin, RCAI–biotin or BSI–biotin at 2 μg/ml in PBS-T for 1 h at 37°C with the addition of 0.1 mM CaCl₂ for the SNA and BSI incubations. The plate was washed and 100 μl of 1 mg/ml streptavidin–HRP: 1:1000 in PBS-T added for 1 h at 37°C, followed by further washes and addition of 100 μl of 1 mg/ml o-phenylenediamine (OPD) in 0.1 M citrate buffer (pH 5.0) with 0.3% H₂O₂. The reaction was stopped after 5 min with 50 μl 3 N H₂SO₄ and absorbances read at 490 nM using a Dynatech MR5000 ELISA plate reader.

Preparation of GlcNAc–pITC–BSA neoglycoprotein

GlcNAc was bound covalently onto BSA using a slight modification of the method of McElroy et al. (1972). BSA, 60 mg of BSA in 1 ml of 0.3 M NaCl with 0.1 M NaHCO₃ (pH 10.0) were added to a 5 mg/ml solution of GlcNAc–pITC (Sigma) in the same buffer and continuously stirred for 18 h at room temperature. The reaction mixture was then extensively dialysed against distilled water and the neoglycoprotein, GlcNAc–pITC–BSA, lyophilized at −50°C, 5–10⁻¹⁴ atmos. and stored desiccated at −20°C. The number of GlcNAc molecules per BSA molecule was estimated using the benzothiazolone hydrazone assay (Manz and Varki, 1993).

Preparation of lymphoplastic cell antibodies

An immunized anti-hepatitis B-secreting human B-cell line, HB-1 (Shepherd et al., 1992), was used as a standard source of lymphoplastic β1,4-GalTase. PB was collected in heparinized tubes and the peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation on Lymphoprep (Nycomed, Denmark). B cells were separated from the PBMC by using anti-CD19-coated magnetic beads according to the manufacturer’s instructions (Dynabeads, Dynal, Wirral, UK). PB and HB-1 B-cell extracts were obtained by cell lysis using 0.2% (w/v) Triton X-100 in PBS for 30 min at 4°C. Following centrifugation at 15 000 g for 30 min at 4°C in an IEC Centra-3R centrifuge, supernatants were collected and stored at −70°C. Prior to assay, the lysates were centrifuged at 122 000 g for 5 min at room temperature. Protein concentrations were determined using the Bio-Rad protein microassay, with BSA as a standard (Bradford, 1976).

ELISA β1,4-GalTase assay

One hundred microlitres of GlcNAc–pITC–BSA, non-derivatized BSA or ovalbumin (chick egg albumin, grade VI) at 50 μg/ml in PBS were coated onto microtitre plates with PBS alone in the border wells, for 2 h at 37°C then overnight at 4°C. Plates were washed four times with PBS-T and then blocked with 200 μl of 1% BSA in PBS for 1 h at 37°C. Plates were washed and then doubling dilutions of partially purified bovine milk β1,4-GalTase (diluted in 0.1 M sodium cacodylate (pH 7.0), 0.5 M NaCl, 20 mM MnCl₂) or the test samples were loaded into the wells. The reaction was started by the addition of buffer to give a total incubation volume of 100 μl and containing final concentrations of 0.1 M sodium cacodylate (pH 7.0), 0.5 M NaCl, 20 mM MnCl₂, 0.5 mM ATP and 0.1 M UDP-galactose. Following incubation for 1 h at 37°C, the reaction mixture was washed out and the plates incubated for a further 1 h with 100 μl 0.5 μg/ml RCAI–biotin diluted in PBS-T. Plates were then washed prior to the addition of 100 μl of streptavidin–HRP at a 1:5000 dilution in PBS-T for 1 h at 37°C. Development of the plates using OPD was as above. The samples were quantified using the bovine milk GalTase standard curve (in lactose synthase units) and activity expressed in pmol/mg of protein.

Radiochemical GalTase assay

Cell lysates were incubated in Eppendorf tubes for 30 min at 37°C with final concentrations of 0.1 M sodium cacodylate (pH 7.0), 0.5 M NaCl, 20 mM MnCl₂, 0.5 mM ATP, 10 μg/ml ovalbumin or GlcNAc–pITC–BSA, and 0.1 mM UDP:β-D-galactose (100 μCi/mmol) in a total volume of 100 μl. Where Kₘ studies were performed, the acceptor substrate concentrations were as stated in Figure 4. Reactions were stopped by the addition of 1 ml of ice-cold 2% phosphotungstic acid in 0.5 M HCl. The precipitate was centrifuged at 4°C for 10 min at 3000 g in a microfuge. The pellet was resuspended and washed a further two times, then dissolved in 1 M NaOH. Samples were neutralized with CH₂COOH and prepared for liquid scintillation counting in a Minaxi Tri-Carb 4000 beta counter (United Technologies, Packard, Berkshire, UK) following the addition of Ecoscint A scintillant (National Diagnostics, Atlanta, GA). Enzyme activity was calculated from a standard curve, as stated for the ELISA assay.

β-Galactosidase assay

Fifty microlitres of cell lysate were pipetted into a microtitre plate and 110 μl of 0.1 M citrate buffer at a range of pH 2–8 were added. Following the addition of 50 μl of o-nitrophenyl β-D-galactosidase (ONPG) at 4 mg/ml, the plates were incubated at 37°C until colour development. The reaction was stopped with 90 μl 1 M NaCO₃ and the optical densities read at 410 nM. Purified jack bean β-galactosidase (Oxford Glycosystems, Oxford) and Escherichia coli β-galactosidase (Sigma) were used as positive controls.

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Abbreviations

BSI, Bandeiraea simplicifolia II; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; β1,4-GalTase, β1,4-galactosyltransferase; GlcNAc, N-acetylgalcosamine; GlcNAc–pITC–BSA, N-acetylgalcosamine–phenylisothiocyanate–bovine serum albumin; HRP, horseradish peroxidase; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PBS-T, phosphate-buffered saline; PBS, 0.15 M PBS (pH 7.4), 0.05% Tween 20; RA, rheumatoid arthritis; RCAI, Ricinus communis agglutinin I; SNA, Sambucus nigra.

References


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The Stability of Lymphocytic β1,4-Galactosyltransferase Expression During Pregnancy and Lactation

P. A. JEDDI, J. KEUSCH, P. M. LYDYARD & P. J. DELVES

Department of Immunology, Division of Pathology and Infectious Diseases, University College London Medical School, London, UK

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The authors used murine pregnancy as a model to investigate the regulation of IgG glycosylation. Pregnancy is associated with decreased levels of circulating IgG. The oligosaccharides on this IgG from late (day 15), but not early (day 8), pregnant Balb/c mice exhibited increased levels of terminal galactose. The levels remained elevated 8 days post-partum in lactating mice. Nonetheless, splenic β1,4-GalTase mRNA and enzyme activity remained relatively constant throughout pregnancy and into lactation. This was in contrast to a pregnancy-associated increase in mammary gland β1,4-GalTase mRNA. Thus the increased IgG galactose levels seen in pregnancy are regulated by mechanisms which are independent of transcriptional control of β1,4-GalTase expression.

INTRODUCTION

Changes in IgG glycosylation have been reported in normal human and murine pregnancy [1-3]. The number of terminal galactose residues on N-linked oligosaccharides increases during normal pregnancy and following delivery, before eventually returning to normal levels. Such changes in IgG Fc associated glycosylation are potentially physiologically significant in that they have been shown to influence the effector function of IgG with respect to interaction with Clq [4], Fcγ receptors [4] and mannose-binding protein [5].

Beta 1,4-galactosyltransferase (β1,4-GalTase) is a widely distributed enzyme which participates in the glycosylation process in the Golgi by transferring galactose to acceptor sugars containing terminal non-reducing N-acetylglucosamine (GlcNac). It is therefore responsible for the addition of galactose to the N-linked oligosaccharides on IgG. The expression of the β1,4-GalTase gene in mammary glands isolated from mid- to late pregnant and lactating mice is ~ 10-fold higher relative to somatic tissue [6], but the expression of this gene in lymphocytes during pregnancy has not previously been investigated.

To investigate whether the increase in IgG galactose levels seen in pregnancy are caused by up-regulation of lymphocytic β1,4-GalTase, we have measured the level of β1,4-GalTase gene expression and enzyme activity in spleen cells from pregnant and lactating mice in relation to the galactose content of their serum IgG.

MATERIALS AND METHODS

Animals. Female Balb/c strain mice (n = 22) were purchased at 10 weeks of age from Charles River Ltd (Margate, Kent, UK). Normal 9-week-old Balb/c males (n = 5) were purchased from the same supplier and used for syngeneic mating. The mice were maintained on a standard diet and housed one per cage.

Experimental design. Six female mice were left as controls and the others were mated with normal Balb/c males. The day of finding a vaginal (copulation) plug was designated the first day (day 1) of pregnancy. The females were separated from the males after detecting the vaginal plug and housed one per cage. Five mice were culled on day 8 and five mice on day 15 of pregnancy. Six mice were left to go through parturition and the litters were not weaned from the mothers until the termination of the studies, on day 8 post-partum. After killing mice by CO2 inhalation, blood was taken by cardiac puncture, spleens removed into ice-cold medium, and mammary glands snap-frozen in liquid nitrogen and kept at −70°C until further analysis. The mating was planned so that the average age of mice in each group was similar (16.5 ± 0.3 weeks) at the termination of the studies. Pregnancy in 8-day and 15-day pregnant mice was confirmed by observing the fetuses in the uterus.

IgG concentration. Serum IgG levels were measured by ELISA essentially as described by Engvall [7]. Ninety-six-well maxisorp
microtitre plates (Nunc) were coated overnight at 4°C with 100 µl/well of 5 µg/ml of affinity purified F(ab') fragment of goat anti-mouse IgG (Jackson Immunoresearch Laboratories, ME, USA) in PBS. After washing four times with PBS-0.05% Tween 20 (PBS-T) the plates were blocked with 200 µl/well 1% BSA in PBS. Calibrated mouse serum (The Binding Site, Birmingham, UK) was diluted to a range of 0.003-3µg/ml of IgG in PBS-T. Sera from the experimental mice, diluted 1:10,000, and the standards were tested in duplicate by adding 100 µl/well to the washed plates and incubating for 1 h at 37°C. After washing four times with PBS-T, bound IgG was detected using 100 µl/well of horseradish peroxidase-conjugated F(ab')2 sheep anti-mouse IgG (Sigma, Poole, UK) in PBS-T incubated for 1 h at 37°C. Plates were again washed four times with PBS-T, and 100 µl/well of the substrate p-nitrophenyl phosphate (Sigma) (2 tablets/10 ml 100 mM bicarbonate buffer pH 9.5) added. The reaction was stopped with 50 µl/well 1 M sodium hydroxide and the optical densities read at 410 nm. The immunoglobulin concentration of each sample was interpolated from the standard OD curve plotted on paper.

IgG terminal galactose. The levels of terminal galactose at two sites were measured by a modified version of a previously published assay [8]. Briefly, microtitre plates were coated with recombinant truncated protein G' (Sigma) and blocked with 0.05% Tween 20, 1% BSA in PBS. Sera were diluted 1:25 in 0.1 M glycine, 0.15 M NaCl pH 8.0 and added in triplicate (50 µl/well) to two identical plates and incubated for 2 h at 37°C. After washing, 50 µl/well PBS was added and the plates floated on a water bath at 85°C for 15 min to partially denature the IgG molecules and thus expose the oligosaccharides. Biotinylated lectin (Vector Laboratories Inc., Cambridgehire, UK), either Ricinus communis agglutinin I (RCAl, detects terminal galactose at 1 µg/ml in PBS-T-BSA, or Bandeiraea simplicifolia II (BSII, detects terminal GlcNAc) at 4 µg/ml in PBS-T-BSA containing 0.1 M calcium chloride, were added at 50 µl/well to the coated plates and incubated at 4°C overnight. Bound lectin was detected using Streptavidin-horseradish peroxidase (HRP, DAKO Ltd., Buckinghamshire, UK). The substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) with 1.200 hydrogen peroxide was read at 410 nm and results expressed as the ratio of RCAl/BSII binding. The samples compared in this study were tested at the same time and controls for the assay included sera with known ratios of RCal/BSII binding previously determined [9] in comparison with murine IgG whose glycoforms had been characterized by Professor T. Rademacher and colleagues at the Department of Biochemistry, University of Oxford, using the hydrazinolysis method.

Preparation of spleen cells. Spleens were removed into RPMI 1640 supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine and 50 µg/ml penicillin-streptomycin. Cell suspensions were prepared by disaggregation of spleen using a 20 µm wire mesh and then centrifuged at 350 g for 5 min. The pelleted cells were resuspended for 5 min in 0.16 M ammonium chloride pH 7.2 to lyse erythrocytes. After two washes in Hanks’ balanced salt solution (HBSS) the viability, which was always >90%, was assessed by acridine orange/ethidium bromide staining. Enzyme activity and gene expression measurements were performed on separate aliquots of the same sample.

3.1.4-GaITase enzyme activity. Enzyme activity was measured using ELISA as described previously [10].

3.1.4-GaITase gene expression. Total RNA was isolated from spleen cells and mammary tissues by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction [11]. A ribonuclease protection assay using the RPA II kit (Ambion) was utilized as described previously [12]. The probe recognizing murine 31.4-GaITase corresponds to nucleotides 704-1263 of the sequence published by Shaper et al. [13], and was made to a specific activity of approximately 8 x 10⁶ cpm/µg. The murine GAPDH probe (Ambion, Austin, TX, USA), used as an internal control to normalize the total amount of RNA, was synthesized to a purposefully lower specific activity of approximately 8 x 10⁶ cpm/µg. The murine β-actin probe (Ambion), used as an internal control in the mammary tissue (in which GAPDH has been reported to fluctuate [14]), was synthesized to a specific activity of approximately 1 x 10⁷ cpm/µg. Protected fragments were analysed on a polyacrylamide-urea gel. The level of 31.4-GaITase mRNA and of steady state control mRNA was assessed by phosphoimaging using a Bio-Rad GS-250 Molecular Imager (Bio-Rad, Richmond, CA, USA). Results are expressed as the ratio of 31.4-GaITase control mRNA signal.

RESULTS

Serum IgG concentration

The total IgG concentration was found to be reduced five fold in 15-days pregnant (P < 0.001) compared to the agematched non-pregnant mice (Fig. 1). Eight-days pregnant mice showed levels of serum IgG that were not significantly different (P = 0.84) from those found in the control non-pregnant mice.

IgG terminal galactose

The level of IgG galactosylation, as measured by the ratio of RCal/BSII binding, increased in 15-days pregnant (P < 0.001) and lactating (P < 0.001) mice compared to the age-matched non-pregnant mice (Fig. 2). Eight-days pregnant mice showed low levels of IgG galactosylation that was not significantly different (P = 0.34) from that found in the control non-pregnant mice. To exclude the possibility that the decreased levels of IgG in the pregnancy and post-partum sera would influence the result, the ELISA plates were coated with a concentration of Protein G' which would be fully saturated with IgG even when using those sera in which the lowest levels of IgG were found.

31.4-GaITase enzyme activity

The level of 31.4-GaITase enzyme activity (Fig. 3) did not change significantly in spleen cells during the gestation period and post-partum compared to the enzyme activity in control non-pregnant mice.

31.4-GaITase gene expression

Spleen cells from non-pregnant, 8- and 15-days pregnant, and lactating mice had comparable levels of 31.4-GaITase mRNA. The level of 31.4-GaITase mRNA in mammary tissue, however, was increased significantly in the 15-days pregnant mice compared with 8-days pregnant and was even higher in the lactating mice (Figs 4 & 5).
**DISCUSSION**

Increased levels of terminal galactose were observed on serum IgG from late (day 15), but not early (day 8), pregnant Balb/c mice. Rook and colleagues [1] detected a similar change in IgG galactose in DBA/1 mice but in this strain the changes were detected as early as the fourth day of pregnancy. Studies in CBA strain mice [2] and in human subjects [1,3] have also shown increased IgG galactose during pregnancy. In the present study the level of IgG galactosylation in the Balb/c mice was shown to be still raised 8 days post-partum in lactating mice.

Fifteen day pregnant and lactating Balb/c mice were shown to have serum IgG levels that were reduced by approximately 80% compared to the non-pregnant mice. Similarly striking decreases in IgG levels are seen in CBA/Ca (71% reduction at 16 days of pregnancy [15]) and C3H/He (approximately 70% reduction at 12 days of pregnancy [16]) mice. Our results show that the decrease in IgG levels in Balb/c mice is temporally related to the increase in IgG galactose levels, i.e. it was seen in late pregnancy and post-partum, but not during early pregnancy. Several factors have been proposed as contributing toward the dramatic decrease in IgG concentration during murine pregnancy, including the pregnancy-associated increased plasma volume [15,17], selective transport of IgG to the fetus [15], increased IgG degradation [15], selective B-cell clonal inactivation [16] and T-cell mediated suppression of immunoglobulin class switching [16]. B-cell development has been found to be suppressed during pregnancy [18] and lactation is known to delay the regeneration of B-cell precursors in the bone marrow of post-partum mice [18]. Serum IgG1 and IgG2 levels have also been shown to be reduced in lactating mice [19]. It is possible that a decreased rate of production of IgG might permit a longer trans-Golgi transit time, with a concomitant increase in the percentage of immunoglobulin heavy chains which become galactosylated.

The addition of galactose to terminal GlcNAc on the IgG oligosaccharide is dependent upon the presence of β1,4-GalTase. We found that during pregnancy expression of β1,4-GalTase mRNA in the spleen, a major source of circulating IgG, did not increase as determined using an extremely sensitive RPA.
technique, an approach which readily detected the known pregnancy-associated increase in the mammary gland [6]. Furthermore, the enzyme activity in the spleen remained relatively constant throughout pregnancy.

In this study we measured \( \beta 1.4 \)-GalTase mRNA levels in total spleen cells rather than in purified B cells. There is no evidence for B-cell specific regulatory elements within the \( \beta 1.4 \)-GalTase gene locus comparable to the mammary cell specific promoter [6] which may be responsive to hormones such as prolactin. Rillema and colleagues have shown that this hormone enhances the activity of, and mRNA accumulation for, \( \beta 1.4 \)-GalTase in mammary tissue [20, 21]. Although B cells also express receptors for prolactin [22] we have found no effect on \( \beta 1.4 \)-GalTase mRNA levels when human PBL were cultured in the presence of prolactin, insulin and hydrocortisone (PA Jeddi et al., unpublished observations); a combination which has been shown to increase \( \beta 1.4 \)-GalTase mRNA levels in mammary tissue [20, 21]. However, when prolactin, in combination with the cellular activators anti-IgM and interleukin-2 which cause increased IgG secretion by B-cells, was added to B cell enriched (non-plastic-adherent, SRBC rosette-depleted) peripheral blood mononuclear cells, there was a small increase in \( \beta 1.4 \)-GalTase mRNA compared to the levels without prolactin, but there was no effect on IgG terminal galactose levels (PA Jeddi et al., unpublished observations).

We have previously reported that splenic B cells purified from MRL-lpr/lpr mice, which have reduced levels of terminal galactose on their IgG [23], show similar levels of \( \beta 1.4 \)-GalTase mRNA relative to age-matched normal control CBA/Ca mice [9]. We have now examined \( \beta 1.4 \)-GalTase gene expression in a situation where there is increased levels of terminal galactose on IgG (i.e. pregnancy). Taken together these observations unequivocally show that significant changes in IgG galactose levels can occur in the absence of concomitant changes to the steady-state levels of lymphocytic \( \beta 1.4 \)-GalTase mRNA. In general, protein glycosylation is thought to be controlled by alterations in the activity of glycosyltransferases and that the amount of these enzymes is mainly regulated at the level of gene transcription, with enzyme activity directly correlating with the level of mRNA [24]. The present study shows, for the first time, that the physiological pregnancy-associated alteration in IgG...
glycosylation is not due to this type of regulation. Thus, protein structure effects related to the internal nature of the Fc-associated oligosaccharide [25] are likely to play a key role in the control of IgG glycosylation. Inter-heavy chain disulfide bond formation can take place either in the endoplasmic reticulum or in the Golgi complex [25], the precise location depending on several factors including the species and tissue origin of the IgG. It is entirely conceivable that above a certain limiting level of β1,4-GalTase gene expression, it is the rate and location of inter-heavy chain disulfide bond formation that determines the level of galactose on the IgG Fc-associated oligosaccharides [27]. This would suggest that reduced levels of galactosyltransferase would not lead to a reduction in IgG galactosylation, so long as the amount of enzyme remained above a threshold level. Such a scenario is supported by the finding that the level of galactose on human monoclonal antibodies is dependent on the culture method and does not correlate with the level of β1,4-GalTase activities in the cell lines producing these antibodies [28].

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REFERENCES

9 Jedd PA. Bodman-Smith KB. Lund T et al. Agalactosyl IgG and β1,4-galactosyltransferase gene expression in rheumatoid arthritis patients and in the arthritis-prone MRL lpr/lpr mouse. Immunology 1996;87:654–9.
12 Jedd PA. Lund T. Bodman KB et al. Reduced galactosyltransferase mRNA levels are associated with the galactosyl IgG found in arthritis-prone MRL-lpr/lpr strain mice. Immunology 1994;83:484–8.

16 Yokoyama M, Koga Y, Taniguchi K et al. Lymphocytes emigrating from the thymus to the spleen during postpartum regulate serum immunoglobulin levels in mice. Immunology 1988;63:151–6.


