# ENGINEERING PROTEIN GLYCOSYLATION IN CHINESE HAMSTER OVARY CELLS

## GENETIC MANIPULATIONS, GLOBAL GLYCOPROTEIN ANALYSIS, AND STUDIES OF ENVIRONMENTAL INFLUENCES

Thesis by

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# Abstract

The ability to engineer the glycosylation of proteins, and particularly recombinant proteins, would be of great benefit in the study and production of glycoproteins. One way to alter glycosylation is to genetically manipulate glycosyltransferase expression in a host cell. Two approaches of genetic manipulation of glycosyltransferase expression were explored: the use of a cell line with a defined alteration in the glycosylation pathway (a glycosylation "mutant") and the introduction by transfection of a new glycosyltransferase activity under the control of an inducible promoter. Additionally, the extent to which a particular genetic manipulation in the glycosylation pathway could be influenced or limited by either protein-specific effects or environmental conditions was evaluated. Optimized methods for globally surveying the response of individual glycoproteins to genetic alterations in glycosyltransferase expression were developed to aid in these evaluations. Among other results, it was demonstrated that by transfecting into a host cell the cloned glycosyltransferase β-galactoside α2,6-sialyltransferase under the control of the inducible MMTV promoter, the oligosaccharides of tissue-type plasminogen activator (tPA) could be altered, under induction, to possess α2,6-linked sialic acid, a modification not detected on tPA of untransfected or uninduced cells. The methods of genetically manipulating glycosylation and evaluating the outcome of such manipulations which were explored in this report should be widely useful in efforts to tailor glycoprotein oligosaccharide structures for specific applications and to control the glycosylation of glycoproteins made in large-scale culture.

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# PROLOGUE

Often, chemical engineers who find themselves dabbling in biology are asked, "So, what does this have to do with chemical engineering?" Since this thesis could easily prompt the same question from both the well-meaning and the cynical, it was thought that this question should be addressed at the outset. To avoid getting into the details of this work before their time, the author would like to consider the question on a general level, beginning with an undocumented, highly speculative account of how chemical engineers came to look like biologists.

Biologists are superb at finding solutions to biological problems – on a small, experimental scale. At some point in the not too distant past, biologists began to realize that they did not have the time or the expertise to deal with the issues of expanding their laboratory scale successes to meet the demands of a world-wide market. It was likely at this time that chemical engineers first entered the biological scene. Probably at first, making a biological product was very similar to making a chemical product, and the application of chemical engineering principles to this new arena was straightforward. As the production of biologicals grew more complex, chemical engineers continued to rise to the challenge, resulting in the development of a whole branch of chemical engineering devoted to bringing biological discoveries to the world. For the most part, these specialized chemical engineers continued to look like traditional chemical engineers because they were using chemical engineering tools to address the challenges they met.

But recently, chemical engineers working with biological products derived from live cells have realized that by virtue of what they put in their reactors, they have access to all of the tools that have been developed in the field of biology. Biochemical engineers are not restricted to the use of traditional chemical engineering methods – they have the option of utilizing biological tools when it seems appropriate. For instance, if cells in a reactor are not getting enough oxygen, a chemical engineer could use traditional chemical engineering methods to increase the amount of oxygen available to the cells, or a chemical engineer could borrow some tools from biology and genetically engineer the cells to better utilize the oxygen that is already present. As another example, if a protein product was difficult to separate from a complex mixture using traditional separation methods, a chemical engineer could attempt to devise a new separation method; alternatively, a chemical engineer could borrow tools from biology and genetically engineer the protein to possess metal binding sites which would allow the protein to be removed from the mixture by using a metal affinity column. Many chemical engineers who are availing themselves of these new approaches are looking less and less like chemical engineers, but in reality they are solving the same problems that chemical engineers have always solved, just with different tools.

So what does this thesis have to do with chemical engineering? The control of glycosylation of recombinant proteins is a challenge which is being addressed by the chemical engineering community, and the author, as a chemical engineer, has joined the search for a solution. According to good engineering principles, the problem was investigated thoroughly, and the author selected the best tools available to bring about a solution – this thesis is the result.

# Chapter 1

# INTRODUCTION

Proteins are essential components of every living thing. They serve living cells and systems in a variety of ways. For many years the underlying concept of protein synthesis was that one gene is transcribed into one mRNA, which is translated into one protein. However, in recent years this model has been challenged by a number of exceptions to the rule, such as gene splicing, mRNA splicing and modification, and post-translational modification. As a result of these various opportunities for modification, proteins of different functionalities can be derived from a single gene.

## Glycoproteins and Glycosylation

One post-translational modification which many cell surface and secreted proteins produced in mammalian cells undergo is termed "glycosylation;" glycosylation is the addition of carbohydrates to a polypeptide backbone to create a glycoprotein. There are two main types of glycosylation. O-linked glycosylation is characterized by an O-glycosidic bond between a carbohydrate and a polypeptide, most commonly at a serine (Ser) or threonine (Thr) residue. N-linked glycosylation is characterized by an N-glycosidic bond between a carbohydrate and a polypeptide, which occurs exclusively at asparagine (Asn) residues in the consensus sequence Asn-X-Thr/Ser, where X is any amino acid. Typically, both N-linked and O-linked glycosylation occur in a cell simultaneously, and a single protein molecule can possess both N-linked and O-linked carbohydrates. Many different aspects of glycosylation and glycobiology have been reviewed (for example, see ref. [25, 79]). While much of what follows concerning the nature, study, and manipulation of glycoproteins can be applied to either type of glycosylation, the focus of this report will be N-linked glycosylation.

## Assembly of N-linked Oligosaccharides

Glycosylation of a protein is not template-based like transcription or translation. N-linked glycosylation of a protein begins in the endoplasmic reticulum when a lipid-linked oligosaccharide is attached to a polypeptide chain through the R-group of an asparagine. This precursor oligosaccharide is then trimmed to remove three terminal glucose residues, resulting in a structure terminating in nine mannose residues. This structure can be further modified to yield three different classes of oligosaccharides [87]. As shown in Figure 1.1, the nine-mannose oligosaccharide, and any oligosaccharide which after further modification retains more than five mannose residues, is classified as high mannose. Oligosaccharides which have been acted on by N-acetylglucosaminyltransferase I (GlcNAc-T I) are labelled "hybrid;" oligosaccharides which have been further modified by N-acetylglucosaminyltransferase II (GlcNAc-T II) are termed "complex."

The complete matrix of pathways by which an oligosaccharide is processed to its final form is extremely complex because each intermediate oligosaccharide structure is likely a substrate for more than one glycosyltransferase, and most glycosyltransferases are capable of acting on more than one oligosaccharide structure. After modification by GlcNAc-T II, the core of a complex oligosaccharide can be further modified as shown in Figure 1.2. This figure is oversimplified in that it does not consider peripheral modifications, such as fucosylation, which can occur while the core structure is being formed, and it does not consider more unusual highly branched structures, such as the bisected pentaantennary oligosaccharides found on hen ovomucoid [149]. Each N-acetylglucosaminyltransferase (GlcNAc-T) shown establishes its own specific branch. The dark arrows with no enzyme listed represent further peripheral modifications, such as galactosylation, which prevent the oligosaccharide from being a substrate for other N-acetylglucosaminyltransferases and further branching. Action by GlcNAc-T III to add a bisecting N-acetylglucosamine also prevents further branching by making an oligosaccharide a non-substrate for GlcNAc-T II, IV, or V [17]. The core structures depicted then have the capacity to undergo a wide range of peripheral and terminal modifications, eventually yielding the completed oligosaccharide [148].

Heterogeneity of Oligosaccharides

One interesting aspect of glycosylation is that there is a tremendous amount of heterogeneity in the structures of glycoprotein oligosaccharides, and heterogeneity can be found at many levels. If one begins by considering a genetically homogenous population of cells grown under a defined set of conditions, three types of oligosaccharide heterogeneity can be observed. First, the oligosaccharides at a single glycosylation site on a single type of polypeptide backbone will probably have a range of structures. For example, any one of four core structures (biantennary, bisected biantennary, triantennary, or tetraantennary) can be found at a single glycosylation site on β-interferon when it is made in the PC8 cell line [81]. Those polypeptides which have identical attached oligosaccharide structures at identical sites are referred to as a single glycoform. A single cell type under constant conditions produces many different glycoforms of the same polypeptide backbone; this phenomenon is referred to as microheterogeneity. Furthermore, a single type of polypeptide backbone with two or more glycosylation sites will likely have a different distribution of oligosaccharide structures attached at each site [130, 147]; this is classified as site-dependent heterogeneity. For example, tissue-type plasminogen activator (tPA) made in human colon fibroblast cells has mostly high mannose structures attached at Asn-117, while mostly highly branched complex oligosaccharides are attached at Asn-448 [130]. A third type of heterogeneity is protein-dependent heterogeneity; in a single cell line, the distribution of oligosaccharide structures present on one type of polypeptide backbone may be significantly different from the distribution of oligosaccharide structures present on another type of polypeptide backbone.

To begin to understand the source of such heterogeneity, it is helpful to consider the environment in which oligosaccharide synthesis takes place. One analogy might be to view the newly formed polypeptide chains as being part of a river which flows through a series of pools; the series of pools would represent the different compartments of the Golgi bodies. One could picture the pools being lined with a variety of glycosyltransferases; each pool would have a different glycosyltransferase composition, which would be related to its position in the string of pools. A protein molecule would move somewhat "randomly" around each pool, encountering the various glycosyltransferases along the sides of the pool in no particular order. If an oligosaccharide did not possess the structural and conformational determinants required for modification by a glycosyltransferase it encountered, it would not be modified by that glycosyltransferase at that time. These interactions between the protein molecule and the glycosyltransferases would continue until the protein molecule was caught up by the river and moved onto the next pool, which would be lined with a different set of glycosyltransferases. The exact time a protein molecule spent in a pool would not be fixed, but would fall within a distribution, so some molecules would be in a particular pool longer than others.

With this picture in mind, some of the underlying causes of microheterogeneity might already be clear. In one extreme, a protein molecule could spend so long in a particular pool that it was modified by every available enzyme; in the other extreme, a protein molecule might get swept right through a pool without even coming near a glycosyltransferase. In between the two extremes there would be a whole host of interaction scenarios between a protein molecule and the glycosyltransferases of the pool. Additionally, one might recall that modification of an oligosaccharide by a particular glycosyltransferase may make that oligosaccharide a non-substrate for other glycosyltransferases. So while the order in which an oligosaccharide encounters various glycosyltransferases is not predetermined by a template or an assembly line, the order of encounter is extremely important in determining the structure of the oligosaccharide. Based on this understanding of the Golgi bodies and protein glycosylation, it is no surprise that the oligosaccharides attached at a single site on a polypeptide backbone are structurally heterogeneous.

Recently, it has been proposed that microheterogeneity could also be influenced by exoglycosidases at work in the Golgi apparatus or extracellularly. Exoglycosidases in the Golgi bodies could be in direct competition with the glycosyltransferases for determining the structure of an oligosaccharide; there might be ongoing addition and removal of sugar residues until the oligosaccharide became a non-substrate for one type of enzyme or the other. Extracellularly, there might be irreversible, but not universal, modification of oligosaccharides after a glycoprotein is secreted into the culture medium. One set of studies has shown that Chinese hamster ovary (CHO) cells and three other industrially-relevant cell lines possess non-negligible quantities of sialidase, β-hexominidase, and fucosidase activities extracellularly in the cell culture medium, activities which are reasonably stable and active at the pH of medium [50, 51]. It is not clear yet whether the

presence of these activities is intentional by the cell, or simply a result of cell lysis or aberrant sorting events. To study the influence of extracellular glycosidase activity on oligosaccharide structures, multiple studies of the effects of culture conditions on glycosylation have included the incubation of purified protein with cell-free conditioned medium or growing cells; these studies found no change in the oligosaccharide structures of the purified proteins as a result of the incubations [1, 27, 62, 131]. Thus, it is not clear in general what role extracellular glycosidase activity could or does play in determining the oligosaccharide structures of glycoproteins.

The understanding of the Golgi bodies and protein glycosylation which was developed to explain the heterogeneity in oligosaccharide structures at a single glycosylation site can be expanded to provide an understanding of site-dependent heterogeneity and protein-specific heterogeneity. In a nutshell, the secondary and tertiary structure of a protein in the local area of a glycosylated asparagine residue will significantly affect the ability of the attached oligosaccharide to be a substrate for a glycosyltransferase. The structure a polypeptide backbone assumes may make an oligosaccharide completely inaccessible to glycosyltransferases, or the polypeptide structure may force a developing oligosaccharide into a conformation which makes it a non-substrate for a glycosyltransferase [34]. The structure of a polypeptide backbone can also influence at what stage of development an oligosaccharide is a substrate for a particular glycosyltransferase. For example, an oligosaccharide which is attached to one type of secondary structure may be a good substrate for GlcNAc-T III after just the action of GlcNAc-T II, while an oligosaccharide attached to a different type of secondary structure may require the presence of one or more GlcNAc branches to bring its structural determinants into the proper conformation to allow modification by GlcNAc-T III [149]. There may even be cases where a glycosyltransferase is capable of interacting directly with the polypeptide backbone near an oligosaccharide to stabilize or destabilize the interaction of the glycosyltransferase and the oligosaccharide [34]. Furthermore, research on two different proteins, tPA and interleukin-6 (IL-6), has indicated that disulfide bond formation can inhibit oligosaccharide processing [4, 124].

Since an oligosaccharide's ability to interact with glycosyltransferases is largely dependent on the *local* secondary and tertiary structure of the attached polypeptide, it is reasonable to expect that the distribution of oligosaccharide structures at one asparagine site will be different from the distribution of oligosaccharide structures at another asparagine site on the same protein; this is the basis for site-dependent heterogeneity. For example, an N-glycosylation site which is attached to a portion of a protein that folds very quickly into a structure which makes the oligosaccharide inaccessible to glycosyltransferases may possess only high mannose and hybrid oligosaccharide structures, while other sites on the protein may remain exposed to glycosyltransferase activity long enough to have their attached oligosaccharides processed into highly branched complex structures. By considering two sites on different proteins, the above explanation for site-dependent heterogeneity easily extends to the case of protein-dependent heterogeneity.

Thus far, the discussion of heterogeneity in oligosaccharide distributions has been limited to the consideration of glycosylation phenomena which are believed to occur within a single cell; in practice, these phenomena are typically studied utilizing a population of genetically identical cells grown under a defined set of environmental conditions. A different type of heterogeneity can be seen when the glycoform distribution of a protein made under certain genetic and environmental conditions is compared with the glycoform distribution of the same protein made under different genetic and/or environmental conditions, such as in a different cell line or culture environment. This type of heterogeneity is referred to as "macroheterogeneity."

One example of macroheterogeneity may be observed when a single type of cell is cultured under different conditions. Researchers found that thyroid cells in primary *in vitro* culture produced glycoproteins in which more than 70% of the oligosaccharides had three or more branches, while glycoproteins from intact thyroid cells possessed less than half that amount of highly branched sugar chains [144]. There have also been extensive studies which have shown that changes in culture processes and conditions can have significant effects on the glycosylation of therapeutic recombinant proteins; this topic will be discussed at length in Chapter 5.

Macroheterogeneity can also be seen in the tissue-specific glycosylation of proteins within a single organism. Yamashita found that γ-glutamyltranspeptidase possessed bisected sugar structures when isolated from the mouse kidney, but not when isolated from the mouse liver [187]. In another study, Parekh, *et al.* compared brain-derived thy-1 with thymocyte-derived thy-1 and found there were no common glycoforms [127]. Glycoform distributions of glycoproteins can also shift as part of development [43, 88] or transformation [32, 99, 173].

Macroheterogeneity can also be observed in the species-specific glycosylation of identical polypeptide backbones. Most of the research on species-specific glycosylation has been done on therapeutically important glycoproteins as genetic engineers have sought to express human cDNA's in immortal mammalian cell lines to achieve large-scale production. Kagawa, *et al.* did a comparative analysis of the oligosaccharide structures present on β-interferon (β-IFN) made in CHO cells, mouse-derived C127 cells, and human lung carcinoma-derived PC8 cells, and on the protein found naturally in human circulation [81]. There were several shared glycoforms among the four samples, but there were marked differences both in the distribution of the glycoforms and also in the presence or absence of certain oligosaccharide features. For example, only β-IFN made in C127 cells and PC8 cells possessed the gal(α1-3)gal sequence, which is antigenic in humans. Similar studies have been done with tPA [128] and erythropoietin (EPO) [169]; each study found differences in the composition and distribution of glycoforms when a glycoprotein was made in different cell lines.

The explanation for microheterogeneity proposed earlier can be extended to explain the observance of macroheterogeneity. Once again, consider the Golgi bodies as pools lined with glycosyltransferases; the amount of substrate modified by a glycosyltransferase would be directly related to the amount of that glycosyltransferase activity present, and therefore, differences in the glycosyltransferase composition of a pool would result in differences in the distribution of oligosaccharide structures. In comparing cells with identical genomes (i.e. cells grown under different conditions or cells from different tissues), the amount of glycosyltransferase activity present in a pool would most likely be controlled at the level of mRNA synthesis [83, 154, 182], although other regulatory mechanisms have been proposed [reviewed in 11, 85]. Under some conditions, a glycosyltransferase present in the genome might not be expressed at all. Some evidence has indicated there can also be temporal changes in the activity of a glycosyltransferase due to a modification such as phosphorylation or even a change in glycosylation [120]. On a species-specific level, differences in the composition of the pool could also result from the presence or absence of a functional glycosyltransferase gene. This explanation for macroheterogeneity has been proposed previously [11, 132].

The hypothesis that the structure of oligosaccharides is determined to a large extent by the levels of expression of the various glycosyltransferase mRNA's and activities has been largely supported by the results of extensive research done on the relationships between oligosaccharides, glycosyltransferase activities, and glycosyltransferase mRNA expression levels. This large body of research has demonstrated direct correlations between: the presence of a particular glycosyltransferase and the presence of the oligosaccharide structure resulting from its action [43, 93, 99]; the level of mRNA and the level of activity of the encoded glycosyltransferase [133]; the level of mRNA and the level of oligosaccharide structures showing modification by the encoded glycosyltransferase [65, 78]; the level of a particular glycosyltransferase activity and the level of oligosaccharide structures showing modification by that glycosyltransferase [35, 111, 114, 121, 173, 189]; and the level of mRNA, the level of encoded glycosyltransferase activity, and the level of oligosaccharide structures showing modification by that glycosyltransferase [39, 106].

Additionally, some studies have demonstrated the importance of the relative levels of glycosyltransferase activities in determining the structure of oligosaccharides [117, 158]. If two glycosyltransferases are capable of modifying the same oligosaccharide, and the action of one will prevent the action of the other, the situation could be viewed as "competitive;" in such a competition, while the absolute amount of a glycosyltransferase would still determine the upper limit of how many oligosaccharides could be modified by that enzyme, the amount of that glycosyltransferase relative to a competing glycosyltransferase would probably determine how many oligosaccharides actually were modified by that enzyme. For example, one study provided evidence that an increase in GlcNAc-T III activity was correlated with the decreased synthesis of fucosylated polylactosaminoglycans, even though the activity levels of the enzymes directly responsible for polylactosaminoglycan synthesis did not change [18].

Despite the large body of evidence showing correlations between mRNA levels, glycosyltransferase activity levels, and oligosaccharide modification levels, there are examples where these correlations do not exist. One experiment demonstrated the total absence of an expected oligosaccharide modification in the presence of high levels of the glycosyltransferase activity and substrates necessary for the modification [44]. Another experiment determined there was no statistically-significant correlation between GlcNAc-T III activity and mRNA levels in a series of cell lines, although high GlcNAc-T III activity seemed to be associated with high levels of GlcNAc-T III mRNA [188]. In both reports, researchers postulated the involvement of other regulatory mechanisms.

Functions of Glycoprotein Oligosaccharides

The oligosaccharide moieties on proteins have been shown to be important in many different ways [37, 47, 79, 123, reviewed in 183]. In a very general sense, N-linked glycosylation has been shown to be essential for the full-term development of mice [76, 104]. However, most of the research on the function of oligosaccharides has been focused on the specific effects of glycosylation on individual proteins and the biological significance of differences in glycoforms.

Glycosylation can affect the physical characteristics of a protein. Glycosylation can increase a protein's solubility and reduce its tendency to aggregate [141, 183]. Glycosylation can also increase the thermostability of a protein [75, 118, 175]. Additionally, the presence of attached oligosaccharides has been shown to be necessary for proper protein folding numerous times [40, reviewed in 64, 74]; often, after a protein is properly folded, the oligosaccharides can be cleaved off with no effect on the protein [8, 30]. Furthermore, glycoproteins which, due either to mutation or chemical inhibition, are not glycosylated can show significant decreases in cell surface expression [167, 184] or secretion [90, 175, 184]. Proper glycosylation may also play a role in the correct cleavage of glycoproteins [28] and polarized secretion [84].

The activity of a glycoprotein can also be influenced by the nature of its attached oligosaccharides [90, 168, 170]. Absence or removal of specific sugar residues or entire oligosaccharides can increase or decrease the activity of a protein. For example, *in vitro* studies with EPO showed that removal of peripheral sialic acid, galactose, and N-acetylglucosamine residues increased the activity of the protein up to five-fold, while further trimming of the oligosaccharides decreased the activity of the protein, and complete removal of the sugar chains abolished all *in vitro* activity [171]. Additionally, a study of a range of naturally occurring glycoforms of plasminogen showed that the second-order rate constant for activation decreased as the sialic acid content increased [138]. Not only can glycosylation affect the quantitative activity of a glycoprotein, but it can also affect the qualitative activity of a protein; this can be seen profoundly in the case of the IgE-binding protein, where the oligosaccharides determine if the glycoprotein will be an enhancer or

suppresser of IgE biosynthesis [71]. Another form of activity, receptor binding and signal transduction, can also be influenced by glycosylation [91, 92]. Receptor-ligand interactions mediated by an Ig superfamily molecule can be significantly influenced just by the level of sialylation [16], and deglycosylated gp120 can bind to a CD4 receptor but is not capable of entering the cell to generate a productive infection [125, 126].

The safety and efficacy of glycoprotein therapeutics is also heavily dependent on glycosylation. First, the structure of a glycoprotein's oligosaccharides can be important in determining the clearance rate of the glycoprotein during *in vivo* circulation [12, 185]. The liver possesses receptors for specific oligosaccharides [7]; if a glycoprotein's oligosaccharide is bound by these carbohydrate-specific receptors, it is internalized and cleared from circulation. One such receptor is specific for asialoglycoproteins, that is, those proteins which have an exposed galactose residue due to the absence of a terminal sialic acid; injected glycoproteins which have exposed galactose residues are cleared from circulation quickly [41] and will not be as therapeutically effective as sialylated counterparts. Other receptors which are specific for N-acetylglucosamine- or mannose-terminated glycoprotein oligosaccharides have also been characterized [7].

Oligosaccharides can also be extremely important in determining the antigenicity of a protein; oligosaccharides can either be the antigenic determinant of a protein or they can mask the antigenic determinant of a protein's polypeptide backbone [2, 150]. As one example of the former, a significant amount of the normal human immunoglobin complement is directed specifically against terminal Gal($\alpha$1-3)Gal residues [42]; a glycoprotein therapeutic which possessed these residues on its oligosaccharides would not be a good choice for human injection. On the other hand, the presence of carbohydrates on the surface of a protein can mask potential antigenic epitopes, especially in the case of viruses attempting to avoid recognition. At least two examples have been studied where viruses have escaped neutralization by antibodies by the addition of a glycosylation site [15, 31]; furthermore, it is theorized that a major function of the twenty or more oligosaccharides on the HIV surface protein gp120 is to protect the protein from antibody recognition by interaction with endogenous lectins [107].

Glycosylation and the Large-Scale Production of Recombinant Glycoproteins

Glycoproteins present a special challenge to those who manufacture recombinant proteins on a large scale, especially for therapeutic use [reviewed in 79, 129]. Since oligosaccharide structure can markedly influence the biological properties of a glycoprotein, it is clear that the glycosylation of a recombinant protein product must be characterized, understood, and controlled to avoid unwanted biological consequences. The distribution of oligosaccharide structures must be considered carefully in initial experiments to create glycoprotein solutions to biological problems; furthermore, given the range of factors which can influence glycoform distributions, constant evaluation of a protein's glycoform distribution throughout product development and manufacture is necessary so that undesirable

variations can be detected and dealt with appropriately. For instance, consider the development of a typical glycoprotein therapeutic; it is developed and characterized utilizing relatively small-scale culture methods, and eventually, the glycoforms produced under those conditions are determined to be safe and effective. As the product is prepared for large scale production, the cell line and culture conditions have to be carefully selected to minimize differences between the small-scale and large-scale glycoform distributions. Additionally, research needs to be done to ensure that the large-scale recombinant protein has the appropriate *in vivo* activity, despite any differences in the small-scale and large-scale glycoform distributions. Once the product is in large scale production, the protein's glycosylation must be monitored to ensure that batch-to-batch variations in cells and culture environments do not result in significantly different glycoform distributions, such that the therapeutic effect of the glycoprotein is altered.

Traditionally, when faced with the challenge of controlling the glycosylation of recombinant proteins, biochemical engineers have taken the approach of assuming the cell to be a set of fixed parameters. With this view, many studies have attempted to control the glycosylation machinery of a cell by controlling the culture environment. This approach has led to the body of research summarized in reviews such as "Environmental Effects on Protein Glycosylation" [46] and others [47, 79]. Many other studies have evaluated the glycoform distributions of a protein of interest made in different mammalian cell hosts [81, 128, 130] to find the host cell which produced a glycoform distribution most similar to that which occurs naturally; this area of research could also be characterized as viewing each type of cell as a set of fixed parameters.

However, the work that follows is based on the view that the cell is a complex factory which can be investigated, understood, and retooled. Based on the model which was proposed earlier for the heterogeneous nature of glycoforms and glycoform distributions, it is proposed that genetic manipulation of glycosyltransferase expression can be used to change the absolute and relative composition of a glycosyltransferase pool in such a way as to produce desired glycoform distributions of cloned heterologous glycoproteins of interest. Defined genetic manipulation of glycosylation would allow one to create and analyze a full range of protein glycoforms to determine which glycoform best served the biological need; it would also allow one to manipulate a recombinant host cell line to generate a desired glycoform distribution in the context of large-scale bioprocess conditions. Thus, this work seeks to demonstrate the feasibility of this approach, that is, the manipulation of the glycosylation of cloned heterologous proteins by the genetic alteration of glycosyltransferase expression.

Genetic Alteration of Glycosyltransferase Expression

Genetic alteration of glycosyltransferase expression might be accomplished in several ways. First, one could select and/or screen for a mutant cell line which produces a high percentage of the desired oligosaccharide modification on its cell surface proteins, and then

introduce a cloned heterologous glycoprotein into this altered genetic and biochemical environment, with the expectation that the cloned protein would be affected in a manner similar to the cell surface proteins [165]. The drawback of this method is that it might require extensive screening to find a mutant cell line which expresses the desired shift in oligosaccharide structure. Second, one could employ the relatively new technique of using anti-sense RNA to complex a particular mRNA and reduce the amount of that mRNA which is translated into protein; this technique has been used to reduce the activity of a glycosylation pathway gene while avoiding the use of irreversible genetic intervention or the use of inhibitors which might exert pleiotropic effects [89]. Third, one might actively and precisely intervene in a cell's genetic code either by abolishing the expression of a specific undesired glycosyltransferase or by adding the genetic information to express a specific desired glycosyltransferase. For example, GlcNAc-T I activity has been abolished in mouse embryos by gene "knock-out" technology [76, 104], while in other research, GlcNAc-T I was successfully added to a mutant plant cell line which previously lacked the activity [45].

For the technique of genetically manipulating the glycosylation of a cloned protein to be widely used, it will be necessary to study the ability of the genetic glycosyltransferase manipulation to override normal protein-specific or environmental effects on glycoform distribution. For instance, it might be expected that in a cell in which the glycosyltransferase pool has been altered, some proteins will respond more or less than others (response being defined as a qualitative or quantitative shift in the structures of the attached oligosaccharides); this expectation is based on the common observation of protein-specific glycoform distributions. This protein-specific effect would be important if one is manipulating the glycosyltransferase expression of a cell with the intent of modifying the glycosylation of a particular protein; one's engineering efforts may be thwarted if that protein's properties preclude it from responding to the change in glycosyltransferase expression. In such a case, analysis of just the protein of interest would lead one to believe that the manipulation of glycosyltransferase expression had been unsuccessful, and further experiments might suffer from this erroneous conclusion. Thus, it would be useful to be able to evaluate quickly on a global level if protein-specific effects were influencing responses to a genetic manipulation in a significant way; this could be accomplished by studying the response of particular glycoproteins against the background of the responses of other glycoproteins. Additionally, it would be useful to develop a general technique for quickly and simultaneously evaluating the global and protein-specific effects on glycoform distributions of changes in environmental conditions in the presence of a genetic manipulation to determine which manipulation, genetic or environmental, takes precedence.

Methods to Analyze Changes in Oligosaccharide Structures

Changes in oligosaccharide structure distributions in response to changes in genetic or environmental conditions have been analyzed by a variety of approaches. Each method has advantages and disadvantages, and the most satisfying papers have utilized a combination

of methods in their investigations. Some methods study the total glycoprotein population of a cell without the discernment of individual proteins; one example of this approach would be the release and analysis of the oligosaccharides from a sample of total cellular proteins [137], and another example would be the use of lectins (proteins which recognize specific carbohydrate epitopes) in cell-binding experiments, followed by histochemical investigation [39, 97, 101, 151] or analysis by fluorescence-activated cell sorting [43, 106, 111]. Other methods begin by purifying a protein of interest made under different conditions; this is followed by detailed analysis of the attached oligosaccharides of the purified protein [81, 128, 130, 131, 169, 178] or by less detailed analyses of changes in the glycoprotein's molecular weight [23, 122] or isoelectric point [98, 178] as a result of changes in conditions. However, a definite disadvantage of these two types of methods is that one cannot look at changes in the glycosylation of a particular glycoprotein relative to changes in the rest of the glycoproteins present in the cell. The first approach gives no information on the oligosaccharide structures of any particular protein; the second approach does not give any information about the effects on glycoproteins other than the purified protein of interest.

A third class of methods studies changes in the glycosylation of mixtures of proteins by first separating the proteins using one-dimensional (1-D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzing changes in glycosylation by using lectins [14, 97, 173]. Separation of total proteins by 1-D SDS-PAGE followed by probing with lectins allows the response of a band at a certain molecular weight to be gauged against the response of bands at other molecular weights. However, this method is severely limited by the fact that one band on a gel at a particular molecular weight could be, and probably is, composed of multiple proteins of the same molecular weight, which may be responding differently to the lectins which are being used as probes; in this situation, lectin binding by a single protein could easily mask the absence of lectin binding to the rest of the proteins of that molecular weight.

Analysis of the response of individual glycoproteins in the context of a global survey of response can be accomplished by the use of two-dimensional (2-D) electrophoresis in combination with lectin binding analysis. In its most common use, 2-D electrophoresis (2-DE) separates proteins on the basis of two intrinsic and independent characteristics, molecular weight and isoelectric point; this technique allows one to separate a mixture of proteins into individual protein spots. When combined with lectin probing, this technique allows one to analyze changes in the glycosylation of a cell's glycoproteins, spot by spot, protein by protein. This approach allows one to easily study protein-specific responses to a genetic manipulation and/or easily survey the response of a field of individual glycoproteins to changes in environmental conditions, and in particular, to changes in environmental conditions on top of a genetic manipulation. There is no question that in many cases, further detailed structural analysis of a protein of interest will be necessary, but the 2-DE-lectin approach addresses questions which cannot be easily addressed by detailed

structural analysis of purified proteins, and, in many cases, should be addressed before detailed structural analysis is begun.

Recently, several studies have been published combining 2-D electrophoresis with lectin binding to analyze glycoprotein mixtures from various sources. These studies have all been interested only in getting a vague idea of the glycoprotein composition of a sample of interest, and have mostly used either the relatively general lectins concanavalin A (ConA) [19, 180, 181] and wheat germ agglutinin [52, 56], or the general glycoprotein stain based on the periodate reaction [105]. One other study used ConA as a general probe, followed by a mixture of *Sambucus nigra L.* lectin (SNA) and *Maackia amurensis* lectin (MAA) [10]; another study claimed to use ConA, *Aleuria aurantia* lectin (AAA), and the lectins from the DIG Glycan Differentiation Kit from Boehringer Mannheim, but only the results for the AAA lectin were shown [135]. These studies have demonstrated the resolving power of 2-D electrophoresis with regard to a sample containing a variety of glycoproteins, but none of these studies involved the comparison of lectin patterns of different samples, nor were any of these studies particularly interested in making statements about the structural nature of the glycoproteins they were studying.

On the other hand, many studies have utilized 2-D electrophoresis to analyze differences in 2-D protein patterns from different cells or from cells under different conditions, but these studies have usually not involved lectins. The journal "Electrophoresis" has an entire section of each issue devoted to 2-D electrophoresis, and a large percentage of the articles in this section are the application of 2-D electrophoresis to study differences or changes in total or specific protein patterns. Some studies of this nature have utilized lectins, but only in a very cursory sense. One study utilized 2-D electrophoresis and lectin staining to compare the lectin reactivities of sulfated glycoprotein-2 produced in related tissues; the only data which was presented in the paper was a chart summarizing the lectin reactivities of the particular proteins of interest (rated as "-," "+," "++," or "+++") and some 2-D blots showing proteins from different tissues which had been stained with a general glycoprotein stain [153]. Another study claimed to use 2-D electrophoresis with ConA and the Glycan Differentiation Kit to study the effects of bile salt exposure on pancreatic duct barrier function, but there were no pictures or charts presenting the results of this probing, and the results were only mentioned very casually in the text [57]. To summarize, while various combinations of lectins, 2-D electrophoresis, and comparisons of 2-D protein patterns have been undertaken previously, to the best of the author's knowledge, no research has been published to date utilizing 2-D electrophoresis in conjunction with lectin probing to analyze differences or changes in total glycoproteins as a result of differences in genetic or environmental conditions.

Outline of this Research

This study will explore the manipulation of glycosylation by genetic alteration of glycosyltransferase expression, and the susceptibility of this technique to protein-specific

and environmental effects, in two different systems. The first system will involve the genetic addition of a glycosyltransferase under the control of an inducible promoter and will study the effect of this manipulation on a specific cloned heterologous glycoprotein of interest. The second system will use a mutant cell line which uniquely expresses a glycosyltransferase of interest and will explore the interaction of this genetic manipulation with changes in environmental conditions. It is hoped that this research will demonstrate the usefulness and unique capabilities of this approach which will enable researchers to exert defined and predictable influences on the glycosylation of specific proteins of interest, and especially on the glycosylation of therapeutic glycoproteins produced on a large scale.

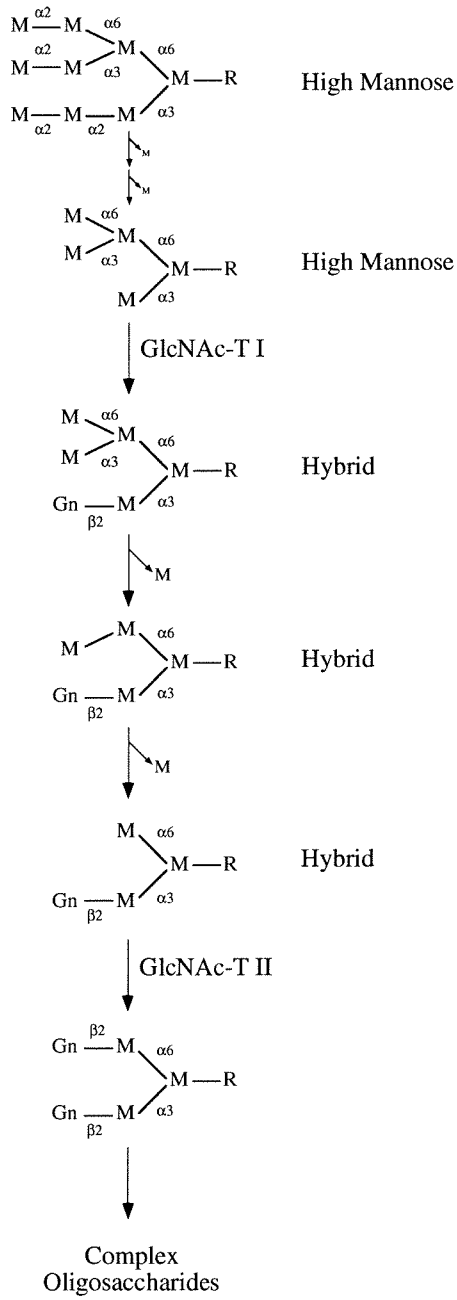


**Figure 1.1.** Pathway to determine the class of oligosaccharide. The following abbreviations are used: R, –β4Gnβ4Gn–Asn; M, mannose; Gn, N–acetylglucosamine; GlcNAc–T, N–acetylglucosamynltransferase.

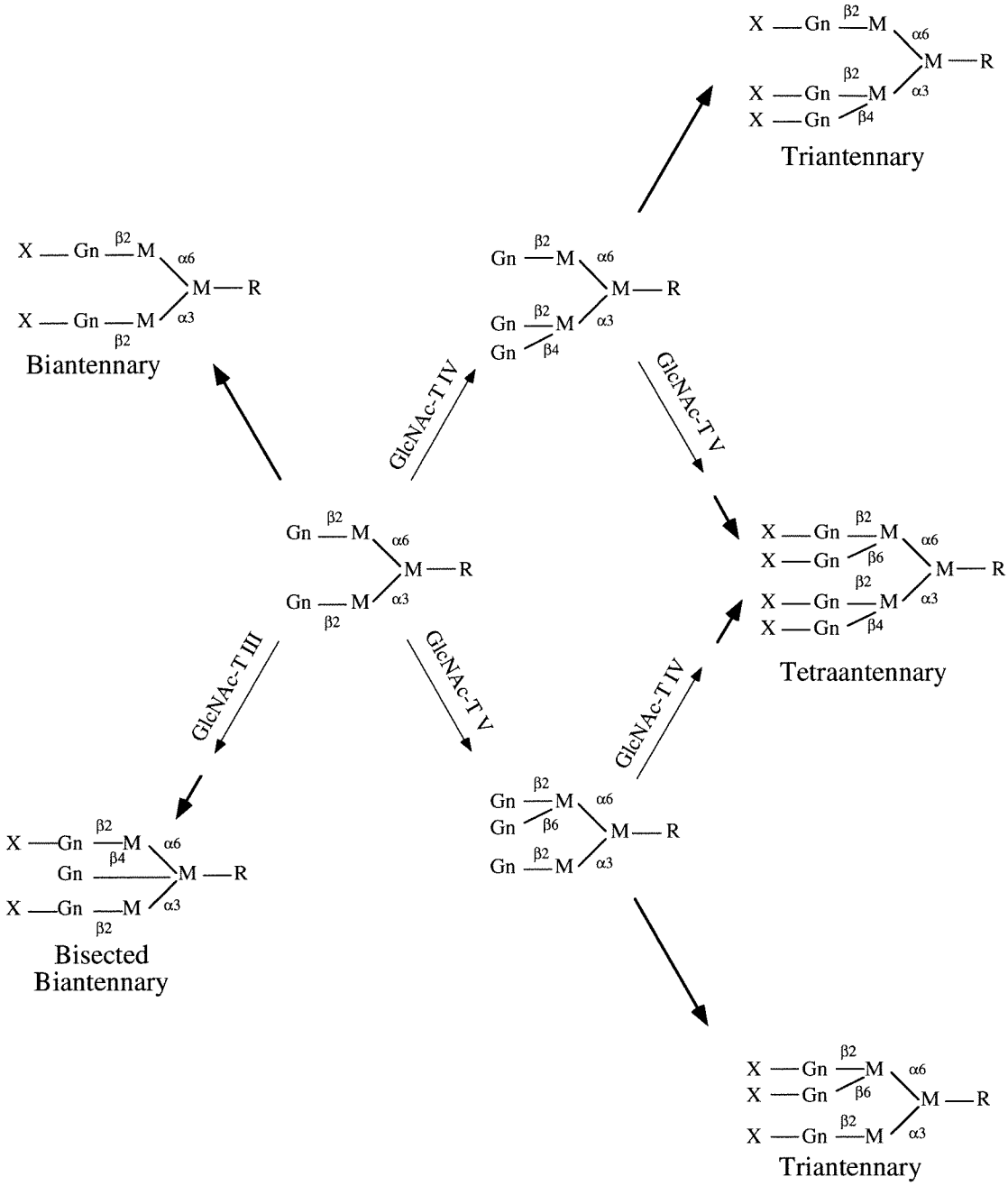


**Figure 1.2.** Formation of the core structure of complex oligosaccharides. The following abbreviations are used: R, –β4Gnβ4Gn–Asn; M, mannose; Gn, N–acetylglucosamine; GlcNAc–T, N–acetylglucosamynltransferase; X, peripheral residues, such as galactose and sialic acid.

# Chapter 2

## Development of Techniques to Perform a Global Survey of Glycoproteins

The first chapter of this research presented the rationale for using 2-D electrophoresis in conjunction with lectin staining to study changes in oligosaccharide structure in response to changes in cellular conditions, either internal or external. Two-dimensional electrophoresis of proteins by definition would be the use of any two electrophoretic protein separation techniques in series. However, the term has come to represent in common usage the use of a charge-based separation in the first dimension followed by a size-based separation in the second dimension. For this work, the term refers specifically to separation by charge utilizing isoelectric focusing (IEF) in the first dimension followed by separation by size utilizing SDS-PAGE in the second dimension. By way of introduction, the underlying principles of each type of separation will be considered in turn.

### Isoelectric Focusing of Proteins

Proteins are composed of amino acids. A single amino acid has a carboxyl group and an amino group, both of which can be proton acceptors or proton donors, depending on pH. An amino acid also has a side group, R, which might be ionizable; the charge of an ionizable R group will depend on the nature of the R group and the pH of the surrounding solution. In a chain of amino acids or a protein, the carboxyl groups and amino groups of all but the first and last amino acid are involved in peptide bonds and can no longer accept or donate protons. Thus, not considering the contribution of the single carboxyl group and the single amino group at the terminus of the polypeptide backbone, which will be the same for all polypeptides, the net charge of a polypeptide will be dependent only on the charges of the R groups, which are dependent on the pH of surrounding solution. For example, in a solution of a certain pH, a polypeptide with more negatively charged R groups than positively charged R groups will have a net negative charge; if the solution is made more acidic, some of the negatively charged and uncharged R groups will be protonated, and the charge of the molecule will become less negative. For every polypeptide backbone, there is a single pH at which the number of negatively charged R groups equals the number of positively charged R groups, and the polypeptide has no net charge. This pH is referred to as the isoelectric point (pI), and it is characteristic for a polypeptide.

Separation by charge is based on the fact that polypeptides are charged molecules at any pH other than their pI. If a mixture of proteins is placed between a positively charged anode and a negatively charged cathode in a solution of constant pH, polypeptides which are negatively charged at that pH will migrate toward the positively charged anode, and

polypeptides which are positively charged will migrate toward the negatively charged cathode. A variation of this simple, non-equilibrium system is isoelectric focusing. Isoelectric focusing occurs in a medium which has a pH gradient between the anode and the cathode; the pH is lowest at the anode and highest at the cathode. As a charged polypeptide moves through the pH gradient toward the terminus of opposite charge, it passes through the pH gradient, and the net charge of the polypeptide gradually decreases as its gains or looses protons; it continues to move until it reaches the pH at which it has no charge, its isoelectric point. At this point, having no charge, the polypeptide no longer has an impetus for movement and it remains stationary; if the polypeptide begins to drift away from the pH at which it has no charge, it regains a charge, which compels it to move in the opposite direction, back to its pI. Theoretically, the system should reach equilibrium and remain there.

This may be made more clear by example. Consider a polypeptide which, at the pH of entry into the separating medium, is positively charged. It begins to move toward the negatively charged cathode, passing through regions of increasing pH as it moves; as the pH increases, some of its protons are released, which causes a decrease in its positive charge, either by the acquisition of negatively charged groups or the loss of positively charged groups. It eventually reaches a pH where it has lost so many of its protons that the number of negatively charged groups equals the number of positively charged groups and it has no net charge, so it stops moving. If it were to drift back the way it came, it would enter a pH where it would gain protons and a positive charge, which would draw it back toward the cathode and its pI. If it were to drift toward the cathode, it would enter a pH where it would loose additional protons and gain a negative charge, which would draw it back toward the anode and its pI.

Isoelectric focusing can occur in several different media. Initially, the most common way to do isoelectric focusing was to establish a pH gradient using mobile carrier ampholytes which begin in a solution of uniform pH, but, when an electric field is applied, move quickly according to charge to set up a pH gradient where each carrier ampholyte is relatively stationary at its pI. However, this method has many problems associated with the mobile nature of the molecules establishing the pH gradient. One problem which is not understood or resolved is the fact that over time, the entire pH gradient shifts toward the cathode; as a result, the absolute positions in the IEF gel are dependent on focusing time, and significant portions of the pH gradient and focused protein can be lost off the end of the gel. Furthermore, the pH gradient can be significantly altered by the nature and concentrations of the proteins in the sample being separated, since the proteins themselves behave in a way similar to carrier ampholytes. Thus, if one is planning to compare samples which are vastly different in composition, there is little hope of being able to compare absolute or even relative positions in an IEF gel from sample to sample or experiment to experiment. For this reason, the experiments in this work were carried out using gels with an immobilized pH gradient (IPG) [24], where the constituents which form the pH gradient are covalently attached to the gel matrix.

SDS-PAGE Separation of Proteins

Separation of proteins by size in the realm of electrophoresis is also based on the movement of charged molecules toward an electrode of opposite charge. Specifically in the SDS-PAGE system, protein molecules are uniformly coated by sodium dodecyl sulphate, a negatively charged detergent, which interacts with polypeptides in a nearly constant ratio according to the molecular weight of the polypeptide. The negatively charged polypeptide is then placed in an electric field where it migrates toward the positively charged anode. The speed of migration is dependent primarily on two things. The first is the molecular weight of the polypeptide, which theoretically determines the amount of incorporated SDS, and thus, the amount of incorporated negative charge; a large molecule would incorporate more negative charge than a small molecule and would move more quickly toward a positively charged anode. The second factor in determining the speed of migration is the nature of the separating medium. SDS-PAGE is done in a medium that is porous, and the size of a polypeptide relative to the size of the pores will determine the speed at which that polypeptide is able to move through the pores; generally, smaller molecules will move through a given size pore more quickly than larger molecules. Overall, it is typically the retardation characteristics of the gel which prevail in determining the speed of migration, and the negative charge imparted by the SDS serves only to provide a motivation for movement; thus typically SDS-PAGE discriminates by size in that large molecules move more slowly through the pores than small molecules, with the result that the distance traveled in a given amount of time is inversely related to the size of the polypeptide. The pore size of a polyacrylamide gel can be chosen to maximize the difference between the migration speeds of the polypeptides one wishes to separate.

Two-Dimensional Electrophoresis of Glycoproteins

While many of the details of this two-dimensional electrophoresis system do not bear going into, there are some aspects which are of particular relevance in the quest to study glycoproteins. As has been mentioned, the separation in the first dimension is based on the migration of the polypeptide to the pH zone where it has an equal number of positive and negative charges across the total of its R groups. What has not been considered in this discussion is that many of the modifications polypeptides undergo as they mature into functional proteins significantly affect the charge of the completed protein. Specifically considering glycoproteins, a very common terminal modification on an attached oligosaccharide is the addition of sialic acid, which typically has a negative charge. Considering a protein with a given polypeptide backbone, one additional sialic acid molecule attached to the protein by way of an oligosaccharide will increase the negative charge on the protein molecule by one; the additional negative charge will cause the protein to move farther toward the positively charged anode to find a pH zone where it has no net charge. Thus the addition of a sialic acid residue to a protein will decrease its pI. Szkudlinski, *et al.* documented an inverse relationship between the sialic acid content of a

glycoform and its pI; they found that the isoforms of thyrotropin with the lowest pIs were richest in sialic acid [168]. Given a series of protein molecules with identical polypeptide backbones and increasing numbers of sialic acid, the protein molecules will separate into multiple zones in an IEF gel, with each zone being occupied by glycoforms having the same number of sialic acids; the species having the most sialic acids will be closest to the anode.

Furthermore, each sialic acid not only brings with it its charge, but also its molecular weight; in fact, often an additional sialic acid residue is just the terminal component of an entire additional oligosaccharide branch. A typical, simple branch consisting of just an N-acetylglucosamine, a galactose, and a sialic acid has a molecular weight of over 700 Daltons. This increase in molecular weight would manifest itself as a slower rate of migration in the second dimension. In addition, sialic acid can affect the binding of SDS to the protein, resulting in a reduction in the amount of incorporated SDS and a decrease in the migration rate [33]. Thus, an additional branch on the oligosaccharide of a protein would be expected to alter the position of that protein on a 2-D gel toward the acidic end of the focusing medium and to a higher molecular weight. Thus, the microheterogeneity of a glycoprotein as discussed in the first chapter would tend to manifest itself on a 2-D gel as a chain of spots which stretch up and to the acidic end of the focusing medium, with the length and angle of the chain being dependent on the protein and its distribution of oligosaccharide structures. This pattern has been demonstrated in published studies (e.g. [10, 157]).

Of course, not all glycoproteins would necessarily have this form. For example, high mannose oligosaccharide structures differ from each other only in the number of mannose residues, with the number of mannose residues ranging from five to nine. Glycoproteins which have only high mannose structures would not be expected to show changes in IEF position as a function of the number of mannose residues because mannose residues typically have no charge. Furthermore, since each mannose residue has a molecular weight of only 180 Daltons, one would not expect to see a wide distribution in molecular weight if a glycoprotein bearing only high mannose structures was separated on a typical SDS-PAGE gel.

Since a glycoform's position on a 2-D gel is dependent on its complement of attached oligosaccharides, 2-D electrophoresis has the potential to be a powerful technique in the study of glycoproteins. This type of separation, combined with immunostaining and/or lectin probing, could provide a large amount of information about the glycoform heterogeneity of a single protein, and even information about the specific oligosaccharide structures attached to a particular glycoform. The nature of this technique also allows the study of multiple proteins in a sample simultaneously.

The use of 2-D electrophoresis, however, is not yet a trivial matter. It is a largely empirical technique, with protocols being developed by optimizing conditions through pilot

experiments [142] rather than by the application of a universal formula or method. The composition of the sample (concentration of detergent, carrier ampholytes, urea, total protein), the nature of the isoelectric focusing gel (physical characteristics such as length and width, as well as biochemical characteristics such as the pH gradient and the composition of the liquid phase), and the physical parameters such as isoelectric focusing temperature and voltage all play important, inter-related roles in determining the effectiveness of a 2-D separation. Furthermore, conditions which facilitate the focusing of one protein may impede the focusing of another.

Extraction of Glycoproteins for Two-Dimensional Electrophoresis

The use of 2-D electrophoresis in the specific study of glycoproteins is complicated further by the fact that many glycoproteins of interest are integral membrane proteins. Integral membrane proteins typically have large regions which are designed to exist in the hydrophobic environment of the cellular lipid bilayer, and as a result, these regions tend to be uncomfortable in aqueous solution. This can lead to difficulties in extracting integral membrane proteins from the lipid bilayer; furthermore, once the proteins are extracted into an aqueous environment, interactions of the exposed hydrophobic regions on multiple protein molecules can cause aggregation.

Of course, difficulties associated with the extraction of glycoproteins from cellular lipid bilayers would be encountered no matter what type of final analysis was planned. However, the use of isoelectric focusing as an analytical tool places some restrictions on the methods which can be used to solubilize these difficult proteins. For example, guanidinium chloride is regarded as a very effective denaturant, but it is not compatible with isoelectric focusing because salt ions significantly increase the conductivity of the system, leading to high currents and excessive heating [5].

Detergents are also effective in solubilizing integral membrane proteins. The non-polar portion of a detergent molecule can bind to the hydrophobic region of a protein, which was previously imbedded in lipid, and the polar portion of the same detergent molecule can interact with the aqueous solution [3]; thus the hydrophobic regions of a protein can be protected from the aqueous environment. However, ionic detergents are not ideal for applications involving isoelectric focusing because the charge of the detergent which is bound to a protein molecule will significantly affect that protein's apparent pI [33]. Another potential problem of ionic detergents in isoelectric focusing is that their charge may compel them to release from the protein in order to migrate to an electrode [33], leaving the hydrophobic portions of the protein unprotected and open to aggregation; this is not a problem in SDS-PAGE because the proteins are immersed in a solution with an excess of SDS. Non-ionic detergents, by their very nature, do not cause such complications, but they are typically not as effective as their ionic relatives in solubilization applications [33]. Zwitterionic detergents are detergents which have both a positive and a negative charge, resulting in a net charge of zero; they have been shown to be effective for

protein solubilization, and one member of this group, CHAPS [69] is commonly used in isoelectric focusing applications.

Another very common solubilization agent, especially in the preparation of samples for isoelectric focusing applications, is urea [33]. Urea works by disrupting hydrogen bonds [33, 55, 72] and is compatible with isoelectric focusing because it does not affect the charge of a protein molecule. A good example of its usefulness was presented in research which showed, by the use of a gradient urea gel for IEF, that at increasing urea concentrations, a smear of orosomucoid resolved into several very distinct bands, with the clarity reaching a plateau at about 6 M urea and higher [3]. However, a common opinion seems to be that urea is not very effective, especially in comparison to SDS [9, 55].

In addition to considering the interaction between the solubilization method and the isoelectric focusing system, one must bear in mind that a solubilization method that works well for one protein may be working against the solubilization of another protein. For example, if a highly hydrophobic solubilization system is chosen to encourage the exit of hydrophobic domains from a lipid bilayer, the more hydrophilic proteins or portions of proteins may find themselves in an unfriendly environment and may have a tendency to aggregate. For all of these reasons, writings on protein solubilization typically recommend that several methods be tried in pilot experiments to determine the best method for each sample of interest [33].

Goal of this Research

Given the wide range of experimental possibilities and the relative lack of formulas and guides for determining optimal conditions for a two-dimensional separation *a priori*, the need to spend time developing optimized protocols for the solubilization and isoelectric focusing of glycoproteins becomes obvious. Furthermore, because the focus of this work is to study a mixture of proteins, the goal is not to optimize the resolution of a single protein, but rather to find a protocol which optimizes the resolution of the mixture of proteins as a whole. Additionally, because the objective of this research is to study the response of *all* of a cell's glycoproteins, it is necessary to determine what subset of the glycoproteins is not visualized by each of the methods. If no method can be found which allows the resolution of most of the glycoproteins at once, it will be necessary to employ multiple methods to ensure that the response of as many glycoproteins as possible is evaluated. The work of this chapter addresses these issues in the development of a protocol to survey cellular glycoproteins.

# Methods

*Reagents:* Most reagents used in electrophoresis were electrophoresis grade and were purchased from Sigma (glycerol, NP-40, iodoacetamide) or BioRad (sodium dodecyl sulfate (SDS), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea, glycine, BioLyte carrier ampholytes, dithiothreitol (DTT), piperazine diacrylamide (PDA), acrylamide, β-mercaptoethanol (BME), N,N,N',N',-tetramethylethylenediamine (TEMED), ammonium persulphate). Dimethylformamide (DMF) was from EM. Primary antibodies to vinculin, tropomyosin, actin, and tubulin were obtained from Sigma. The antibody to elongation factor-II (EF-2) was a kind gift of Professor A. C. Nairn (Rockefeller University, NY, NY 10021). Secondary antibodies to rabbit and mouse IgG were from Promega.

*Cell culture:* Pro-5 (ATCC CRL 1781) and LEC10 Chinese hamster ovary cells were grown in adherent monolayer culture in α-MEM (GIBCO) supplemented with 5% dialyzed fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO) in a humidified incubator with 5% $CO_2$. They were routinely subcultured at a 1:10 dilution every 2 to 3 days, before reaching over-confluency. The LEC10 cells were generously provided by Professor Pamela Stanley.

*Extraction of cellular proteins:* 100 mm dishes were seeded with $1.5 \times 10^6$ cells; 24 hours later they were harvested. The plates were washed once with warm PBS, and then 3 mL of Sigma (non-enzymatic) Cell Dissociation Reagent in PBS was added to each plate. Plates were incubated at 37°C for 10 minutes, after which they were tapped on the side to free the cells from the plate. The cells were pipetted off the plate into a 50 mL centrifuge tube, and the plate was washed with 2 mL of warm PBS, which was added to the cell suspension. The tube with the cells was kept on ice for the duration of the processing. The cells were spun at 1000 rpm for 10 minutes in a Beckman TJ 6 centrifuge at 4°C. The supernatant was removed and the cells were washed two more times. After the final spin, cells were resuspended at $5.8 \times 10^7$ cells/mL in 100 mM Tris pH 6.8, 2% BME, and either 2% SDS or 25% DMF. Cells were sonicated for 2 minutes and then heated to 100°C for 5 minutes. Extracts were frozen at this point.

*Harvest of supernatant proteins:* $7.5 \times 10^6$ cells were seeded into a T175 flask in 5% serum. After 24 hours, the serum-containing medium was replaced with α-MEM with no serum; the medium was replaced an additional four times over the following 27 hours. The supernatant was harvested 36 hours after the final replacement and again after an additional 36 hours. Harvested supernatants were frozen immediately. Supernatants were concentrated for loading onto IPG strips by using Centriplus and Microcon concentrators from Amicon.

*Sample preparation:* The optimized method of sample preparation was as follows: to 125 μL of either cell extract or concentrated supernatant was added 5 μL of 0.1% bromophenol

blue, 7.5 µL of 40% BioLyte 3-10 carrier ampholytes, 15.0 µL of 20% CHAPS, and 0.14 g urea; this yielded about 250 µL of a solution containing 1.2% carrier ampholytes, 1.2% CHAPS, and about 9.5 M urea. This particular quantity of sample was used to load two IPG strips, and sample preparation was adjusted proportionately up or down to load a different number of IPG strips. In the case of the cell extracts, there were typically urea crystals left in the sample, indicating saturation. For the serial loading experiments, the final volume was scaled up to 450 µL; either 225 µL of cell extract or extract plus water was used. For the unoptimized method of sample preparation which was used in some experiments, cell extracts were diluted with an equal volume of a solution containing 9M urea and 2% BioLyte 3-10 carrier ampholytes.

*Isoelectric focusing:* Immobiline DryStrip IPG strips, pH 3-10L, 18 cm, were used. Unless otherwise noted, they were rehydrated in 8 M urea, 0.5% NP-40, 1.5% CHAPS, 0.2% BioLyte 3-10 carrier ampholytes, 0.2% DTT, 10% DMF. Electrode strips were soaked in either 0.05% NaOH or 0.0595% phosphoric acid. After placing the IPG strips in an Immobiline DryStrip tray, mineral oil was added to the tray to a depth which covered the strips but not the sample cups. 100 µL of sample was loaded at the extreme cathodic or anodic end as indicated. After the sample was added to the cup, mineral oil was added to completely cover the sample cups. The typical program for isoelectric focusing was 2 hours at 360 V (20 V/cm), 2 hours at 1440 V (80 V/cm), and 35-40 hours at 2970 V (165 V/cm), all at a controlled temperature of 25°C. For experiments in which the sample was serially loaded, 100 µL of sample was loaded initially, and an additional 100 µL was added after 24, 48, and 72 hours for a total of 400 µL of loaded sample; after the first three loadings, the strips were focused for 2 hours at 360 V and 22 hours at 1440 V, and after the final loading, the strips were focused for 2 hours at 360 V, 2 hours at 1440 V, and 61 hours at 2970 V.

*IPG strip equilibration:* Strips were incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2% w/v DTT, 50 mM Tris pH 6.8 on a shaking platform for 10 minutes. They were rinsed briefly with water and then incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2.5% w/v iodoacetamide, 50 mM Tris pH 6.8, and a dash of bromophenol blue on a shaking platform for 5 minutes. Each strip was touched along its edge briefly on wet filter paper before being trimmed to fit onto the SDS-PAGE gel; the gel was trimmed approximately 1 cm on the end at which it was loaded and typically about 1.5 cm on the opposite end.

*Second dimension SDS-PAGE:* For the second dimension, 10% T polyacrylamide gels crosslinked with 0.27% PDA of dimensions 19 cm × 16 cm × 1.5 mm were used. The running buffer was 28.8 g/L glycine, 6 g/L Tris base, 1 g/L SDS unless otherwise specified. Up to six gels were run in parallel using the BioRad Protean II Multicell. 5 µL of broad range molecular weight standards (New England BioLabs) was loaded between the spacer and the basic end of the IPG strip. Gels were run at 4°C at 40 mA per gel for approximately 5 hours.

*Transfer of proteins onto a solid support:* The Pharmacia Multiphor II NovaBlot semi-dry blotting unit was used for blotting. Proteins were blotted onto Immobilon P membranes (Millipore) wetted in 100% MeOH and equilibrated in blotting buffer (39 mM glycine, 48 mM Tris base, 20% MeOH). Gels were transferred from the glass plates into water briefly, after which they were stacked in units (anode to cathode) of filter paper (Whatman 3 mm chromatography paper), membrane, and gel, with two pieces of filter paper on both sides of the stack. Gels were blotted for 6 hours at 200 mA. After blotting, the membranes were either processed immediately as described below, or transferred to water until needed.

*Detection of E-PHA-binding glycoproteins:* This protocol for the detection of glycoproteins recognized by the erythroagglutinating lectin of *Phaseolus vulgaris* (E-PHA) was based on the "Applications of digoxigenin-labeled lectins in glycoconjugate analysis" technical sheet from Boehringer Mannheim. Membranes (approx. 15 cm × 16 cm) with bound proteins were rinsed in 100 mL TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 75 mL 0.5% blocking reagent (Boehringer Mannheim) in TBS for 1 hour on a rocker platform. This was followed by two ten-minute rinses in 100 mL TBS and one ten-minute rinse in buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, in TBS, pH 7.5). The blots were then incubated in 35 mL of 4 µg/mL E-PHA-alkaline phosphatase (E-PHA-AP, E-Y Labs) in buffer 1 for one hour on a rocker platform. This was followed by one ten-minute rinse in 150 mL TBS on a rocker platform and two ten-minute rinses in TBS on a shaking platform; the blots were switched to clean trays before the final rinse. The developing solution was 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5 with 187.5 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 500 µg/mL 4-nitro blue tetrazolium chloride (NBT); 40 mL of developing solution was used per membrane. The membranes were incubated with developing solution without shaking for approximately 1 hour, 45 minutes, after which they were rinsed thoroughly with water and dried on filter paper.

*Staining of proteins with gold particles:* Membranes with transferred proteins were rocked in 50 mL PBST (2 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 150 mM NaCl, 0.3% Tween 20, pH 7.4) twice for 5 minutes, once for 2 hours, and three times for 5 minutes. They were then rinsed in 150 mL water twice for 5 minutes, after which they were incubated in 35 mL of gold protein staining solution (Zymed) until protein spots were clearly visible. They were then rinsed in water.

*Immunostaining:* Membranes with transferred proteins were rinsed in TBST (10 mM Tris, 150 mM NaCl, 0.05%, pH 8.0) and blocked for 30 minutes in TBSTM (TBST plus 5% dried nonfat milk). They were then incubated with primary antibody in TBSTM for one hour, followed by three ten-minute washes in TBSTM. This was followed by incubation with secondary antibody diluted 1:7500 in TBSTM for one hour, followed by one ten-minute wash in TBSTM and two ten-minute washes in TBST. Blots probed with

horseradish peroxidase-labelled secondary antibodies were detected with ECL Western blotting detection reagents and protocol (Amersham) using Hyperfilm ECL (Amersham). Blots probed with alkaline phosphatase-conjugated secondary antibodies were developed in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5 with 165 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 333 µg/mL 4-nitro blue tetrazolium chloride (NBT).

# Results

Pilot experiments to develop an optimized method for glycoprotein solubilization and isoelectric focusing were done using LEC10 cells. These cells produce glycoproteins which have bisecting N-acetylglucosamine residues incorporated into their attached oligosaccharides [20]. This oligosaccharide structure is easily detected by the *Phaseolus vulgaris* erythroagglutinating lectin (E-PHA) [26, 53, 113, 186]. By using a lectin to probe the 2-D separated samples, protocols could be evaluated and optimized based specifically on the solubilization and resolution of glycoproteins.

## Extraction of Glycoproteins for Two-Dimensional Electrophoresis

Initially, extraction of the cellular proteins was done in the presence of 2% SDS, 100 mM Tris pH 6.8, and 2% BME. The cells were sonicated and heated to 100°C for 5 minutes, yielding a clear but viscous solution. When the cell extract was analyzed by 2-D electrophoresis, the E-PHA-binding proteins were visualized as a smear across the higher molecular weight region of the blot (Figure 2.1B); while there were regions of higher or lower staining intensity, there were no distinguishable spots. A colloidal gold stain of a similar sample revealed that gold-staining proteins were able to focus well over the whole gel (Figure 2.1A); this result confirmed the need to evaluate potential protocols based strictly on the ability of the protocol to solubilize and resolve E-PHA-binding proteins.

A possible explanation for the smear was that the SDS used in the extraction, because of its negative charge, was being stripped away from the proteins during isoelectric focusing, leaving the proteins unprotected from aggregation. To avoid this complication of ionic detergents, dimethylformamide (DMF) was chosen as an extraction agent; DMF has previously been shown to be useful in the isoelectric focusing of hydrophobic proteins [59, 60, 191]. Extraction of cells in 25% DMF, 100 mM Tris pH 6.8, and 2% BME yielded, after sonication, a white, cloudy suspension, which was not rendered clear by heating, but could be separated into a white, fluffy pellet and a clear supernatant by simply allowing the sample to stand for a few minutes. Working under the assumption that the pellet contained a significant fraction of the glycoproteins of interest, great care was taken throughout the procedure, up to and including loading, to make sure the sample was well-mixed before any aliquots were removed. Despite the presence of particulates in the sample, when the DMF-extracted cells were analyzed by 2-D electrophoresis, spots were clearly visible and the high molecular weight smear was no longer present (Figure 2.1C). The smear which was associated with the loading site was distinctly broader and darker in the DMF-extracted sample than in the SDS-extracted sample; it was difficult to discern if the same amount of lectin-staining protein had entered each gel, with the darker smear resulting from more of that protein getting stuck at the loading site, or if the darker smear represented an additional amount of glycoprotein which had not entered the gel using the SDS extraction method.

Entrance of Glycoproteins into Focusing Gel

The presence of significant amounts of lectin-staining glycoprotein in the loading site smear prompted research into techniques which would enable those glycoproteins to enter the IPG strip; henceforth, the term "enter the IPG strip" (and variations thereof) will refer to the movement of glycoproteins out of the loading site smear and into the rest of the IPG strip. It was first theorized that the glycoproteins were having difficulty moving from a 25% DMF solution into a gel with no DMF. To address this, DMF-extracted proteins were loaded onto IPG strips rehydrated in solutions containing 0 to 25% DMF. Initial experiments suggested that DMF in the IPG strip significantly increased the entry and resolution of lectin-staining proteins (data not shown); however, in later experiments, comparisons of samples resolved in IPG strips with DMF and without DMF gave inconsistent results (data not shown).

Other experiments focused on altering the composition of the sample which was loaded onto the IPG strip. Up to this point, samples had been prepared for 2-D separation by diluting cell extracts with an equal volume of a solution containing 9 M urea and 2% carrier ampholytes. To investigate this variable, identical aliquots of cell extract were prepared for loading by the addition of different reagents. A higher concentration of Tris in the sample did not improve entry or resolution, and in fact, the gels showed indications of burning (data not shown). Addition of urea to saturating levels caused a significant increase in the amount of lectin-staining glycoprotein which entered the IPG strip; this was seen as a general increase in the staining intensity of spots which had been seen previously and by the appearance of new staining regions, either as spots or smears (data not shown). Similar but less pronounced improvements were seen when CHAPS was added to the sample to a final concentration of 1.2% (data not shown). A combination of 1.2% CHAPS and saturating urea yielded the best results in terms of number of visible spots, resolution of spots, and staining intensity of spots (data not shown).

Application of this new method of sample preparation to cells extracted with 2% SDS resulted in a significant increase in spot resolution over the previous method (Figure 2.2A, compare with Figure 2.1B). However, better results were obtained using 1.2% CHAPS and saturating urea in conjunction with DMF-extracted cell samples (Figure 2.2B). Overall the spots were more intensely stained, and in some cases, better resolved, on the blot of the DMF-extracted cells. The loading site smear was substantially broader and darker in the SDS-extracted sample, which is likely due to a higher percentage of the total lectin-staining protein not entering the IPG strip, considering the overall lower staining intensity of the spots in the SDS-extracted sample.

Influence of Sample Loading Site

The influence of the sample loading site on sample entry and resolution was also studied. If a protein is loaded at a site which is very close to its isoelectric point, it will have very

little impetus for movement based on its net charge. Furthermore, it will also be fighting the tendency to precipitate, as proteins are more likely to precipitate near their isoelectric point. In pH regions away from a protein's pI, protein molecules have an excess of positive or negative charge which repels other like protein molecules with similar charge excesses; however, at the pI, not only is there not an excess of like charge to cause repulsion, but there is a balance of positive and negative charge, which could lead to electrostatic attraction between like molecules. The choice of loading site can also be important if a potential loading site is in the same pH region as a protein of interest, as dense vertical smearing at the loading site could prevent detection or study of individual proteins which focus to that pH region.

To study the effect of the sample loading site on the resolution and sample entry of cell extracts, DMF-extracted samples were prepared with 1.2% CHAPS and saturating urea, and loaded at either end of an IPG strip (Figure 2.3A and B). Comparing the patterns of overall lectin-staining of samples loaded at the acidic (Figure 2.3A) and basic (Figure 2.3B) ends of the IPG strips, it appeared that approximately the same amount of lectin-staining glycoprotein entered and focused in each IPG strip, independent of the loading site, although the basic loading site had a broader, darker loading site smear. However, there were definite differences in the resolution of proteins in different parts of the gel depending on where the sample was loaded. Lectin-staining proteins which focused toward the basic end of the gel were resolved well when loaded at the acidic end, but they appeared more like a smear, with only hints of spots, when loaded at the basic end. Lectin-staining proteins in the middle of the gel also were resolved better when loaded at the acidic end. Lectin-staining proteins which focused toward the acidic end of the gel were resolved better when loaded at the basic end, but some spots in this region were also resolved to some extent when loaded at the acidic end. Overall, loading at the acidic end of the gel gave much better resolution over 80% of the gel, while loading at the basic end allowed the resolution of the lectin-staining proteins in the most acidic 20% of the gel. It is important to note that if only one loading site had been used, a substantial fraction of the lectin-binding spots would not have been seen. For example, when cell extract from a cell line which expressed lower quantities of E-PHA-binding proteins was analyzed, a spot in the high molecular weight acidic region which stained very intensely on gels loaded at the basic end was not visible at all on gels loaded at the acidic end (Figure 2.3C and D).

Similar results were obtained when 2-D separated cell extracts were stained with gold (data not shown). Gold-stained proteins were not resolved in the most acidic 30% of the gel when cell extracts were loaded at the acidic end; it is interesting that lectin-stained proteins loaded at the acidic end were able to resolve well in an additional 10% of the gel. Gold-stained proteins were not resolved in the most basic 20% of the gel when cell extracts were loaded at the basic end. However, gold-stained proteins loaded at the basic end were able to resolve well across the other 80% of the gel; it is interesting that lectin-stained proteins

loaded at the basic end were resolved well only in the most acidic 20% of the gel. All of these observations are consistent with the concept that proteins which are loaded away from their pI will focus better; however, the extent of the effect of the loading site on resolution is different for lectin-stained and gold-stained proteins.

Identification of Marker Proteins

Using the optimized methods of cell extraction and sample preparation and other techniques described in the Methods section, antibodies were used to identify five families of spots; two of these identifications were confirmed and three additional identifications were made by comparing the gold-stained pattern of 2-D separated cellular extracts to published maps of CHO cell proteins [95]. These identifications are shown on Figure 2.4. In trying to make identifications by comparison to a published map, the influence of the sample loading site on the overall pattern became extremely apparent. The published map had been loaded at the basic end of an IPG strip, while the blot presented in Figure 2.4 was loaded at the acidic end of an IPG strip. Initially, the overall gold-staining patterns of the two blots appeared to be very different, but having studied the influence of the sample loading site in previous experiments, it was realized that the apparent difference between the two maps was a result of different portions of the gel not being resolved, due to their proximity to the loading site; while this difference significantly altered the overall pattern of the gel, spot-by-spot comparison revealed that in the regions where both gels had resolved spots, the patterns were very similar.

Studies to Increase Amount of Sample Loaded

Experiments were also done to increase the amount of sample which could be loaded onto a gel. Typical IPG-IEF protocols employ a small plastic cup for sample loading; this cup has a maximum volume of 100 µL. Bjellqvist, *et al.* used a larger sample cup to increase the volume of sample that could be loaded at one time [13]; while this research was a success, it is not yet generally useful because both the gels and the cups had to be made in-house and are not commercially available.

Other labs have reported rehydrating the IPG strip in the sample itself, after the sample is adjusted properly to include the normal constituents of rehydration buffer [140, 152]; this method avoids any sample cup limitations. Initially, the use of this method resulted in a moderately uniform, high intensity lectin stain across the higher molecular weight region of the blot (Figure 2.5A ); this indicated that the lectin-staining protein had entered the IPG gel, but had been, for the most part, unable to focus. Multiple changes to the running conditions (new electrical program, IPG strip temperature increased from 15°C to 25°C) greatly improved the resolution of the lectin-staining proteins, especially at lower molecular weights (Figure 2.5B); however, the intensely staining smear in the higher molecular weight region of the gel only partially diminished in intensity, and the smear still represented an obstacle to the study of any individual lectin-binding spots in that region of

the gel. It was interesting to note that within the smear, there were multiple well-defined, well-focused negative staining regions; this suggested that non-glycoproteins were capable of focusing well under the same conditions which left the glycoproteins as a smear.

The higher running temperature and different program also seemed to improve the resolution and sample entry of a control IPG strip, which had been prepared like a normal IPG strip and loaded using a sample cup (data not shown). A study on the effect of temperature on IPG-IEF has been published, and the conclusion of the researchers was that IPG strips focused at 20°C had better sample entry and resolution compared to those run at 10 or 15°C; they also pointed out that consistency in temperature is important, as the temperature can have significant effects on the mobility and focused position of proteins in IPG strips [48]. All subsequent IPG isoelectric focusing was done at a controlled temperature of 25°C.

Experiments were also conducted in which the sample cup was filled multiple times over a period of several days. Varying amounts of cell extracts (increasing by two-fold) were prepared for isoelectric focusing in the same final volume containing 1.2% carrier ampholytes, 1.2% CHAPS, and saturating urea. Sample cups were filled daily with 100 µL of sample for four days, for a total volume of 400 µL. The two samples with the smallest quantities of cell extract per final volume (25/500 and 50/400) had no sample pellet left when they were loaded, while the two samples with larger quantities of cell extract per final volume (100/400 and 200/400, 200/400 being equal in terms of cell extract per final volume to a "typical" single load) had increasing amounts of pellet left in the presence of saturating urea; "typical" sample preparations did have substantial pellets left in the presence of saturating urea.

The E-PHA-binding patterns of the serially loaded 2-D gels showed a general increase in the total amount of lectin-staining protein, a specific increase in the lectin-staining intensity of individual spots, and an increase in the total number of distinguishable spots with increasing amounts of cell extract per final volume (Figure 2.6). However, the sample with the highest amount of cell extract showed a complete absence of a chain (indicated by an arrow) which had been consistently present and increasing in intensity across the three lower protein loads. This disappearance of certain spots when large amounts of protein are loaded may be a common phenomenon; Merrick, *et al.* studied the effect of increased protein loads on resolution and staining intensity, and their results showed that some protein spots which were seen at lower levels of protein loading were not visible at higher levels of protein loading [103].

It is interesting to note that the sample with the lowest concentration of cell extract per final volume, which had no particulates in the presence of saturating urea, had a significant smear at the loading site. This indicated the loading site smear problem could not be solved simply by lowering the concentration of cell extract in the loaded sample, or by lowering the total quantity of extract on the gel. This also indicated that the smear was not

completely related to the presence of particulates in the sample. Furthermore, while generally there was far less visible smearing in the E-PHA-binding pattern of this sample, it was interesting to note that there were still problems in horizontal resolution and vertical smearing, even when only this small amount of extract was separated.

Supernatant Samples

Some of the above techniques were applied to the 2-D separation of supernatant samples. First, a brief set of experiments explored the effect of sample preparation on supernatant glycoprotein entry and resolution. Concentrated supernatant was prepared for loading either by diluting it with an equal volume of 9 M urea, 2% carrier ampholytes or by adding urea to saturation in the presence of 1.2% CHAPS and 1.2% carrier ampholytes; the former was focused in a non-DMF IPG gel, while the later was focused in an IPG gel containing 10% DMF. Substantially more lectin-staining protein entered the IPG gel in the presence of saturating urea and 1.2% CHAPS, and, to the extent that it was possible to compare, given the significant difference in overall and per-spot lectin staining, the spots were better resolved in the 1.2% CHAPS/saturating urea sample (data not shown).

To study the effect of the sample loading site on the resolution of spots in a supernatant sample, supernatant samples were prepared in 1.2% carrier ampholytes, 1.2% CHAPS, and saturating urea, and then loaded at the extreme basic or acidic end of IPG gels. In all cases, when supernatant samples were loaded at the acidic end of an IPG gel, there was a significant breakdown of the polyacrylamide media structure in the region 1 to 5 cm from the basic end of the gel (Figure 2.7); the exact location and width of the region of breakdown varied from gel to gel. This breakdown resulted in a complete loss of proteins in the region of the gel breakdown, and there was no focusing in any region of intact gel to the basic side of the breakdown site. This phenomenon is likely sample dependent, in that it was never observed with cell extracts from the same cells, nor is it commonly noted in published reports describing 2-D experiments or techniques.

In initial sample loading site experiments using relatively small quantities of supernatant proteins, lectin-stained proteins in the basic two-thirds of the gel (up to the point where the gel broke down) were better resolved when loaded at the acidic end, and the lectin-stained proteins in the acidic third of the gel were resolved to about the same extent independent of the loading site (data not shown). However, in later experiments with much higher protein loads (Figure 2.8), lectin-staining proteins in the acidic third of the gel were not resolved well when the sample was loaded at the acidic end, and several lectin-binding chains at the extreme acidic end of the gel were visible only when the sample was loaded at the basic end. Lectin-stained proteins in the middle third of the gel were resolved slightly better when the sample was loaded at the acidic end. No judgment can be made about comparative resolution of spots in the basic third of the gel since that region of the IPG gel disintegrated when supernatant samples were loaded at the acidic end.

# Discussion

The results of these experiments clearly affirm the importance of evaluating multiple methods for sample solubilization, sample preparation for isoelectric focusing, and the loading and focusing of the IPG strips. These experiments also demonstrated the interdependence of the different parameters, especially with regard to optimization. When methods of sample solubilization were initially explored using an unoptimized method of sample preparation for isoelectric focusing, extraction in 25% DMF clearly allowed for the solubilization and resolution of E-PHA-binding proteins which appeared only as a smear when extracted with 2% SDS; however, when the comparison was repeated using an optimized method of sample preparation, the SDS extraction method gave results that were almost as good as the DMF extraction. With so many inter-related variables to consider, optimizing a procedure of this nature could become a never-ending task; it is necessary to choose, based on careful consideration, only a few variables to consider, and to at some point, choose the best method based on the completed experiments.

Extraction in 25% DMF and extraction in 2% SDS solubilized roughly the same complement of proteins when the samples of extracted cells were compared using optimized sample preparation protocols. However, extraction in 25% DMF did result in better resolution of and higher E-PHA binding to most spots and chains. Unlike many other examples in the literature (e.g. [33]), there were very few examples of proteins which were solubilized by only one method or were solubilized to a significantly greater extent by one method compared to the other. This indicated there was not a need to regularly use multiple solubilization methods in parallel to solubilize and study different complements of proteins.

In contrast, significantly different complements of proteins were resolved when the two choices for sample loading site were compared. When the sample was loaded at the acidic end, E-PHA-binding proteins which had previously resolved in the acidic third of the gel were not discernible as chains or spots, and when the sample was loaded at the basic end, E-PHA-binding proteins which had previously resolved in the basic third of the gel were not discernible as chains or spots. This observation was not specific to glycoproteins; when gold-stained blots of proteins loaded at either end of the IPG strip were compared, up to one-third of the gel on the end corresponding to the loading site was devoid of resolved spots (data not shown). This indicated that to study all of the glycoproteins in a sample, it would be necessary to use both loading sites in parallel to resolve the different subsets of proteins. However, in supernatant experiments, use of the acidic loading site was not possible because the polyacrylamide media structure of the basic end of the gel broke down.

Experiments in serial loading also demonstrated the inability of one method to capture simultaneously all of the chains and spots. While most of the E-PHA-binding proteins increased in staining intensity with increased sample loading, and new spots and chains

became visible at higher loads, some E-PHA-binding proteins lost resolution at higher loads and other E-PHA-binding proteins disappeared altogether. Thus, a thorough study of the total complement of glycoproteins would necessitate a range of loading levels to ensure that each protein was considered under its particular optimal conditions.

These experiments enabled the development of a protocol for surveying cellular glycoproteins, while also highlighting the complexity of attempting to study a mixture of glycoproteins, or even non-glycoproteins, using 2-D electrophoresis. While 2-D electrophoresis is an extremely powerful technique for studying mixtures of proteins, good visualization of all of the proteins in a sample may require the parallel use of multiple methods of extraction and separation.

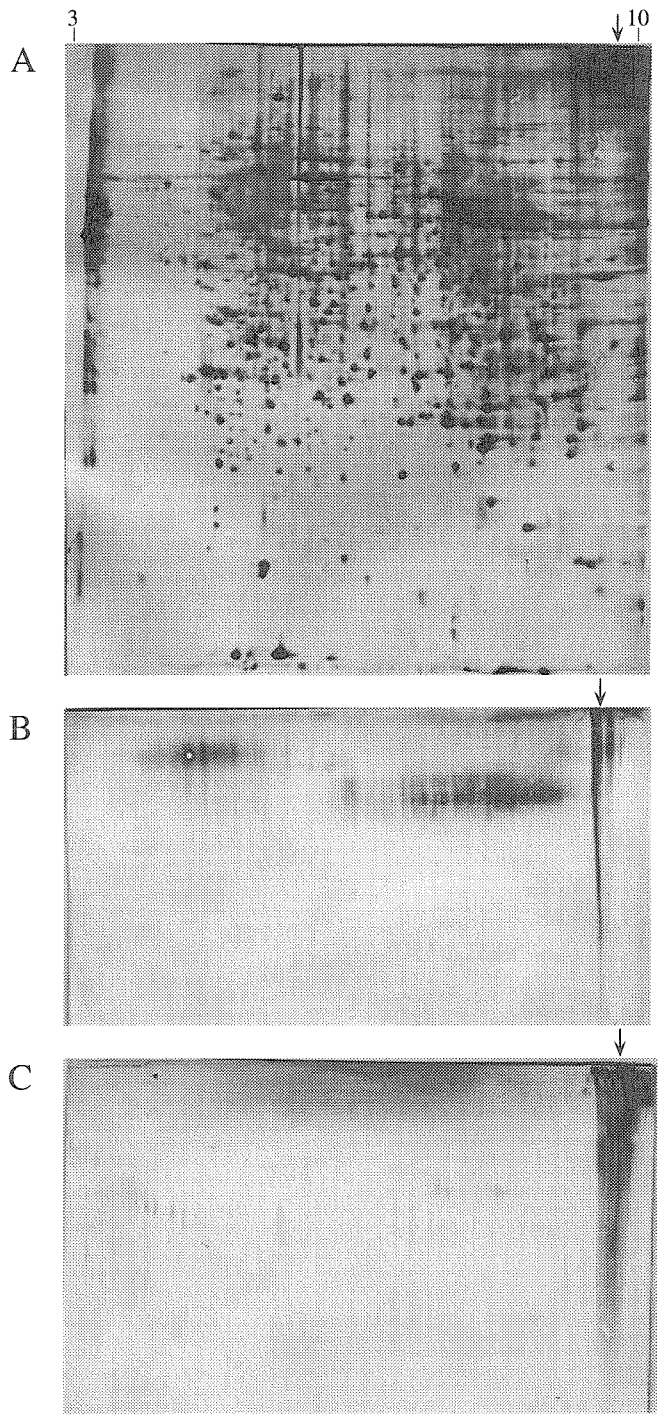


**Figure 2.1.** LEC10 cell extracts diluted with equal volume of 9 M urea/2% carrier ampholytes, loaded at the basic end of the IPG strip, and separated by IEF and SDS-PAGE on a 12% T gel. (A) and (B) Cells extracted in 2% SDS; (A) gold stain, (B) detection by E-PHA-AP. (C) Cells extracted in 25% DMF, detected by E-PHA-AP. The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

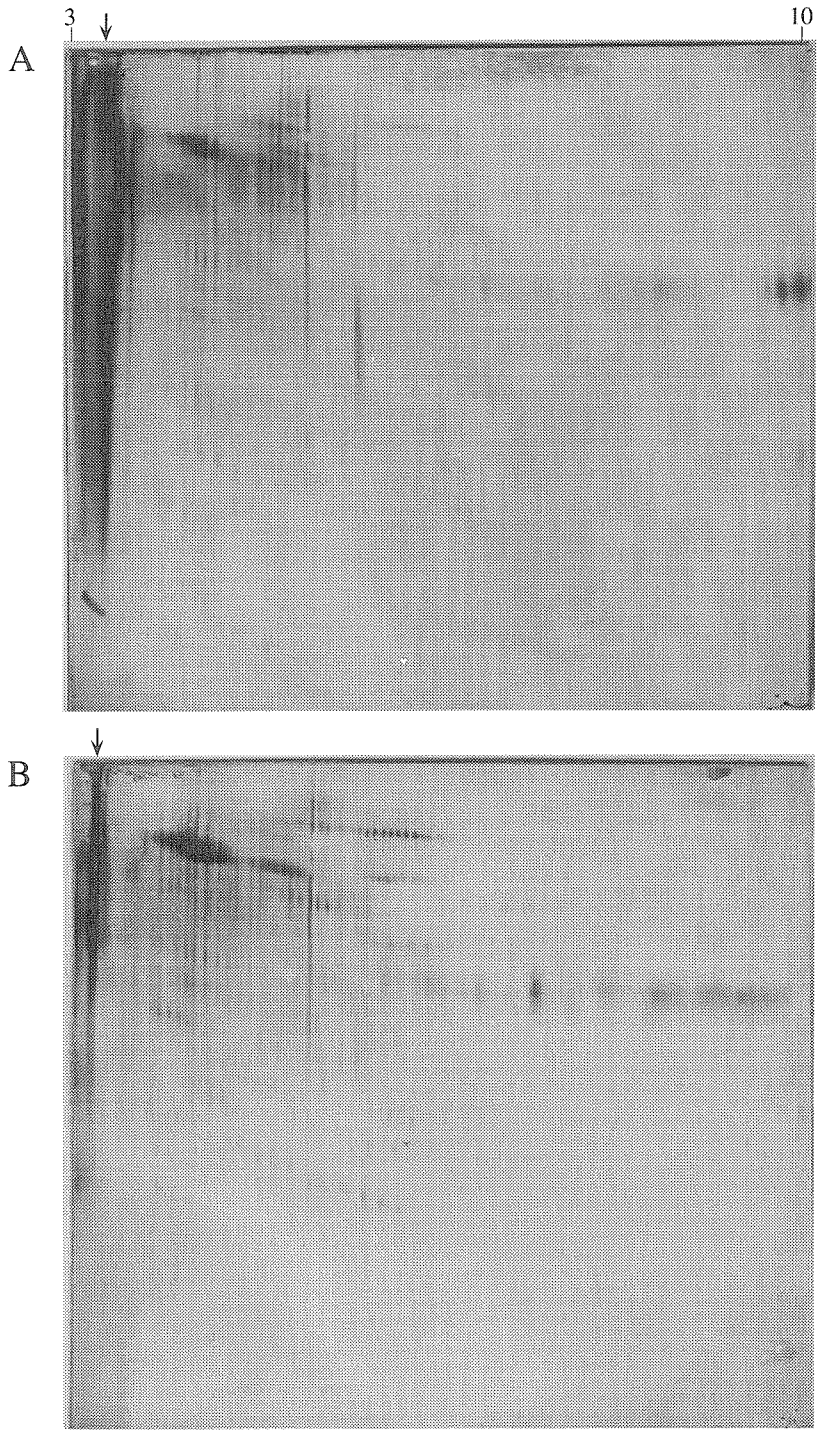


**Figure 2.2.** Comparison of extraction protocols. LEC10 cell extracts prepared in 1.2% CHAPS, 1.2% carrier ampholytes, and saturating urea, loaded at the acidic end of the IPG strip, separated by IEF and SDS-PAGE on a 10% T gel, and detected with E-PHA-AP. (A) Cells extracted in 2% SDS. (B) Cells extracted in 25% DMF. The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

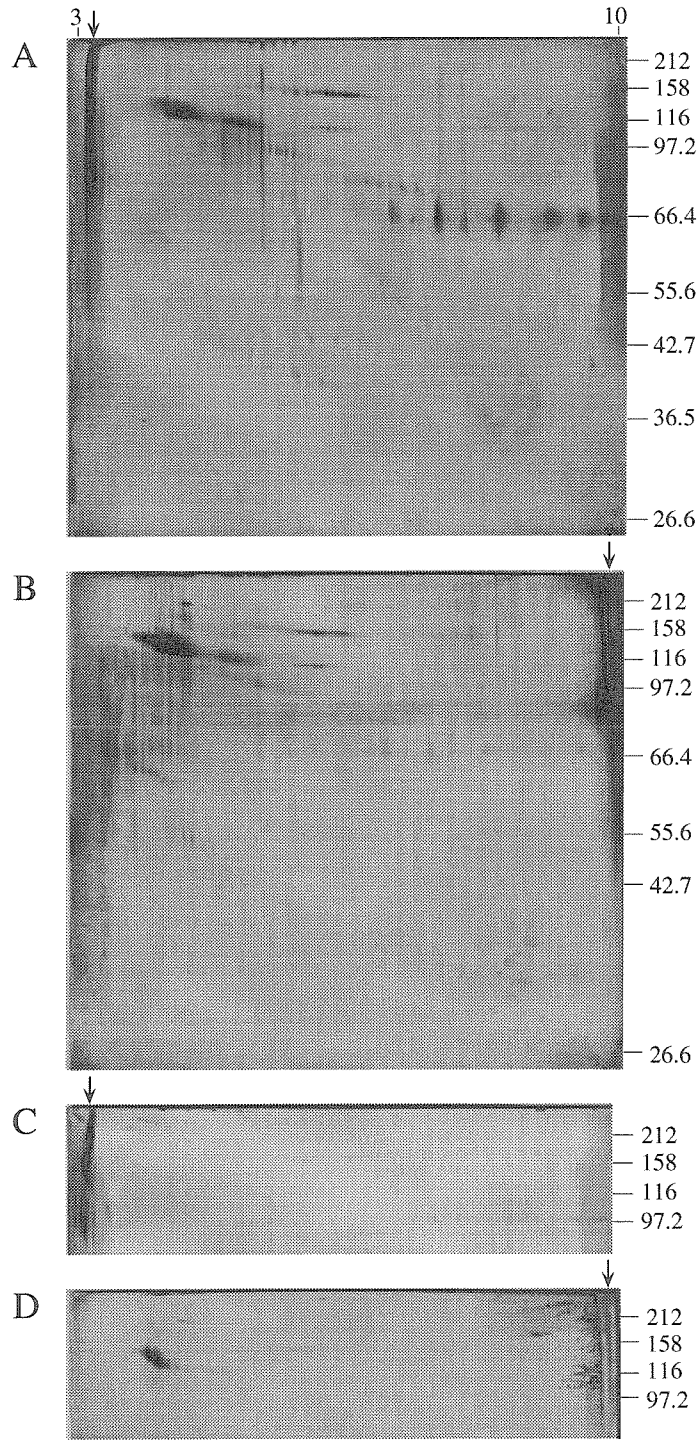


**Figure 2.3.** Comparison of loading sites. Cells extracted in 25% DMF; sample prepared in 1.2% CHAPS, 1.2% carrier ampholytes, and saturating urea, separated by 2-DE, and detected with E-PHA-AP. (A) and (B) LEC10 cells; (A) loaded at the acidic end of the IPG strip, (B) loaded at the basic end of the IPG strip. (C) and (D) Pro-5 cells; (C) loaded at the acidic end of the IPG strip, (D) loaded at the basic end of the IPG strip. Positions of molecular weight (MW) markers in kilodaltons (kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

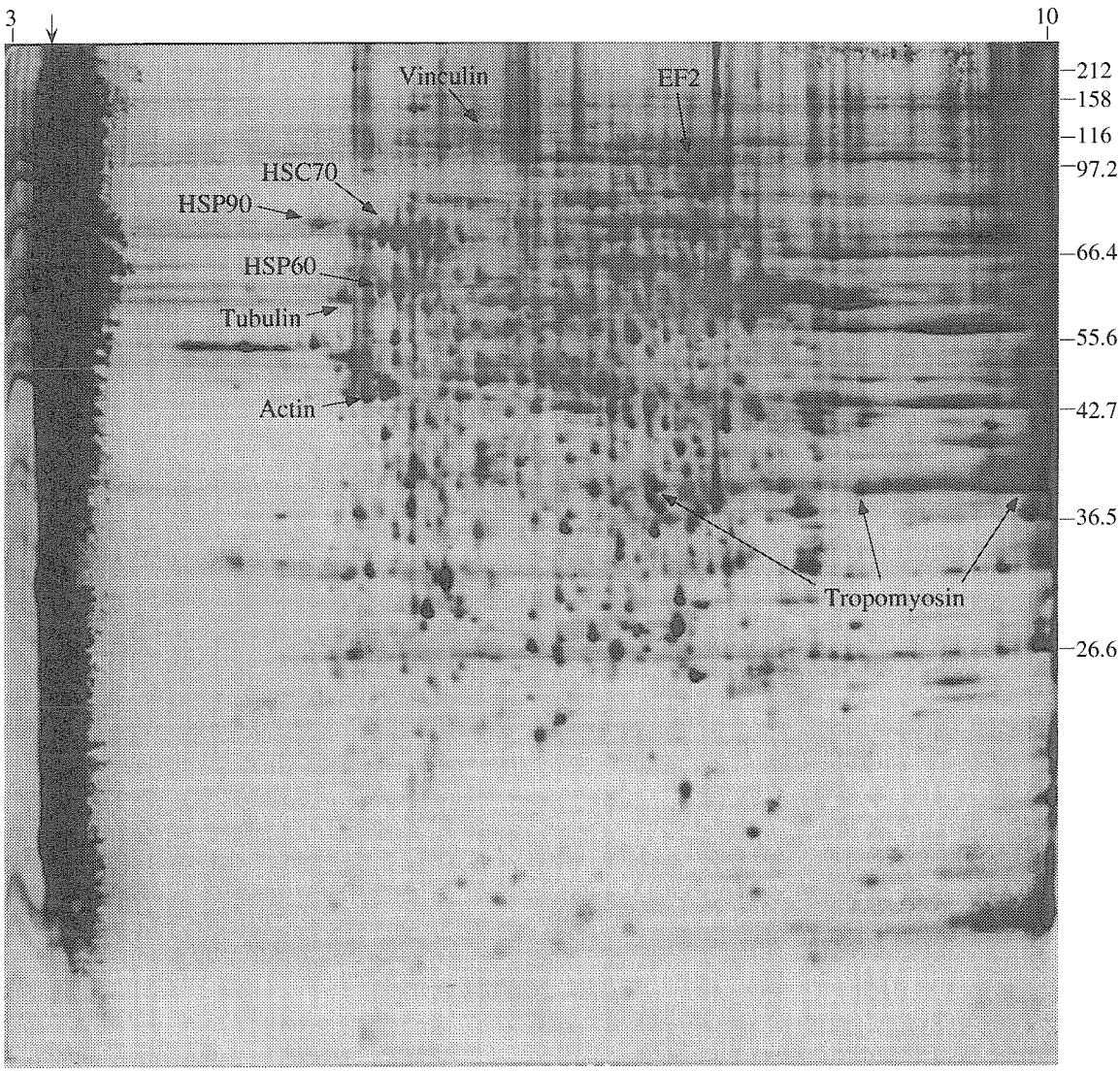


**Figure 2.4.** Colloidal gold stained pattern of Pro-5 cell extract with identified proteins marked. The cell extract was separated by IEF on a 3-10L IPG strip and SDS-PAGE on a 12% T gel and detected with colloidal gold. Proteins were identified by immunostaining (I) or comparison to published maps (C) as follows: tubulin – I, C; actin – I, C; vinculin – I; tropomyosin – I; EF2 – I; HSC70 – C; HSP90 – C; HSP60 – C. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of the gel indicates the sample loading site.

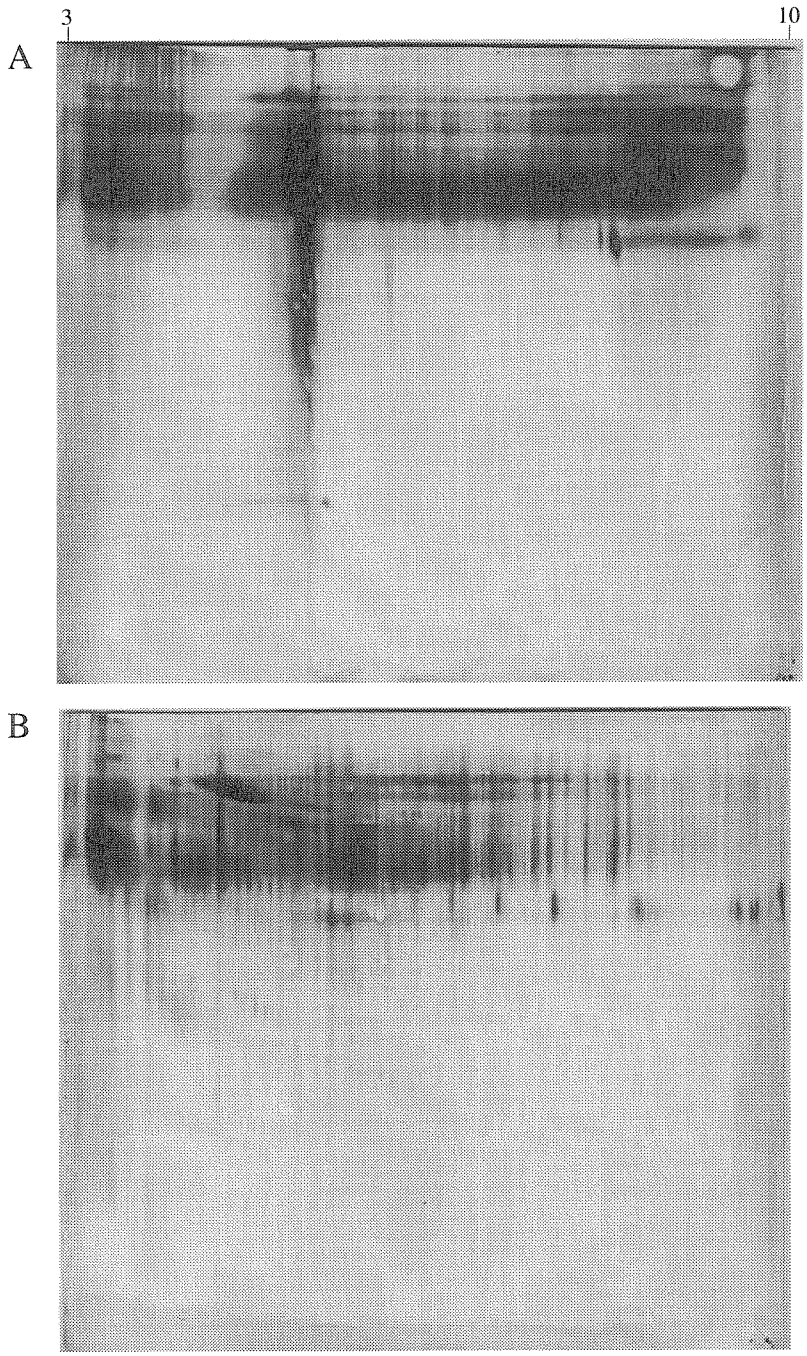


**Figure 2.5.** Rehydration of IPG strip in sample. LEC10 cells extracted in 25% DMF. (A) Initial experiment. (B) Subsequent experiment using a different electrical progam and an increased focusing temperature. The orientation of the pH gradient is shown at the top.

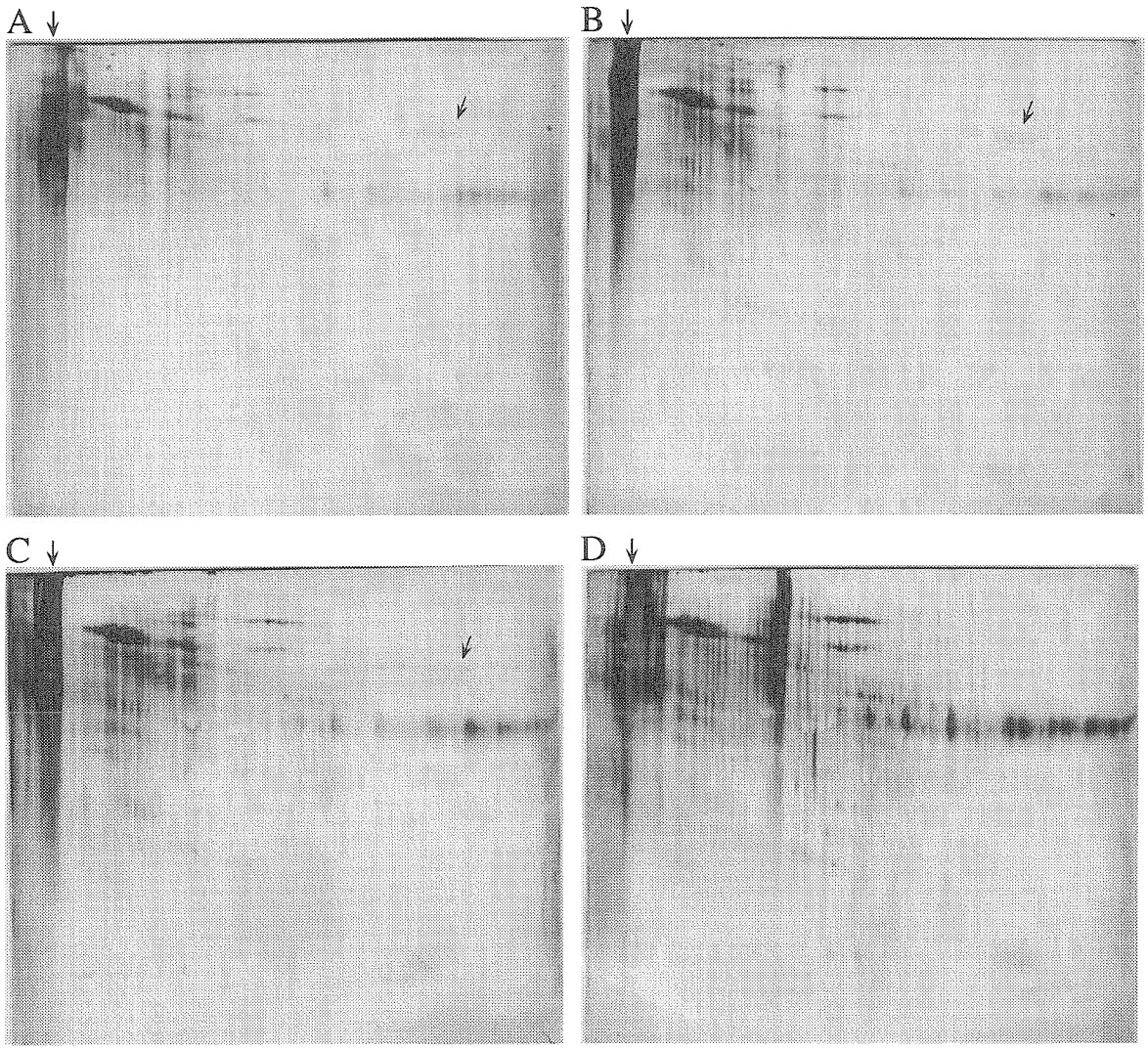


**Figure 2.6.** Effect of increasing sample load using serial loading technique. LEC10 cells extracted in 25% DMF. Samples were prepared in 1.2% CHAPS, 1.2% carrier ampholytes, and saturating urea; 400 µL of sample was loaded at the acidic end of the IPG strip as described in text, separated by 2-DE, and detected with E-PHA-AP. (A) 25 µL of cell extract. (B) 50 µL of cell extract. (B) 100 µL of cell extract. (D) 200 µL of cell extract. Note the absence of the chain indicated by an arrow in panel (D). The arrow at the top of each gel indicates the sample loading site.

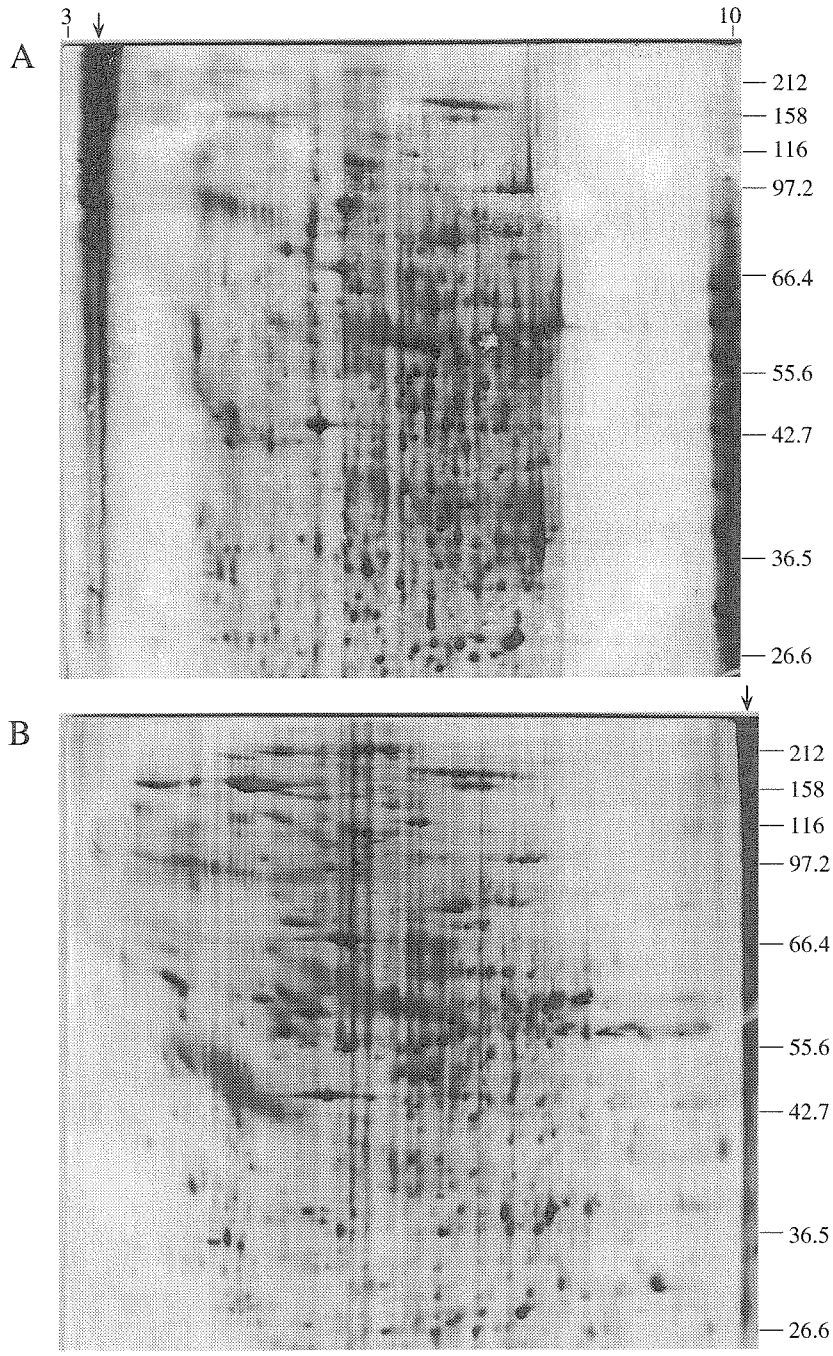


**Figure 2.7.** Effect of loading site on gold-staining pattern of supernatant samples. Concentrated LEC10 supernatant samples prepared in 1.2% CHAPS, 1.2% carrier ampholytes, and saturating urea, separated by 2-DE, and detected with colloidal gold. (A) Loaded at the acidic end of the IPG strip. (B) Loaded at the basic end of the IPG strip. Note in (A) the absence of gold-staining protein in the basic third of the gel; this is the region which suffered from gel deterioration. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

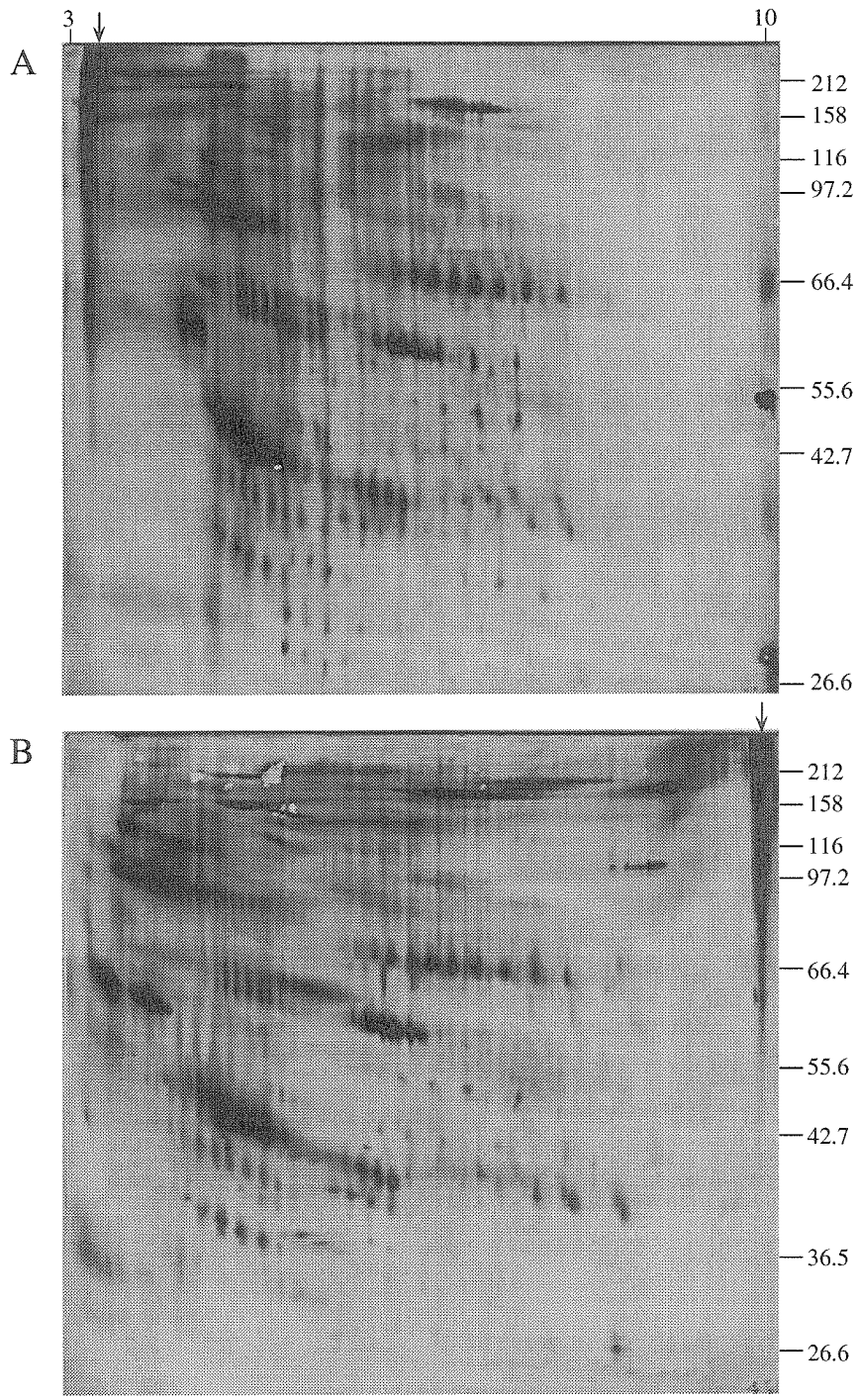


**Figure 2.8.** Effect of loading site on E-PHA-binding pattern of supernatant samples. Concentrated LEC10 supernatant samples prepared in 1.2% CHAPS, 1.2% carrier ampholytes, and saturating urea, separated by 2-DE, and detected with E-PHA-AP. (A) Loaded at the acidic end of the IPG strip. (B) Loaded at the basic end of the IPG strip. Note in (A) the absence of E-PHA-binding proteins in the basic third of the gel; compare with parallel blot shown in Figure 2.7A. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

# Chapter 3

## Inducible Alteration of the Glycosylation of a Heterologous Glycoprotein Achieved by Genetic Manipulation

In Chapter 1, it was proposed that genetic alteration of glycosyltransferase expression could be used to manipulate the glycoform distribution of a cloned heterologous protein of interest. One way to genetically alter glycosyltransferase expression in a cell line would be to transfect into the cell line a gene encoding a new glycosyltransferase activity. While in some cases it would be advantageous to place this new activity under the control of an extremely strong, constitutive promoter, in other cases it might be useful to place this new activity under the control of an inducible promoter; the use of an inducible promoter would allow one to study protein glycosylation in the presence of varying levels of the new glycosyltransferase activity. To demonstrate the feasibility of modifying the glycosylation of a protein of interest by the introduction of a new glycosyltransferase gene and corresponding activity, β-galactoside α2,6-sialyltransferase under the control of an inducible promoter was transfected into a Chinese hamster ovary (CHO) cell line expressing tPA.

### Sialyltransferases and Chinese Hamster Ovary Cells

Sialyltransferases catalyze, among other things, the addition of sialic acid residues to galactose residues at the ends of branches of complex oligosaccharides. Oligosaccharides on human proteins usually contain a mixture of both α2,6-linked and α2,3-linked sialic acid; the two different linkages are formed by two distinct enzymes, β-galactoside α2,6-sialyltransferase (α2,6ST) and β-galactoside α2,3-sialyltransferase (α2,3ST), respectively. However, CHO cells, which are commonly used for the manufacture of human therapeutics, do not attach α2,6-linked sialic acid to their oligosaccharides and produce glycoproteins which have only α2,3-linked sialic acid [22, 70, 81, 115, 128, 146, 159, 160, 169]; furthermore, Northern blots and activity assays indicate that CHO cells do not express α2,6ST [93].

There has been one report of the attachment of α2,6-linked sialic acid to glycoproteins made in CHO cells; this is found in the work of Davidson and Castellino, who studied the glycosylation of human plasminogen expressed in CHO cells [29]. They conclusively demonstrated the presence of α2,6-linked sialic acid on the purified recombinant protein. They claimed that the transfected protein had directed its own glycosylation by stimulating α2,6ST at the level of transcription or translation and/or by containing within its protein or gene structure some determining factor which directed protein-specific α2,6-sialylation. However, this work was quite incomplete in that there was no proof that random

integration of the transfected material did not "accidentally" allow for expression of a silent α2,6ST gene; this possibility could have been addressed by presenting another unrelated clone, as proven by Southern analysis, which produced plasminogen with α2,6-sialylation. Furthermore, there was no analysis of other cell-derived glycoproteins to prove that only the human plasminogen had received this highly unusual modification.

The only other indication that CHO cells might be capable of attaching α2,6-linked sialic acid to oligosaccharides was found in a study of vampire bat plasminogen activator made in CHO cells. In that work, purified protein was analyzed by a lectin-based 96-well binding assay, and this assay showed binding of a lectin specific for α2,6-linked sialic acid to the purified protein [136]. The authors made no comment regarding the unusual nature of this result (in fact, they didn't even mention the result in the text of the results or discussion); the focus of the paper was very much simply the demonstration of the ability to make active plasminogen activator in large quantities in CHO cells, so it is extremely difficult to comment on the validity or implications of this one experiment.

Thus it would be of theoretical and practical significance to establish the ability to produce α2,6-linked sialic acid on cloned proteins made in CHO cells. Experimenting with α2,6ST was particularly suitable for this work because the cDNA encoding this enzyme was available [179], and normal CHO cells provided an excellent genetic background in that they do not normally express endogenous α2,6ST activity. Additionally, this gene had already been expressed in CHO cells, and α2,6-linked sialic acid was detected on cell surface glycoproteins; it should be noted that this study did not analyze the glycosylation of any specific protein, heterologous or otherwise [93]. Thus it could be expected that the protein would be transcribed, translated, and translocated properly. A further benefit of this system was the existence of a lectin which is specific for α2,6-linked sialic acid; this lectin, *Sambucus nigra* agglutinin (SNA) [156], is available commercially in a wide-range of conjugates. A complementary lectin, *Maackia amurensis* agglutinin (MAA), specific for α2,3-linked sialic acid [176], is also readily available.

Tissue-type plasminogen activator (tPA) was chosen as a model protein. tPA has four potential N-glycosylation sites, and there are two naturally occurring forms, one which is glycosylated at three sites and another which is glycosylated at only two sites [130]. However, the complexity of the glycosylation was not a major concern since these experiments were concerned only with the presence or absence of α2,6-linked sialic acid at the terminal end of complex branches. A CHO cell line expressing tPA was readily available, and procedures for the purification, detection, and quantitation of tPA were well established.

Inducible Promoters

In order to study the feasibility of inducibly altering the glycosylation of a cloned protein, it was necessary to choose an inducible promoter element. The range of inducible promoters

available to mammalian cell engineers is quite limited, and those that are available typically have serious drawbacks associated with their use [reviewed in 82]. For example, metallothionein promoters, which are induced by heavy bivalent metal ions, glucocorticoid hormones, and interferon, have high basal levels of expression and exhibit poor inducibility [82]. The use of heat shock promoters requires exposing the cells to extreme environmental conditions which, in addition to being generally detrimental, activate an entire series of endogenous heat shock promoters and their associated genes [134]. The β-interferon promoter is also available, but activation requires the initiation of a viral infection or the addition of large quantities of double stranded RNA [96]; either method would be difficult to sustain at steady state long enough to generate the required amount of sample.

The glucocorticoid-responsive mouse mammary tumor virus LTR (MMTV) is an inducible promoter [77, 80, 94] which does not suffer from the problems listed above. Previous research has shown that reasonable levels of expression and induction can be achieved at non-toxic concentrations of the glucocorticoid analog dexamethasone [80]. One potential drawback of this promoter is directly related to the choice of α2,6ST as the experimental glycosyltransferase. Work done with a rat hepatoma line demonstrated an increase in α2,6ST transcription and activity after exposure of the cells to dexamethasone; it appears that *in vivo*, glucocorticoids are involved in stimulating hepatic response to acute systemic injury, which results in the production of several sialylated serum glycoproteins [177]. It is highly likely that this dexamethasone sensitivity is restricted to the liver and possibly a few other organs; a study has been done on the tissue-specific expression of sialyltransferases, and it was determined that the liver had the highest expression of α2,6ST of the seven tissues studied, including the ovary, by over ten-fold ([133]; similar results in [83]). However, the results of two studies published after the initiation of the experiments described in this chapter seem to suggest otherwise. One report demonstrated an increase in α2,6ST transcript and an increase in α2,6-linked sialic acid on glycoproteins when rat fibroblasts were exposed to dexamethasone [174]; another report demonstrated that the glycosylation of insulin receptor isolated from both lymphocytes and hepatoma cells was altered in multiple ways, including degree of sialylation, when the host cells were treated with dexamethasone [36]. With this potential complication in mind, the MMTV promoter was selected as the inducible promoter and appropriate controls were included in the experiments to reveal if dexamethasone was exerting any influence on cellular glycosylation unrelated to the expression of the transfected gene.

# Methods

*Reagents:* Most reagents used in electrophoresis were electrophoresis grade and were purchased from Sigma (glycerol, NP-40, iodoacetamide) or BioRad (sodium dodecyl sulfate (SDS), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea, glycine, BioLyte carrier ampholytes, dithiothreitol (DTT), piperazine diacrylamide (PDA), acrylamide, β-mercaptoethanol (BME), N,N,N',N',-tetramethylethylenediamine (TEMED), ammonium persulphate). Dimethylformamide (DMF) was from EM.

*Cells and plasmids:* Chinese hamster ovary cell line CHO.1-15 500, constitutively producing tPA under non-selective conditions, was obtained from ATCC (CRL 9606). Cells were grown in Ham's F12 medium (Gibco) with 5% dialyzed fetal bovine serum (Gibco); serum was filtered to remove particulates. Plasmid pMSG was obtained from Pharmacia. The α2,6ST gene was a gift from Dr. James Paulson.

*Plasmid construction:* Plasmid isolations, restriction enzyme digests, ligations, and transformations were all done according to standard protocols; isolation of fragments from agarose gels was accomplished using the GeneClean II kit (Bio 101 Inc.). The α2,6ST-containing plasmid was cut with EcoRI to yield a 1.6 kb fragment which included the whole gene. This was inserted into the polylinker site of pBluescript II SK +/- and transfected into DH5α. Clones positive for possession of a plasmid with insert, as indicated by white color when plated on IPTG, were screened by restriction digestion to confirm orientation of the inserted gene. A plasmid containing the gene in proper orientation was cut with SmaI and XhoI to yield a 1.6 kb fragment which was inserted into the polylinker site of pMSG, located at the 3' end of the MMTV promoter. This plasmid was used to transform HB101; clones surviving ampicillin selection were screened by plasmid restriction digest to find the correct, complete plasmid, termed pMSG-ST. DNA for transfection was purified by a cesium chloride gradient.

*Transfection:* CHO cells were transfected with pMSG-ST according to the standard protocol of Graham and Van der Ebb [49]. 8.8 μg of purified, uncut pMSG-ST plasmid DNA was precipitated with calcium chloride and added to a 60 mm dish inoculated with $7 \times 10^5$ cells 24 hours before. Cells were grown in non-selective medium for 48 hours, after which they were replated into ten 100 mm dishes in non-selective medium. After 24 hours, the medium was replaced with selective medium (250 μg/mL xanthine, 15 μg/mL hypoxanthine, 10 μg/mL thymidine, 2 μg/mL aminopterin, 25 μg/mL mycophenolic acid, 10% fetal calf serum in F12 [110]). After seven days, 2-5 colonies per plate were visible; colonies were picked on days 15, 16, and 17 post-transfection. Clones were picked and transferred with a cotton swab dipped in trypsin. A total of 108 colonies were transferred to 24-well plates. Selection was applied for four weeks before screening began.

*Screening of cell lines for expression of α2,6ST:* Cells were split into duplicate 60mm dishes containing coverslips in selection medium. After 24 hours of growth,

dexamethasone was added to one dish to a final concentration of 2 μM. After an additional 24-48 hours of growth, cover slips were removed and lectin stained according to the protocol of Lee [93]. Cover slips were fixed in 2% paraformaldehyde in PBS pH 7.4 for one hour at room temperature; they were then blocked with 50 mM ammonium chloride in PBS for 30 min. The coverslips were stained for 45 minutes with 25 μg/mL fluorescein isothiocyanate-SNA (FITC-SNA, E-Y Labs) in PBS in the dark, after which they were briefly washed in PBS and mounted in 15% polyvinol alcohol, 33% glycerol in 100mM Tris, pH 8.5. Duplicates were randomly separated during mounting; the slides were kept in the dark until viewing.

*Flow cytometry:* Cells from a confluent 100 mm dish were split into two 100 mm dishes 24 hours before harvesting for cytometry; when appropriate, dexamethasone to a final concentration of 2 μM was added at this time. Cells were harvested with Sigma Cell Dissociation Reagent (Sigma) at 37°C. Harvested cells were spun for 10 minutes at 1000 rpm and resuspended in PBS to a final cell concentration of $4 \times 10^6$ cells/mL. The cells were incubated for 2 hours in the presence of 2.5 μg/mL SNA-biotin (E-Y Labs) on ice with occasional shaking. After washing in PBS, the cells were resuspended to a concentration of $8 \times 10^6$ cells/mL; they were then incubated in the presence of 8 μg/mL streptavidin-R-phycoerythrin (Gibco) for 30 minutes on ice with occasional shaking. The cells were washed in PBS and run through the Caltech Cell Sorting Facility under the direction of Dr. Rochelle Diamond.

*Isolation of tPA:* For tPA harvesting, one confluent 100 mm dish was used to inoculate each T175; the transfected cell lines which had been growing in selective medium were inoculated into non-selective medium. After 24 hours, the serum-plus medium was removed and replaced with non-selective, serum-free medium; the medium was changed with non-selective, serum-free medium at time points 3, 6, 9, and 24 hours after the initial change. Following the final change, dexamethasone was added to the "induced" flasks at a final concentration of 2 μM. The medium was harvested 36 hours later and fresh medium was added, with or without 2 μM dexamethasone. The medium was harvested again 36 hours later. Samples were centrifuged and frozen immediately.

The purification procedure was modified from the method of Harkas [58]. Samples from the two harvests were combined and dialyzed for 18 hours at 4°C against dialysis/start buffer (1 M NaCl, 20 mM Tris, 0.01% Tween 80, pH 7.5). The concentrated retentate was then run through a 1 mL HiTrap chelating column (Pharmacia) charged with 0.1 M $ZnCl_2$. Approximately 30 mL of each sample was applied, after which the column was washed with 10 mL of start/dialysis buffer. Initially, elution was accomplished with 1 M NaCl, 20 mM Tris, 0.01% Tween 80, 50 mM Imidazole, pH 7.5; 0.25 mL fractions were collected. The optimized elution protocol was as follows: (all elution buffers were start buffer plus the indicated concentration of imidazole): 5 mL 5 mM imidazole; 3 mL 7 mM imidazole, 8 mL 9 mM imidazole, and 3 mL 50 mM imidazole. The fractions were then concentrated

to a final volume of approximately 60 µL using Centricon-10 microconcentrators (Amicon).

*Sample preparation for 2-D electrophoresis:* The method of sample preparation was as follows: to 125 µL of either cell extract or concentrated supernatant was added 5 µL of 0.1% bromophenol blue, 7.5 µL of 40% BioLyte 3-10 carrier ampholytes, 15.0 µL of 20% CHAPS, and 0.14 g urea (to yield a final concentration of about 9.5 M urea in about 250 µL); this particular quantity of sample was used to load two IPG strips, and sample preparation was adjusted proportionately up or down to load a different number of IPG strips.

*Isoelectric focusing:* Immobiline DryStrip IPG strips, pH 3-10L, 18 cm, were used. The strips were rehydrated in 8 M urea, 0.5% NP-40, 1.5% CHAPS, 0.2% BioLyte 3-10 carrier ampholytes, 0.2% DTT, 10% DMF. Electrode strips were soaked in either 0.05% NaOH or 0.0595% phosphoric acid. After placing the IPG strips in the Immobiline DryStrip tray, mineral oil was added to the tray to a depth which covered the strips but not the sample cups. Samples were loaded at the extreme cathodic or anodic end as indicated. After the sample was added to the cup, mineral oil was added to completely cover the sample cups. The typical program for isoelectric focusing was 2 hours at 360 V (20 V/cm), 2 hours at 1440 V (80 V/cm), and 35-40 hours at 2970 V (165 V/cm), at a controlled temperature of 25°C.

*IPG strip equilibration:* Strips were incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2% w/v DTT, 50 mM Tris pH 6.8 on a shaking platform for 10 minutes. They were rinsed briefly with water and then incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2.5% w/v iodoacetamide, 50 mM Tris pH 6.8, and a dash of bromophenol blue on a shaking platform for 5 minutes. Each strip was touched along its edge briefly on wet filter paper before being trimmed to fit onto the SDS-PAGE gel; the gel was trimmed approximately 1 cm on the end at which it was loaded and typically about 1.5 cm on the opposite end.

*Second dimension SDS-PAGE:* For the second dimension, 10% T polyacrylamide gels crosslinked with 0.27% PDA of dimensions 19 cm × 16 cm × 1.5 mm were used. The running buffer was 28.8 g/L glycine, 6 g/L Tris base, 1 g/L SDS. Up to six gels were run in parallel using the BioRad Protean II Multicell. 5 µL of broad range molecular weight standards (New England BioLabs) were loaded between the spacer and the basic end of the IPG strip. Gels were run at 4°C at 40 mA per gel for approximately 5 hours.

*Transfer of proteins onto a solid support:* The Pharmacia Multiphor II NovaBlot semi-dry blotting unit was used for blotting. Proteins were blotted onto Immobilon P membranes (Millipore) wetted in 100% MeOH and equilibrated in blotting buffer (39 mM glycine, 48 mM Tris base, 20% MeOH). Gels were transferred from the glass plates into water briefly, after which they were stacked in units (anode to cathode) of filter paper,

membrane, and gel, with two pieces of Whatman 3 mm chromatography paper on both sides of the stack. Gels were blotted for 6 hours at 200 mA. After blotting, the membranes were either processed immediately as described below, or transferred to water until needed.

*1-D separation of proteins using SDS-PAGE:* Concentrated samples were run on 8.75% acrylamide gels using a BioRad Mini-Protean II cell at 160 mV for 50 minutes. After desalting in transfer buffer for 15 minutes, gels were blotted onto Nitrocellulose at 1.1 mA for 45 minutes or 3 hours, depending on the thickness of the gel.

*Detection of MAA-binding and SNA-binding glycoproteins:* This protocol was based on the "Applications of digoxigenin-labeled lectins in glycoconjugate analysis" technical sheet from Boehringer Mannheim; a full account of this method has been published [61]. Membranes with bound proteins were rinsed in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 0.5% blocking reagent (Boehringer Mannheim) in TBS for 1 hour on a rocker platform. This was followed by two ten-minute rinses in TBS and one ten-minute rinse in buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, in TBS, pH 7.5). The blots were then incubated in either 1 µg/mL SNA-digoxigenin (Boehringer Mannheim) or 5 µg/mL MAA-digoxigenin (Boehringer Mannheim) in buffer 1 for one hour. This was followed by three ten-minute rinses in TBS. The blots were then incubated with 1 µL/mL anti-digoxigenin-alkaline phosphate (Boehringer Mannheim) in TBS for one hour, followed by three more rinses in TBS. The developing solution was 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5 with 187.5 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 500 µg/mL 4-nitro blue tetrazolium chloride (NBT). The membranes were incubated with developing solution without shaking for approximately 1 hour for SNA-probed membranes and 2 or more hours for MAA-probed membranes, after which they were rinsed thoroughly with water and dried on filter paper. In some experiments, as indicated in the figure legend, membranes were incubated with 1 µg/mL SNA-alkaline phosphatase (E-Y Labs) instead of SNA-digoxigenin; these membranes were rinsed three times in TBS and developed.

*tPA immunostaining:* Immunostaining using anti-tPA was carried out by one of two methods, as indicated in the figure legends. Immunostaining using the "XPBS method" was as follows. Membranes with transferred proteins were blocked for at least 30 minutes in 1% dried nonfat milk in XPBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/L $KH_2PO_4$), either directly after blotting, or after lectin detection. The blots were then incubated for 1 hour with 50 µL of rabbit IgG anti-tPA (Organon Teknika) in 10 mL of blocking buffer. After three ten-minute washes in XPBS, blots were incubated for thirty minutes with 25 µL of horseradish peroxidase (HRP)-anti-rabbit IgG (Organon Teknika) in blocking buffer. Following three ten-minute washes in XPBS, tPA bands were detected with ECL Western blotting detection reagents and protocol (Amersham) using Hyperfilm ECL (Amersham).

Immunostaining using the "TBST method" was as follows. Membranes with transferred proteins were rinsed in TBST (10 mM Tris, 150 mM NaCl, 0.05%, pH 8.0) and blocked for 30 minutes in TBSTM (TBST plus 5% dried nonfat milk). They were then incubated with 5 μL/mL rabbit IgG anti-tPA (Organon Teknika) in TBSTM for one hour, followed by three ten-minute washes in TBSTM. Then the blots were incubated with horseradish peroxidase (HRP)-anti-rabbit IgG (Promega) diluted 1:7500 in TBSTM for one hour, followed by one ten-minute wash in TBSTM and two ten-minute washes in TBST. Bands were detected with ECL Western blotting detection reagents and protocol (Amersham) using Hyperfilm ECL (Amersham).

# Results

## Isolation of Cells Expressing α2,6ST

Initially, DH5α was used as the host to screen for correctly formed pMSG-ST; however several rounds of restriction digests of eighteen different clones revealed that the plasmid was rearranging when maintained in this cell line. Pharmacia confirmed that rearrangement had been reported to them previously as a problem with this plasmid in certain hosts; they suggested using HB101 as a host. The plasmid was then grown and isolated from HB101.

Initial selection of clones from transfected cells was based on their ability to make guanine from xanthine in the presence of inhibitors of *de novo* purine nucleotide synthesis; this ability was conferred on them by the expression of the *Ecogpt* gene included on pMSG. The screening of clones surviving selection for expression of α2,6ST was accomplished by incubating uninduced and induced cells with FITC-SNA; cells expressing α2,6ST should possess α2,6-linked sialic acid on their cell surface proteins and thus should bind the SNA and its conjugated fluorescent label. After screening both uninduced and induced samples of each clone, it was determined that twelve clones did not bind SNA under induction, eleven clones bound significant amounts of SNA even when not under induction, and only two clones bound SNA only when under induction. The parental line, grown in non-selection media, but otherwise handled identically, consistently was completely negative for SNA binding, even when cultured in 2 μM dexamethasone. Some clones which were constitutive for SNA binding bound significantly more SNA than the inducible clones under induction; one such clone and one inducible clone were chosen for further study.

Flow cytometry analysis of cells labelled with SNA-biotin and streptavidin-PE confirmed that cells expressing α2,6ST had been isolated (Figure 3.1). In virtually all analytical runs, the percentage of cells showing high SNA-binding was at least 75% for the cells which were putative constitutive expressers of α2,6ST. The fact that only 75% of the supposedly clonal cells possessed α2,6-linked sialic acid on their cell surface glycoproteins could be explained two ways; the population might not have been clonal to begin with, as clones were not obtained by limiting dilution, or the gene might be unstable and lost at some significant rate.

## Analysis of tPA Sialylation by 1-D SDS-PAGE

To analyze the effect of the transfected glycosyltransferase gene on the sialylation of tPA, supernatant proteins were separated by SDS-PAGE and transferred onto a solid support, which was then probed with lectins or antibodies to tPA. Analysis was complicated by the fact that the serum which was added to the cell culture was rich in SNA-binding glycoproteins, which made analysis of the cell-derived glycoproteins extremely difficult.

The options for solving this problem were limited by the fact that the cells required the presence of serum for attachment. Initial experiments were done to determine if the serum glycoproteins could be removed by simple medium exchanges into serum-free medium after the cells had attached; SNA-binding analysis of the supernatant showed that even after as many as six changes over a 24-hour period (allowing for equilibration between the new medium and the cell-associated medium retained from the previous change), there were still significant amounts of serum proteins in the supernatant. Additional experiments attempted to purify tPA directly from supernatant containing 5% serum by the use of metal affinity chromatography; however, there were several serum proteins which purified at the same time to much higher concentrations than the tPA, and the presence of these serum proteins made visualizing the tPA extremely difficult. A series of purifications and Western analyses was conducted to find culture and elution conditions which allowed the glycosylation of tPA to be analyzed in the absence of serum proteins. This led to the development of the protocol listed in the methods section; this protocol combined a series of media exchanges with the use of an optimized metal affinity purification procedure.

Analysis of SNA-binding to SDS-PAGE-separated purified supernatant samples from both untransfected and transfected cells revealed that tPA made in the α2,6ST-transfected cells did possess α2,6-linked sialic acid, while tPA made in the untransfected cells did not (Figure 3.2). Probing with an antibody to tPA confirmed that tPA was present in both samples at similar concentrations (Figure 3.2B). Serial probing of a single membrane, first with SNA and then with an antibody to tPA, confirmed that the bands of protein in the region of 66 kD which were recognized by SNA were identical to the bands which were recognized by anti-tPA antibodies (Figure 3.2C and D).

Analysis of tPA Sialylation Using an Inducible Promoter

Glycoproteins made in the cell line which inducibly expressed α2,6ST were also studied. Despite the published reports of dexamethasone influencing glycosylation [36], and specifically, the expression of α2,6ST [177], once the purification protocol had been developed to remove the SNA-binding serum glycoproteins from samples, glycoproteins from the untransfected cell line consistently showed the absence of SNA binding when grown with and without 2 μM dexamethasone (Figure 3.3). One can also see in this same blot that there is definite SNA binding in the lane containing the supernatant from the putative constitutive expresser of α2,6ST, and that there is no SNA binding in the lanes of supernatant from the putative inducible α2,6ST expresser when $2 \times 10^{-10}$ M or less dexamethasone was used for induction. However, there is clearly SNA binding in the lanes of supernatant derived from cells cultured with $2 \times 10^{-8}$ and $2 \times 10^{-6}$ M dexamethasone. Several other 1-D experiments demonstrated a similar jump in the level of SNA binding when the concentration of dexamethasone was increased from $2 \times 10^{-10}$ to $2 \times 10^{-8}$ M. One such example is shown in Figure 3.4, which shows supernatants harvested from cells grown in the presence of different amounts of dexamethasone; these supernatants were concentrated, but not metal affinity purified. The top panel, which was

probed with the lectin MAA, shows that approximately the same amount of sialylated glycoprotein was present in each lane, and the bottom panel, which was probed with the lectin SNA, shows that at very high molecular weights (~200 kD), SNA binding increased significantly when the level of dexamethasone was increased from $2 \times 10^{-10}$ to $2 \times 10^{-8}$ M . Published studies have shown that induction of this promoter is dose-dependent over the range of $1 \times 10^{-9}$ to $1 \times 10^{-6}$ M dexamethasone [80] or more [68]. More highly resolved studies would be required to determine the dose-dependent nature of induction in this particular cell line.

Figure 3.4 also shows the presence of SNA-binding proteins in all lanes in the region of 66 kD and lower; these bands are present because this supernatant was not metal-affinity purified to remove serum glycoproteins, and the presence of these bands makes it difficult to determine the nature of tPA glycosylation in these samples. However, when these unpurified supernatants were analyzed by 2-D electrophoresis, the serum-derived SNA-binding glycoproteins localized to the acidic region of the IPG strip, while the tPA spots localized to the basic region of the IPG strip (Figure 3.5). Thus, 2-D separation made it possible to see that tPA made in cells induced with $2 \times 10^{-6}$ M dexamethasone did bind SNA, while tPA made in cells which were not induced did not (Figure 3.6).

# Discussion

This research clearly demonstrates the constitutive and inducible modification of the glycosylation of a heterologous glycoprotein by the genetic introduction of a cloned glycosyltransferase gene. By demonstrating that both SNA and antibodies to tPA bind to the same protein bands and spots, it has been conclusively shown that in transfected cells, tPA has been modified to possess α2,6-linked sialic acid; α2,6-sialylation of tPA occurred constitutively in one transfected cell line and only under induction with 2 μM dexamethasone in a separate cell line. Furthermore, it is clear that tPA is not the only glycoprotein being modified by the transfected glycosyltransferase. One-dimensional and two-dimensional separations of supernatant samples allowed visualization of many other SNA-binding spots and bands, indicating that addition of α2,6-linked sialic acid is not specific to tPA.

In the case of the constitutive clone, it would be difficult to prove conclusively that the expression of α2,6-linked sialic acid on tPA is a direct result of expression of the glycosyltransferase cDNA which was introduced. In this cell line, α2,6-sialylation could be the result of a site-specific insertion event which activated a silent endogenous gene or an insertion-site independent phenomenon which resulted in the activation of a silent gene. The latter possibility has been documented in CHO cells, where independent clones expressing a transfected human cDNA all expressed a novel glycosyltransferase activity [6].

However, in the case of the inducible clone, the results definitively support the conclusion that the presence of α2,6-linked sialic acid on tPA is the direct result of dexamethasone induction of the MMTV promoter controlling the expression of the transfected α2,6ST gene. First, uninduced transfected cells did not make tPA with α2,6-linked sialic acid, indicating that the insertion or presence of the transfected material in the CHO genome did not cause a specific or non-specific activation of an endogenous α2,6ST gene. Second, untransfected cells did not make tPA with α2,6-linked sialic acid when cultured with 2 μM dexamethasone, indicating that the presence of dexamethasone was not specifically or non-specifically causing the expression of a silent, endogenous α2,6ST gene. Thus, the presence of α2,6-linked sialic acid on tPA must be the result of the induction of the transfected α2,6ST gene.

These results demonstrating α2,6-sialylation of tPA would be enhanced by detailed oligosaccharide characterization of the purified protein. This type of analysis would determine the extent to which α2,6-sialylation was occurring; this analysis would also determine if α2,6-linked sialic acid was being added in place of or in addition to α2,3-linked sialic acid. It would also be of great interest to determine the dose-dependent nature of the dexamethasone induction of α2,6ST in these cells. Assays of α2,6ST activity and mRNA expression could paint a more complete picture of the pathway by which α2,6-sialylation occurs in response to dexamethasone. And determination of the α2,6ST

activities and sialic acid distributions of cells under different levels of induction would lead to a better understanding of how the level of expression of α2,6ST and level of expression of α2,6-linked sialic acid are related.

Although the ability to manipulate the glycosylation of a heterologous protein by genetic intervention in the host cell line has been demonstrated in this work by the introduction of a new glycosyltransferase activity under the control of an inducible promoter, genetic manipulation of glycosyltransferase expression in host cell lines is by no means limited to this one type of genetic intervention. One could add into a cell line the gene for an activity which was already being expressed with the goal of increasing its expression. Additionally, as has been mentioned previously, a new glycosyltransferase activity could be added under the control of a strong constitutive promoter, or a glycosyltransferase activity could be eliminated by homologous recombination (gene "knock-out"). One might even eliminate a glycosyltransferase activity by homologous recombination and add it back in under the control of an inducible promoter. One could envision as a pinnacle of glycosylation engineering the creation of a cell line in which every potentially useful glycosyltransferase activity is expressed only under the direct control of a highly inducible promoter capable of generating a full range of physiologically relevant expression levels. Such a cell line could be used to generate a full spectrum of glycoforms of a protein of interest, each of which could be evaluated for therapeutic safety and efficacy; furthermore, the glycosyltransferase expression of such a cell line could be finely adjusted to optimize the expression of a desired glycoform.

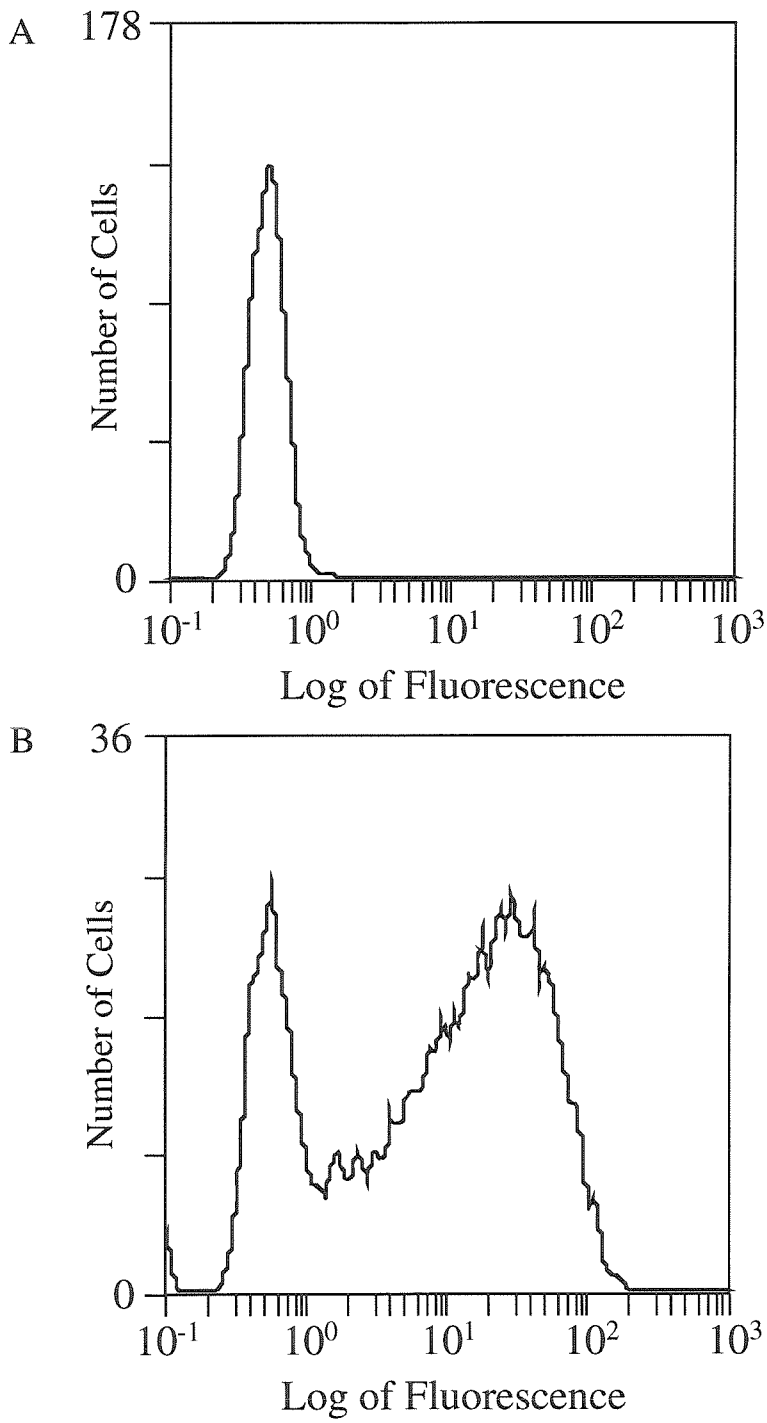


**Figure 3.1.** Flow cytometry histrogram of CHO cells labeled with SNA-biotin and streptavidin-PE. (A) Untransfected cells; (B) cells transfected with α2,6ST.

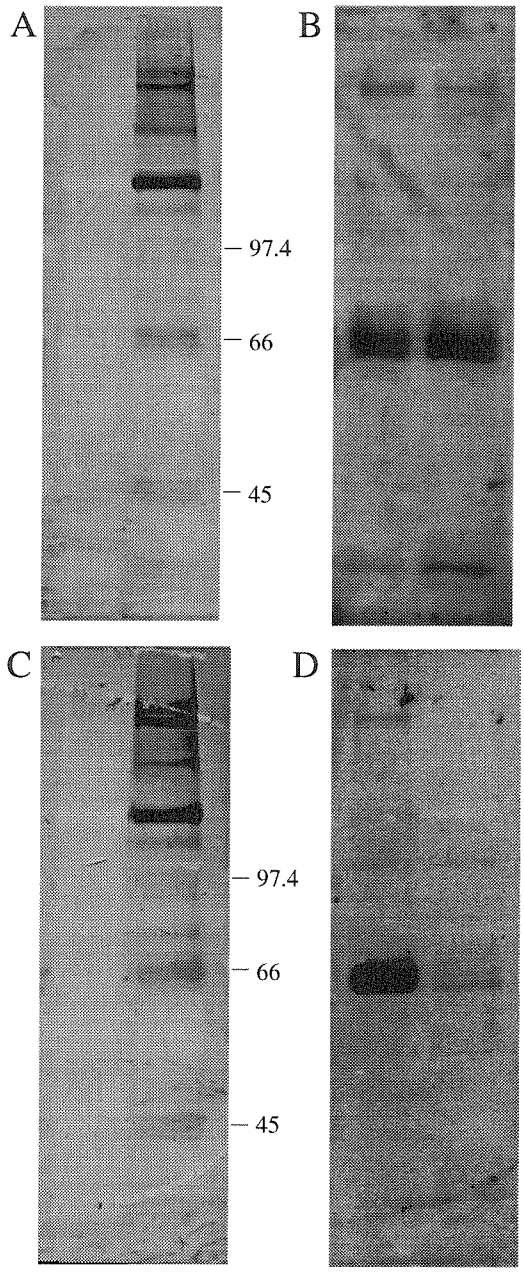


**Figure 3.2.** Purified supernatants from untransfected parental cells (left lane) and cells transfected with α2,6ST (right lane). Samples were separated by SDS-PAGE on an 8.75% T gel and detected with SNA-AP (A and C) or anti-tPA using the XPBS method (B and D). The membrane shown in (C) and (D) was probed first with SNA-AP (C) and then with anti-tPA (D). Note that only proteins from the α2,6ST-transfected cell line bind SNA and that the protein bands at 66 kD which bind SNA are also recognized by anti-tPA. Positions of MW markers (in kD) are shown on the right in (A) and (C).

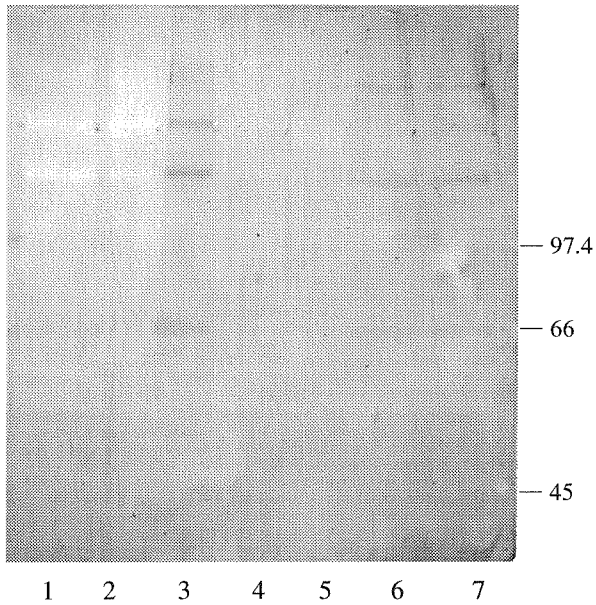


**Figure 3.3.** SNA binding to purified supernatant at different levels of induction with dexamethasone (dex). Supernatant was harvested from cells cultured in different concentrations of dexamethasone as indicated; supernatants were purified as described in the Methods section. Samples were separated by SDS-PAGE on an 8.75% T gel and detected with SNA-AP. Lanes 1 and 2 – untransfected parental CHO cells; (1) 0 μM dex, (2) 2 μM dex. Lane 3 – constitutive α2,6ST-expressing cells. Lanes 4 through 7 – inducible α2,6ST-expressing cells; (4) 0 μM dex, (5) $2 \times 10^{-10}$ M dex, (6) $2 \times 10^{-8}$ M dex, (7) 2 μM dex. Positions of MW markers (in kD) are shown on the right.

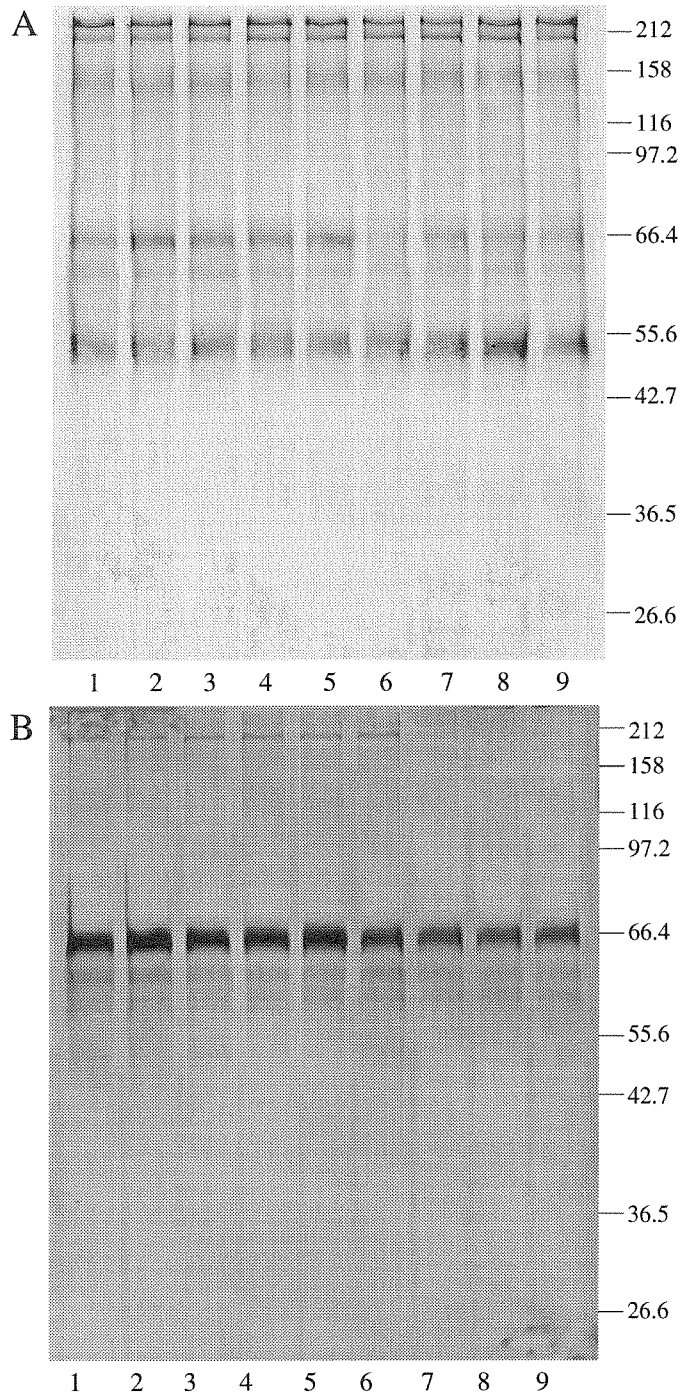


**Figure 3.4.** SNA binding to concentrated supernatant at different levels of induction with dexamethasone (dex). Supernatant was harvested from inducible α2,6ST-expressing cells cultured in different concentrations of dexamethasone as indicated; supernatants were concentrated using Centriplus concentrators. Samples were separated by SDS-PAGE on a 10% T gel and detected with MAA-digoxigenin (A) or SNA-digoxigenin (B). Samples were derived from cells induced with the following concentrations of dexamethasone: (1) $2 \times 10^{-6}$ M, (2) $8 \times 10^{-7}$ M, (3) $3.2 \times 10^{-7}$ M, (4) $1.28 \times 10^{-7}$ M, (5) $5.12 \times 10^{-8}$ M, (6) $2 \times 10^{-8}$ M, (7) $2 \times 10^{-10}$ M, (8) $2 \times 10^{-12}$ M, (9) 0 M. Note the acquisition of SNA binding by the high molecular weight band (~200 kD) at concentrations of $2 \times 10^{-8}$ M dex and higher. Positions of MW markers (in kD) are shown on the right.

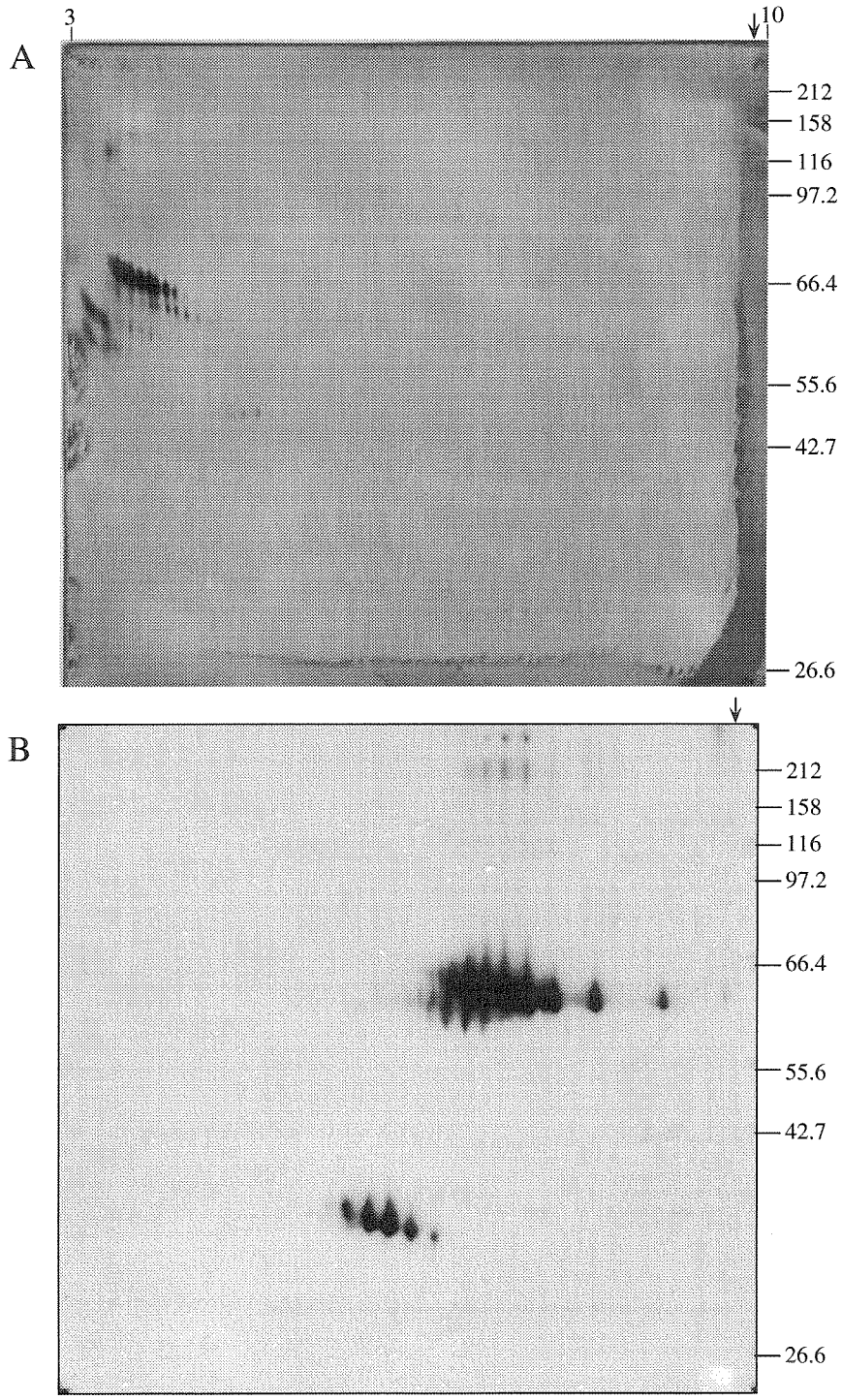


**Figure 3.5.** 2-D separation of concentrated supernatant from uninduced inducible α2,6ST-expressing cells. Supernatant was harvested as described in the Methods section. Supernatant was concentrated using Centriplus and Microcon concentrators, separated by 2-DE as described in the Methods section, and detected with SNA-digoxigenin (A) or anti-tPA using the TBST method (B). Note that the contaminating serum proteins localize to the acidic end of the IPG strip, while the tPA localizes to the basic end. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

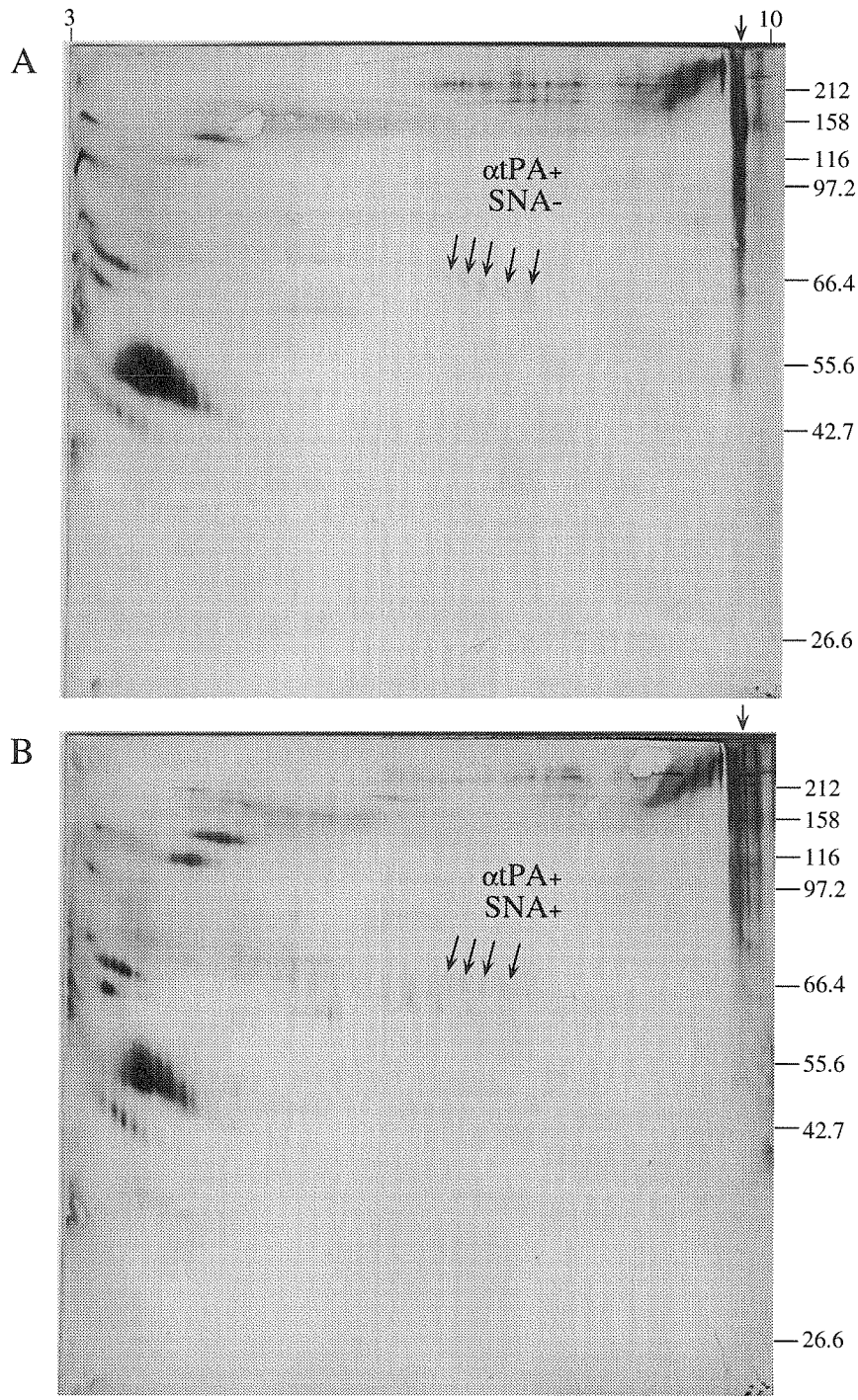


**Figure 3.6.** 2-D separation of concentrated supernatant from inducible α2,6ST-expressing cells with and without induction. Supernatant was harvested as described in the Methods section. Supernatant was concentrated using Centriplus and Microcon concentrators, separated by 2-DE as described in the Methods section, and detected with MAA-digoxigenin. Cells cultured without dexamethasone (A) and with 2 μM dexamethasone (B). Arrows indicate spots which were visualized when parallel blots were stained with anti-tPA using the TBST method. Text indicates if the same spots were visualized on parallel blots stained with SNA-digoxigenin. Note that only the cells cultured with 2 μM dexamethasone show SNA binding to tPA. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

# Chapter 4

# USE OF GLYCOSYLATION MUTANTS TO GENERATE GLYCOPROTEINS WITH DEFINED CHANGES IN THE STRUCTURE OF ATTACHED OLIGOSACCHARIDES

## Glycosylation "Mutants"

Many cell lines which express alterations in the glycosylation pathway have been characterized [reviewed in 161, 162, 163]; for the purposes of this discussion, these cell lines will be referred to as glycosylation "mutants." Cell lines with mutations in the glycosylation pathway are typically isolated by virtue of their ability to survive in culture in the presence of one or more carbohydrate-binding proteins, such as lectins, which are either directly toxic or linked to a toxic agent. It should be noted that this method selects for a particular phenotype at the cell surface, and all such selected cell lines must be characterized carefully to determine the biochemical nature of the alteration in the glycosylation pathway. Furthermore, because one way for a cell to survive in the presence of a toxic carbohydrate-binding protein is to not express the oligosaccharide structure which the toxic agent recognizes, the nature of this strategy tends to select for cell lines which are deficient in the activity of a glycosylation pathway enzyme.

## The Use of Mutant Cell Lines for Manipulating Glycosylation

While it may be very attractive to manipulate the glycosylation of a protein of interest by the insertion or deletion of a particular glycosyltransferase, such an approach may not be possible in all situations. In some cases, the necessary glycosyltransferase cDNA might not be available, or in other cases, achievement of the desired shift in glycoform distribution may require the manipulation of several different glycosyltransferases. In these situations, it would be useful to express a protein of interest in a mutant cell line which already synthesized a high percentage of the desired oligosaccharide structures [165]; one might reasonably expect that the protein of interest, when expressed in the mutant cell line, would have the desired distribution of oligosaccharide structures without the need for any further genetic manipulation.

Introducing a protein of interest into a mutant cell line to achieve a desired glycoform distribution is somewhat different than the common practice of introducing a protein into a variety of cell lines in a search for a cell line that produces an acceptable glycoform distribution. First, a mutant cell line could be viewed as a cell line which has already undergone a characterized genetic alteration in glycosyltransferase expression; a mutant cell line could be similar in phenotype to a cell line which has been genetically altered by using a cloned glycosyltransferase. Thus, the protein is being introduced into a more defined

glycosyltransferase environment, and the resulting glycoform distribution will be more predictable than if it was transfected into an arbitrary cell line. Further, while there can be significant differences in the glycoform distributions of a single protein expressed in different cell lines, expression of a protein in a mutant cell line will typically result in a radical alteration of the distribution of oligosaccharide structures. Common glycosylation mutations are complete inactivation of an activity or the novel expression of an activity. Thus, a protein expressed in a mutant cell line would not merely exhibit subtle shifts in the frequencies of the various oligosaccharide structures; rather, entire sets of structures might appear or disappear from the distribution. For example, a protein expressed in the CHO Lec1 cell line, which does not express GlcNAc-T I, would likely possess only high mannose oligosaccharides [143].

The use of glycosylation mutants for producing glycoproteins with controlled and defined oligosaccharide structures has been proposed previously [164]. This paper discussed the feasibility and value of expressing glycoproteins in CHO cell mutants which were defective in one or more portions of the glycosylation pathway and, as a result, produced very truncated, but homogenous, attached oligosaccharides. Homogenous attached oligosaccharides would be beneficial to those in the biotechnology industry because potential problems associated with the microheterogeneity of glycoforms and variations in the glycoform distribution could be avoided. The attachment of truncated oligosaccharides would not cause problems in cases where the actual structure of the attached oligosaccharide was not a determining factor in the proper folding, transport, or activity of the glycoprotein of interest; however, as was discussed in Chapter 1, the biological activity of many glycoproteins is strongly influenced by the structure of their attached oligosaccharides. This paper also discussed the use of glycosylation mutants to produce specific attached oligosaccharides for targeting glycoproteins to specific tissues via carbohydrate-binding receptors.

The use of a mutant cell line which is devoid of a glycosylation pathway enzyme is rather straightforward in that one would expect that any protein molecule expressed in that cell line would be subject to the limitations imposed on oligosaccharide structure by the absence of that enzyme. However, many mutant cell lines are not characterized by a complete inactivation of a glycosylation pathway enzyme; other types of mutations are a qualitative alteration in activity, a reduction but not total loss of activity, or an increase in or appearance of an activity. In these cases, the effect of the genetic alteration on glycoform distributions would be mediated through the heterogeneous nature of the interactions between glycosylation pathway enzymes and glycoproteins, with the result that not all glycoprotein molecules expressed in the mutant cell line would be affected or affected to the same extent. Further, one might expect to see protein-specific responses to such changes in glycosyltransferase activities. Efforts to manipulate the glycosylation of a protein of interest could be thwarted if that protein's oligosaccharide distribution was significantly influenced by a protein-specific effect, or if the resulting oligosaccharide distribution did not attain a sufficient level of homogeneity. To better understand the

feasibility of using glycosylation mutants to alter the oligosaccharide structures of a protein of interest in a controlled and defined way, it was of interest to determine the extent to which the outcome of a change in the glycosylation pathway, other than the total inactivation of a portion of the pathway, could be influenced by protein-specific effects and the heterogeneous nature of glycosylation.

The LEC10 Chinese Hamster Ovary Cell Line

The LEC10 CHO cell line was chosen for these studies. The LEC10 CHO cell line was originally isolated from the Pro-5 cell line by its resistance to the toxic lectin ricin, and was later characterized to possess a dominant alteration which resulted in the expression of GlcNAc-T III, an enzyme activity not detected in normal CHO cells [20]. GlcNAc-T III catalyzes the β(1,4) addition of one N-acetylglucosamine (GlcNAc) molecule to the central mannose residue of an oligosaccharide [112]; this GlcNAc molecule is referred to as the "bisecting" GlcNAc residue to distinguish it from other GlcNAc molecules which are attached to the central mannose core of an oligosaccharide. GlcNAc-T III has been cloned from both rat [116] and human [73].

The oligosaccharide structures of several purified proteins produced in normal CHO cells have been carefully analyzed, and in these structural characterizations, the presence of oligosaccharides possessing a bisecting GlcNAc residue has not been detected [29, 70, 81, 107, 115, 128, 146, 147, 159, 160, 169, 190]. For three of the proteins, gp120, β-IFN, and tPA, the research group which characterized the attached oligosaccharides of the protein when it was made in CHO cells also analyzed the attached oligosaccharides present when the protein was made in other cells, and these three proteins were shown to possess bisecting GlcNAc residues when made in other cells [81, 108, 130]; this would indicate that bisecting GlcNAc residues were detectable by the methods used and also that the lack of bisecting residues on these proteins when made in CHO cells was not due to a protein-specific limitation which precluded entirely the addition of the bisecting residue. The LEC10 cell line has been shown to assemble oligosaccharides with bisecting GlcNAc residues [20].

The LEC10 phenotype is suspected to arise from the novel expression of a silent endogenous gene [20]. Novel expression of silent endogenous genes is becoming a well-documented phenomenon in CHO cells. One research group has characterized at least three separate mutant CHO cell lines which express novel glycosyltransferase activities as a result of mutational events [20, 21] and has also noted the isolation of several CHO cell lines which expressed a novel glycosyltransferase activity after a transfection procedure [139]. Additionally, another group reported the novel detection of terminal α-galactose residues, antigenic in humans, on recombinant soluble CD4 made in CHO cells [6]; it was reasonably hypothesized that a silent endogenous gene had been activated by the transfection process or the transfected material because an α-galactosyltransferase gene could be detected by Southern analysis in CHO cells which did not express the

corresponding mRNA or activity [158]. One could also argue that the detection of α2,6-linked sialic acid on plasminogen made in CHO cells [29], discussed in Chapter 3, was the result of the activation of a silent endogenous gene. The expression of these activities without the addition of genetic materials encoding these activities would suggest that all of these glycosyltransferase genes, and possibly many more, are present in the CHO genome but not expressed in normal CHO cells.

The presence of silent endogenous genes is not at all surprising considering the tissue-dependence of glycosylation that has already been discussed. In the particular case of GlcNAc-T III, it has been shown that bisecting residues are not detectable on the attached oligosaccharides of γ-glutamyltranspeptidase made in the livers of mice, rats, and bovines, but are easily detectable on the oligosaccharides of γ-glutamyltranspeptidase made in the kidneys of the same animals [187]. Furthermore, another group found that bisected residues were not present on glycoproteins made in a normal liver, but that bisected structures did occur on glycoproteins made in cells isolated from a hepatoma [86]. It can be hypothesized from these results that the GlcNAc-T III gene is present ubiquitously throughout these animals, but completely silent in some tissues.

GlcNAc-T III is of particular interest to this research since it plays a key role in determining the branching structure of oligosaccharides. GlcNAc-T III utilizes as a substrate for modification a range of oligosaccharide structures which have had at least the first GlcNAc branch added by GlcNAc-T I. Modification of an oligosaccharide by GlcNAc-T III blocks the subsequent action of GlcNAc-T II, IV, and V and several other glycosyltransferases [148, 149]. Recalling the discussion of pathways in Chapter 1, action of GlcNAc-T III before GlcNAc-T II will restrict an oligosaccharide to a hybrid form, and the action of GlcNAc-T III on a complex oligosaccharide core structure will prevent further branching, limiting the oligosaccharide to the number of branches it possessed prior to GlcNAc-T III modification. Thus, the introduction of GlcNAc-T III activity into a glycosyltransferase pool is expected to significantly affect the number of branches of glycoprotein oligosaccharides.

Use of the Erythroagglutinating Lectin of *Phaseolus vulgaris*

The LEC10 cell line also is well suited to this study because the erythroagglutinating lectin of *Phaseolus vulgaris* (E-PHA) interacts strongly with oligosaccharides containing a bisecting residue [26, 53, 113, 186]; thus, it interacts strongly with oligosaccharides which have been modified by GlcNAc-T III. The subtleties of E-PHA binding have been studied extensively. E-PHA interaction is highest when the bisected oligosaccharide also possesses a terminal galactose at the nonreducing terminus of the α-D-Man-p-(1→6) branch [53, 113, 186]. The removal of the terminal galactose on the 1→6 branch, resulting in a terminal GlcNAc, reduces the interaction with E-PHA [26, 53, 113, 186]. The influence on binding of α2,6-linked sialic acid on the galactose residue is somewhat in question; three studies reported that the presence of α2,6-linked sialic acid results in no

interaction [53, 113, 186], but one study reported E-PHA binding to oligosaccharides with α2,6-linked sialic acid and no change in binding when the sialic acid was removed [26]. Bisected oligosaccharides with either four branches (tetraantennary) or with only one branch (hybrid) do not interact well with E-PHA [186]. E-PHA will also weakly interact with non-bisected oligosaccharides which possess one [113], two [53, 113], three [53, 113], or four [53] terminal galactose residues. Additionally it was found that the addition of two α2,3-linked sialic acids to a non-bisected biantennary structure with two terminal galactose residues (showing weak interaction with E-PHA) significantly improved the interaction of E-PHA with the oligosaccharide, while the addition of two α2,6-linked sialic acids to the same base structure completely abolished the weak interaction [53]. It should be noted that the influence of α2,6-linked sialic acid on E-PHA binding is not of concern in these experiments because CHO cells do not normally express the enzyme responsible for that linkage. It should also be noted that all of these analyses were performed using lectin affinity chromatography with immobilized lectins.

With these subtleties of E-PHA interaction in mind, it is expected that E-PHA will be useful in analyzing changes in oligosaccharide structure as a result of expression of GlcNAc-T III in CHO cells. If one considered only bisected-oligosaccharide-specific E-PHA binding, one would expect that normal CHO cell glycoproteins would not bind E-PHA. However, considering the other weak interactions which E-PHA can have with terminal galactose and α2,3-linked sialic acid (the only linkage attached by normal CHO cells), it would not be surprising if the glycoproteins of Pro-5 cells, the control cells for these experiments, did bind E-PHA. Previous experiments done in this lab using fluorescence microscopy demonstrated that even at a very low concentration of E-PHA-FITC, Pro-5 cells did bind significant amounts of E-PHA, although LEC10 cells bound substantially more (T. McAdams, personal communication). Additionally, when purified myeloperoxidase produced in CHO cells was blotted onto nitrocellulose and probed with several lectins, the protein did show E-PHA binding [109]; these results are slightly questionable in that the binding was only rated as plus or minus, and the binding was not indicated as being different after treatment of the purified protein with β-galactosidase, which should have abolished binding, although binding was abolished after treatment with glycopeptidase F, which would indicate the lectin was not binding non-specifically to the protein. The authors did not comment on the significance of observing E-PHA binding to a CHO cell protein and indicated that the presence or absence of E-PHA binding after various exoglycosidase treatments confirmed the specificity of the interaction, when in fact, based on the published studies of E-PHA specificity discussed above, the data actually argue against the specificity of the interaction in this assay.

Thus, to investigate the extent to which protein-specific effects and the heterogeneous nature of glycosylation can influence the outcome of a genetic manipulation of glycosyltransferase expression, the oligosaccharide structures of glycoproteins made in Pro-5 and LEC10 cells were compared by using the lectin E-PHA to probe 2-D separated cell extracts and supernatants.

# Methods

*Reagents:* Most reagents used in electrophoresis were electrophoresis grade and were purchased from Sigma (glycerol, NP-40, iodoacetamide) or BioRad (sodium dodecyl sulfate (SDS), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea, glycine, BioLyte carrier ampholytes, dithiothreitol (DTT), piperazine diacrylamide (PDA), acrylamide, β-mercaptoethanol (BME), N,N,N',N',-tetramethylenediamine (TEMED), ammonium persulphate). Dimethylformamide (DMF) was from EM. Antibodies to rough endoplasmic reticulum glycoprotein, lysosomal membrane glycoproteins 1 and 2, polymorphic major cell adhesion glycoprotein, alpha-2-macroglobulin receptor, and medial Golgi cisternae membrane glycoprotein were obtained from the Developmental Studies Hybridoma Bank (Johns Hopkins University). Antibody to galectin-1 was a gift of Professor R. D. Cummings (University of Oklahoma), antibody to β-glucuronidase was a gift of Professor W. S. Sly (St. Louis University), and antibody to arylsulphatase A was a gift of Professor A. R. Polten (Christian-Albrecht-Universitat Kiel).

*Cell culture:* Pro-5 (ATCC CRL 1781) and LEC10 Chinese hamster ovary cells were grown in adherent monolayer culture in α-MEM (GIBCO) supplemented with 5% dialyzed fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO) in a humidified incubator with 5% $CO_2$. They were routinely subcultured at a 1:10 dilution every 2 to 3 days, before reaching over-confluency. The LEC10 cells were generously provided by Professor Pamela Stanley.

*Extraction of cellular proteins:* 100 mm dishes were seeded with $1.5 \times 10^6$ cells; 24 hours later they were harvested. The plates were washed once with warm PBS, and then 3 mL of Sigma (non-enzymatic) Cell Dissociation Reagent in PBS was added to each plate. Plates were incubated at 37°C for 10 minutes, after which they were tapped on the side to free the cells from the plate. The cells were pipetted off the plate into a 50 mL centrifuge tube, and the plate was washed with 2 mL of warm PBS, which was added to the cell suspension. The tube with the cells was kept on ice for the duration of the processing. The cells were spun at 1000 rpm for 10 minutes in a Beckman TJ 6 centrifuge at 4°C. The supernatant was removed and the cells were washed 2 more times. After the final spin, cells were resuspended at $5.8 \times 10^7$ cells/mL in 100 mM Tris pH 6.8, 2% BME, 25% DMF. Cells were sonicated for 2 minutes and then heated to 100°C for 5 minutes. Extracts were frozen at this point.

*Harvest of supernatant proteins:* $7.5 \times 10^6$ cells were seeded into a T175 flask in 5% serum on Day 0. After 24 hours, the serum-containing medium was replaced with α-MEM with no serum; the medium was replaced an additional four times over the following 27 hours. The supernatant was harvested 36 hours after the final replacement and again after an additional 36 hours. Harvested supernatants were frozen immediately.

Supernatants were concentrated for loading onto IPG strips by using Centriplus and Microcon concentrators from Amicon.

*Sample preparation:* The method of sample preparation was as follows: to 125 µL of either cell extract or concentrated supernatant was added 5 µL of 0.1% bromophenol blue, 7.5 µL of 40% BioLyte 3-10 carrier ampholytes, 15.0 µL of 20% CHAPS, and 0.14 g urea (to yield a final concentration of about 9.5 M urea in about 250 µL); this particular quantity of sample was used to load two IPG strips, and sample preparation was adjusted proportionately up or down to load a different number of IPG strips. In the case of the cell extracts, there were typically urea crystals left in the sample, indicating saturation. For the serial loading experiments, 225 µL of cell extract was prepared in a final volume of 450 µL.

*Isoelectric focusing:* Immobiline DryStrips IPG strips, pH 3-10L, 18 cm, were used. The strips were rehydrated in 8 M urea, 0.5% NP-40, 1.5% CHAPS, 0.2% BioLyte 3-10 carrier ampholytes, 0.2% DTT, 10% DMF. Electrode strips were soaked in either 0.05% NaOH or 0.0595% phosphoric acid. After placing the IPG strips in the Immobiline DryStrip tray, mineral oil was added to the tray to a depth which covered the strips but not the sample cups. Samples were loaded at the extreme cathodic or anodic end as indicated. After the sample was added to the cup, mineral oil was added to completely cover the sample cups. The typical program for isoelectric focusing was 2 hours at 360 V (20 V/cm), 2 hours at 1440 V (80 V/cm), and 35-40 hours at 2970 V (165 V/cm), all at a controlled temperature of 25°C. For experiments in which the sample was serially loaded, 100 µL of sample was loaded initially, and an additional 100 µL was added after 24, 48, and 72 hours for a total of 400 µL of loaded sample; after the first three loadings, the strips were focused for 2 hours at 360 V and 22 hours at 1440 V and after the final loading, the strips were focused for 2 hours at 360 V, 2 hours at 1440 V, and 61 hours at 2970 V.

*IPG strip equilibration:* Strips were incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2% w/v DTT, 50 mM Tris pH 6.8 on a shaking platform for 10 minutes. They were rinsed briefly with water and then incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2.5% w/v iodoacetamide, 50 mM Tris pH 6.8, and a dash of bromophenol blue on a shaking platform for 5 minutes. Each strip was touched along its edge briefly on wet filter paper before being trimmed to fit onto the SDS-PAGE gel; the gel was trimmed approximately 1 cm on the end at which it was loaded and typically about 1.5 cm on the opposite end.

*Second dimension SDS-PAGE:* For the second dimension, unless otherwise noted, 10% T polyacrylamide gels crosslinked with 0.27% PDA of dimensions 19 cm × 16 cm × 1.5 mm were used. The running buffer was 28.8 g/L glycine, 6 g/L Tris base, 1 g/L SDS. Up to six gels were run in parallel using the BioRad Protean II Multicell. 5 µL of broad range molecular weight standards (New England BioLabs) were loaded between the spacer and the basic end of the IPG strip. Gels were run at 4°C at 40 mA per gel for approximately 5 hours.

*Transfer of proteins onto a solid support:* The Pharmacia Multiphor II NovaBlot semi-dry blotting unit was used for blotting. Proteins were blotted onto Immobilon P membranes (Millipore) wetted in 100% MeOH and equilibrated in blotting buffer (39 mM glycine, 48 mM Tris base, 20% MeOH). Gels were transferred from the glass plates into water briefly, after which they were stacked in units (anode to cathode) of filter paper, membrane, and gel, with two pieces of Whatman 3 mm chromatography paper on both sides of the stack. Gels were blotted for 6 hours at 200 mA. After blotting, the membranes were either processed immediately as described below, or transferred to water until needed.

*Detection of E-PHA-binding or ConA-binding glycoproteins:* This protocol for the detection of E-PHA-binding glycoproteins was based on the "Applications of digoxigenin-labeled lectins in glycoconjugate analysis" technical sheet from Boehringer Mannheim. Membranes (approx. 15 cm × 16 cm) with bound proteins were rinsed in 100 mL TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 75 mL 0.5% blocking reagent (Boehringer Mannheim) in TBS for 1 hour on a rocker platform. This was followed by two ten-minute rinses in 100 mL TBS and one ten-minute rinse in buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, in TBS, pH 7.5). The blots were then incubated in 35 mL of either 4 µg/mL E-PHA-alkaline phosphatase (E-Y Labs) or 20 µg/mL ConA-alkaline phosphatase (E-Y Labs) in buffer 1 for one hour on a rocker platform. This was followed by one ten-minute rinse in 150 mL TBS on a rocker platform and two ten-minute rinses in TBS on a shaking platform; the blots were switched to clean trays before the final rinse. The developing solution was 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5 with 187.5 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 500 µg/mL 4-nitro blue tetrazolium chloride (NBT); 40 mL of developing solution was used per membrane. The membranes were incubated with developing solution without shaking for approximately 1 hour, 45 minutes for E-PHA-probed membranes and 20 minutes for ConA-probed membranes, after which they were rinsed thoroughly with water and dried on filter paper.

*Staining of proteins with gold particles:* Membranes with transferred proteins were rocked in 50 mL PBST (2 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 150 mM NaCl, 0.3% Tween 20, pH 7.4) twice for 5 minutes, once for 2 hours, and three times for 5 minutes. They were then rinsed in 150 mL water twice for 5 minutes, after which they were incubated in 35 mL of gold protein staining solution (Zymed) until protein spots were clearly visible. They were then rinsed in water.

*Immunostaining:* Membranes with transferred proteins were rinsed in TBST (10 mM Tris, 150 mM NaCl, 0.05%, pH 8.0) and blocked for 30 minutes in TBSTM (TBST plus 5% dried nonfat milk). They were then incubated with primary antibody in TBSTM for one hour, followed by three ten-minute washes in TBSTM. This was followed by incubation with secondary antibody diluted 1:7500 in TBSTM for one hour, followed by

one ten-minute wash in TBSTM and two ten-minute washes in TBST. Blots probed with horseradish peroxidase-labelled secondary antibodies were detected with ECL Western blotting detection reagents and protocol (Amersham) using Hyperfilm ECL (Amersham). Blots probed with alkaline phosphatase-conjugated secondary antibodies were developed in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5 with 165 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 333 µg/mL 4-nitro blue tetrazolium chloride (NBT).

# Results

General Protein and Glycoprotein Stains

To confirm that differences in E-PHA binding were not the result of significant differences in the overall glycoprotein content of the samples, and to generate a baseline picture of the glycoprotein content of a sample for determining what percentage of glycoproteins in a sample had been altered by GlcNAc-T III, it was necessary to have a method for independently revealing all of the glycoproteins in a sample. The first candidate for such a role was the DIG Glycan Detection Kit from Boehringer Mannheim; this kit is based on the oxidation of adjacent hydroxyl groups in oligosaccharides to aldehyde groups by a mild periodate treatment, followed by the conjugation of digoxigenin to these groups and the use of an enzyme-conjugated anti-digoxigenin antibody. This kit was used to stain blots of 2-D-separated Pro-5 and LEC10 cell extracts; these blots were generated in parallel with blots which were stained with E-PHA-AP (Figure 4.1). The results were somewhat useful in that the Pro-5 and LEC10 samples had extremely similar staining patterns, both with regard to spot position and spot staining intensity (data not shown); this indicated that the overall glycoprotein composition of the samples was similar. However, there were only two sets of spots which could be seen in the same position on both the periodate-stained and E-PHA-stained membranes, and those two sets of spots were visible only when the sample was loaded at the basic end of the IPG strip; the rest of the periodate spots showed no correlation to any spots detected by E-PHA. Based on these observations, it was clear that the periodate-based stain would not serve well as a method for determining the total glycoprotein composition of a sample.

The lectin from *Canavalia ensiformis* (ConA) was also evaluated as a general glycoprotein stain for this purpose. ConA typically recognizes oligosaccharides which are high mannose, hybrid, or biantennary in structure. ConA-AP was used to probe blots of 2-D-separated cell extracts from both cell lines, and the staining pattern was compared to that of blots which were run in parallel and probed with E-PHA-AP (Figure 4.2). ConA-AP stained more glycoproteins over a wider region of the gel than the periodate-based stain. The ConA-probed blots of the two different cell extracts were very similar (data not shown); there were a significant number of ConA-stained spots in the two samples which were nearly identical in position, shape, size, and intensity . However, there was not much similarity between the positions of the ConA-stained spots and the positions of the E-PHA-stained spots, indicating that for the most part, the two lectins were recognzing different subsets of glycoproteins. The ConA stain confirmed the baseline similarity of the samples with respect to ConA-binding glycoprotein concentration and composition, but it would not serve well as a method for determining the total glycoprotein composition of a sample.

A general protein staining method utilizing positively-charged gold particles was used to generate an overall picture of the protein composition of the samples, to ensure that there

were no gross differences in the samples which might affect the outcome of the more specific staining methods. It was of note that most of the proteins which were stained with E-PHA-AP were not detected with the gold stain (Figure 4.3); for example, the high molecular weight region which is densely populated with E-PHA-staining proteins appears devoid of protein when stained with gold.

Immunostaining to Identify Immunoreactive Glycoproteins

With the expectation that it was going to be difficult to conclusively determine related spots on a single blot and related spots on a series of blots without the use of antibodies, a panel of antibodies to glycoproteins was used to probe 2-DE separated Pro-5 cell extracts in the hope of finding antibodies which recognized E-PHA staining proteins. The preliminary results are summarized in Figure 4.4; although the immunostaining was done using Pro-5 cell extracts, the locations of the immunostained spots and chains have been shown on a blot of LEC10 cells to show the E-PHA-binding capabilities of the recognized proteins.

Antibodies to rough endoplasmic reticulum glycoprotein, lysosomal membrane glycoprotein 2 (LAMP-2), polymorphic major cell adhesion glycoprotein, and β-glucuronidase did not recognize, at a level that could be detected, any protein spots or chains in the Pro-5 cell extract. Antibodies to the putative alpha-2-macroglobulin receptor (α2MR) and arylsulphatase A (ASA) did recognize spots and chains of the expected molecular weights (180 – 190 kD and 50 kD respectively), and these spots and chains corresponded with spots and chains which were capable of binding E-PHA. Antibody to galectin-1 (gal-1) recognized proteins which had run at the dye front and also a high molecular weight protein chain; galectin-1 would be expected to run at the dye front on 10% T gels as its molecular weight is only 14 kD. The supplier of the antibody to galectin-1 was confident that it did not cross-react with anything, so the high molecular weight chain recognized by galectin-1 is likely an oligomer of galectin-1 or a complex of galectin-1 and another protein; this high molecular weight chain is particularly interesting because it appears to coincide with a chain of E-PHA-binding proteins. The antibody to lysosomal membrane glycoprotein 1 (LAMP-1) recognized protein spots which were approximately 66 kD instead of the expected molecular weight of 110 kD; these spots also coincided with E-PHA binding spots. The antibody to medial Golgi cisternae membrane glycoprotein (MGCMG) recognized a chain of the appropriate molecular weight (125 kD), but this chain did not coincide with any E-PHA binding chains.

E-PHA-Binding Patterns of Cell Extracts

LEC10 and Pro-5 cell extracts were separated by 2-D electrophoresis and the blots were probed with E-PHA-AP to evaluate the effect of the expression of GlcNAc-T III on the presence of E-PHA-binding glycoproteins (Figure 4.5). Very few E-PHA-binding proteins could be detected on blots which were loaded with the extract of $2.9 \times 10^6$ Pro-5 cells (in a sample volume of 100 μL); there were only four chains which could be observed

at this loading level, and the presence of all four chains on one blot was not reproducible. In contrast, at this loading level, many chains (over fifteen) of E-PHA-binding glycoproteins could consistently be detected in the LEC10 cell extract samples, and three of the four chains which could be seen on the Pro-5 blots were stained much more darkly on the LEC10 blots.

To determine if there were other E-PHA-binding glycoproteins present in the Pro-5 cell extract, but at levels too low to be detected, the amount of extract loaded onto one IPG strip was increased by using the technique of serial loading. E-PHA-binding spots and chains increased in staining intensity in both cell extracts when the load was increased by a factor of four to a total of $1.16 \times 10^7$ cells per IPG strip (Figure 4.6); additionally, a few new chains and spots became visible in both extracts at this higher load. Overall, the level of E-PHA binding was clearly higher in the LEC10 cell extract than in the Pro-5 cell extract; the LEC10 cell extract had more E-PHA binding chains and spots than the Pro-5 extract, and, in the cases where spots could be compared between blots, the LEC10 cell extract had a higher level of E-PHA binding per chain or spot.

A detailed comparison of the E-PHA binding patterns was made by making transparencies which duplicated the E-PHA-binding patterns on different blots, and then overlaying the transparencies to make observations. Analysis of the patterns was complicated by the fact that attached oligosaccharides can significantly influence a protein's 2-D spot position, as mentioned previously; thus, it can be expected that glycoproteins made in the Pro-5 cells will not be in the same position on a 2-D blot as their counterparts made in the LEC10 cells. The only way to definitively group related spots and match them to related spots on a different membrane would be to use a polypeptide-specific antibody; in the absence of such antibodies, correlations of spots and chains were based on the size, shape, and position of the spots, the trajectories of chains of putatively related spots, and the positions of spots relative to experimentally defined, internal reference spots.

When the E-PHA-binding patterns of the serially-loaded LEC10 and Pro-5 cell extracts were compared by superposition, several chains could be seen for which the bottom right portion of the chain on the Pro-5 blot appeared to lead directly to or overlap with the upper left portion of a similarly shaped chain on the LEC10 blot (note especially Figure 4.6, chain A); this could be viewed as a shift to a lower molecular weight and more basic pI when glycoproteins were made in the LEC10 cells. Chain B, located on the most basic third of the gel and at a relatively high molecular weight (Figure 4.6), was interesting because it was consistently present in extracts from both cell lines, and it was usually stained to about the same intensity in about the same pattern on blots of LEC10 and Pro-5 cells harvested at the same time. This chain could be derived from the serum or it could be a protein chain which was not modified by GlcNAc-T III; in either case, one would expect such chains to bind E-PHA at similar levels in a similar position regardless of the cell source, as is seen in the case of chain B. There were also many chains (for example, chains 1 and 2 of Figure 4.6) present in the LEC10 cell extract which were not present in the Pro-5 cell extract.

E-PHA-Binding Patterns of Supernatant Glycoproteins

The lectin binding patterns of 2-D-separated supernatant glycoproteins from Pro-5 and LEC10 cells were also analyzed to determine the effect of expression of GlcNAc-T III. ConA-probed blots were very similar in overall patterns and intensities, indicating that the quantity and composition of the ConA-binding glycoproteins in the two samples was similar (data not shown). However, the E-PHA-binding patterns of the two samples were significantly different; the LEC10 sample had significantly more E-PHA binding spots and more E-PHA binding per spot when compared to the Pro-5 sample (Figure 4.7).

In contrast to the cell extracts, the supernatant proteins of the Pro-5 cell line did contain a significant number of E-PHA-binding proteins (Figure 4.7A). One might argue that these proteins represent serum proteins which were not removed from the supernatant by the media exchanges. As mentioned above, if the E-PHA staining proteins in the Pro-5 sample were serum proteins, two things would be expected: corresponding chains should be seen in the same positions on blots of LEC10 supernatants, and the corresponding chains on the LEC10 blots should bind E-PHA at about the same level as on the Pro-5 blots. However, a comparison of the blots revealed that there were no obvious examples of chains which stained to the same intensity in both samples; therefore it is reasonable to assume that the E-PHA binding glycoproteins in the Pro-5 supernatant were glycoproteins which bound E-PHA by virtue of exposed galactose residues rather than the presence of a bisecting GlcNAc.

As with the cell extracts, a more detailed analysis of changes in glycosylation brought about by the expression of GlcNAc-T III was made possible by overlaying transparencies. In all cases where a comparison could be made, LEC10 chains were stained significantly more than their Pro-5 counterparts. Additionally, there were many E-PHA-binding spots in the LEC10 supernatant which had no visible E-PHA-binding counterparts in the Pro-5 supernatant. Furthermore, there were many examples of protein chains which shifted to lower molecular weights and more basic pIs when made in the LEC10 cells. The actual overlay comparison is shown in Figure 4.7C, where the blue spots represent the E-PHA-staining pattern of the Pro-5 cell extract and the red spots represent the E-PHA-staining pattern of the LEC10 cell extract; five examples proteins which shifted in position are highlighted. In some cases, the Pro-5 chain of spots was a subset of the LEC10 chain of spots, while in other cases, the Pro-5 chain possessed E-PHA-binding spots (above and to left of overlap region) which were not detectable on the LEC10 blot.

There was also one chain of note which clearly did not shift in position, but did change significantly in E-PHA binding (chain C of Figure 4.7C). The positions of all of the spots comprising this chain were nearly identical in the two samples; however, the spots of this chain bound significantly more E-PHA per spot when made in the LEC10 cells. When

similar blots were probed with ConA, the spots of chain "C" were in the same position and were stained by ConA-AP to similar intensities in the two samples (data not shown).

# Discussion

The E-PHA binding patterns of cellular extract proteins and supernatant proteins show significant changes when the proteins are made in LEC10 cells. This is seen in a qualitative change in the presence or position of E-PHA-binding chains and also in a quantitative change in E-PHA binding per spot or chain. Thus, the expression of GlcNAc-T III in CHO cells appears to significantly alter the structures of glycoprotein oligosaccharides in a wide-spread way.

Unfortunately, in the absence of a method to detect all of the hybrid and complex glycoproteins in the samples, it was not possible to determine if there was a subset of glycoproteins which were not modified by GlcNAc-T III due to protein-specific effects. Further, in the absence of more detailed oligosaccharide analysis, very little can be said regarding the glycoproteins which were detected by ConA, but not detected by E-PHA when made in the LEC10 cells. It is possible that the oligosaccharides of these glycoproteins are high mannose structures; high mannose structures would be recognized by ConA, but would not be substrates for GlcNAc-T III.

A more detailed consideration of the quantitative and qualitative differences in E-PHA binding patterns of Pro-5 and LEC10 glycoproteins leads to some interesting observations. Returning for a moment to consider the biochemical result of action by GlcNAc-T IIII, one recalls that modification by GlcNAc-T III makes an oligosaccharide a non-substrate for GlcNAc-T II, IV, and V; additionally, GlcNAc-T III competes directly with these other GlcNAc-T's for the same substrates. Thus, the presence of GlcNAc-T III activity would likely reduce the number of oligosaccharides which are modified by GlcNAc-T II, IV, and V. Since these enzymes add branches which are capable of being further extended by various other glycosyltransferases, by adding a single bisecting GlcNAc residue, the oligosaccharide potentially looses the ability to receive three additional branches and all of the extending and terminal sugar residues they could each receive, including sialic acid. An exchange of a bisecting GlcNAc residue for one simple three-sugar branch (GlcNAc, Gal, SA) on one oligosaccharide of a protein would result in a decrease in molecular weight of the protein of 500 Daltons; an exchange of a bisecting GlcNAc residue for three simple three sugar branches could result in a decrease in molecular weight of close to 2 kD. These changes in a protein's molecular weight could easily be increased by the loss of oligosaccharide branches containing repeating N-acetyllactosamine units or by exchanges at multiple glycosylation sites on a single protein molecule. Additionally, the loss of an oligosaccharide branch would also likely result in the loss of the negative charge of the sialic acid residue which typically terminates a branch; a reduction in the negative charge on a protein would increase its pI.

Therefore one could expect that the general result of expression of and oligosaccharide modification by GlcNAc-T III would be a shift in the glycoform distributions of proteins to lower molecular weights and more basic pIs, because GlcNAc-T III-modified

oligosaccharides would likely have fewer branches. This is the pattern which was seen in comparisons of 2-D separated proteins from Pro-5 and LEC10 cells. Thus, both the shift in the chain positions and the changes in E-PHA binding per spot suggest that a large portion of E-PHA binding proteins in made in Pro-5 cells do become modified by GlcNAc-T III when expressed in LEC10 cells.

The caveat in this particular analysis is that there is no conclusive way, aside from an antibody, to demonstrate that any chain in the Pro-5 samples is the same protein at the peptide level as the chain it appears to be related to in the LEC10 samples. For those chains in which there is a significant overlap of spots between samples, the assumption that the same peptide backbone is involved is reasonable. However, in the cases where the overlap is not as clear, the interpretation becomes more speculative. Clearly, the ability to use antibodies to group and track proteins based on common peptide backbones would significantly enhance and substantiate this analysis.

The ability to use antibodies would also resolve questions about E-PHA-binding proteins made in the Pro-5 cells which do not show an observable shift in position when made in LEC10 cells. In some cases, the shift might not be observable because it is not clear which spots in the LEC10 sample correspond to spots in the Pro-5 sample. If antibodies could be found which recognized all of the different peptide backbones which are E-PHA-binding when made in Pro-5 cell, additional examples of shifts due to modification by GlcNAc-T III might become obvious. Even with the use of antibodies, in some cases, the shift in 2-D position of a protein chain or spot made in the LEC10 cells may be so small that it is not distinguishable from gel-to-gel variation. This complication might be overcome by pending improvements in 2-D gel technology.

Changes in E-PHA-binding patterns can be used to speculate on the actual structure of the attached oligosaccharides of E-PHA-binding proteins made in Pro-5 and LEC10 cells. Continuing with a discussion of the expected effects of GlcNAc-T IIII expression on the positions of a protein chain on a 2-D gel, one could hypothesize that oligosaccharides which had more branches in the absence of GlcNAc-T III activity would be more prone to significant shifts in molecular weight and pI than those oligosaccharides with fewer branches because the more highly-branched oligosaccharides would have more to lose, so to speak. In the comparison of cell supernatants presented in Figure 4.7, chain C was highlighted as a chain which did not shift position, but did show a dramatic increase in lectin binding when made in LEC10 cells. Following the above logic, it might be reasonable to infer that the structures of the attached oligosaccharides of chain C were largely of a biantennary or hybrid nature in the absence of GlcNAc-T III. If that were the case, addition of a single bisecting N-acetylglucosamine would not result in any significant changes in molecular weight (less than 0.5% of a 60 kD protein) or in pI, but would significantly increase the protein molecule's E-PHA affinity; thus the spots would stay in the same position, but would greatly increase in staining. This hypothesis regarding chain C's oligosaccharide structure is supported by the fact that chain C was recognized well in

both cell lines by ConA, which is commonly regarded to recognize oligosaccharides of a high mannose, hybrid, or biantennary structure.

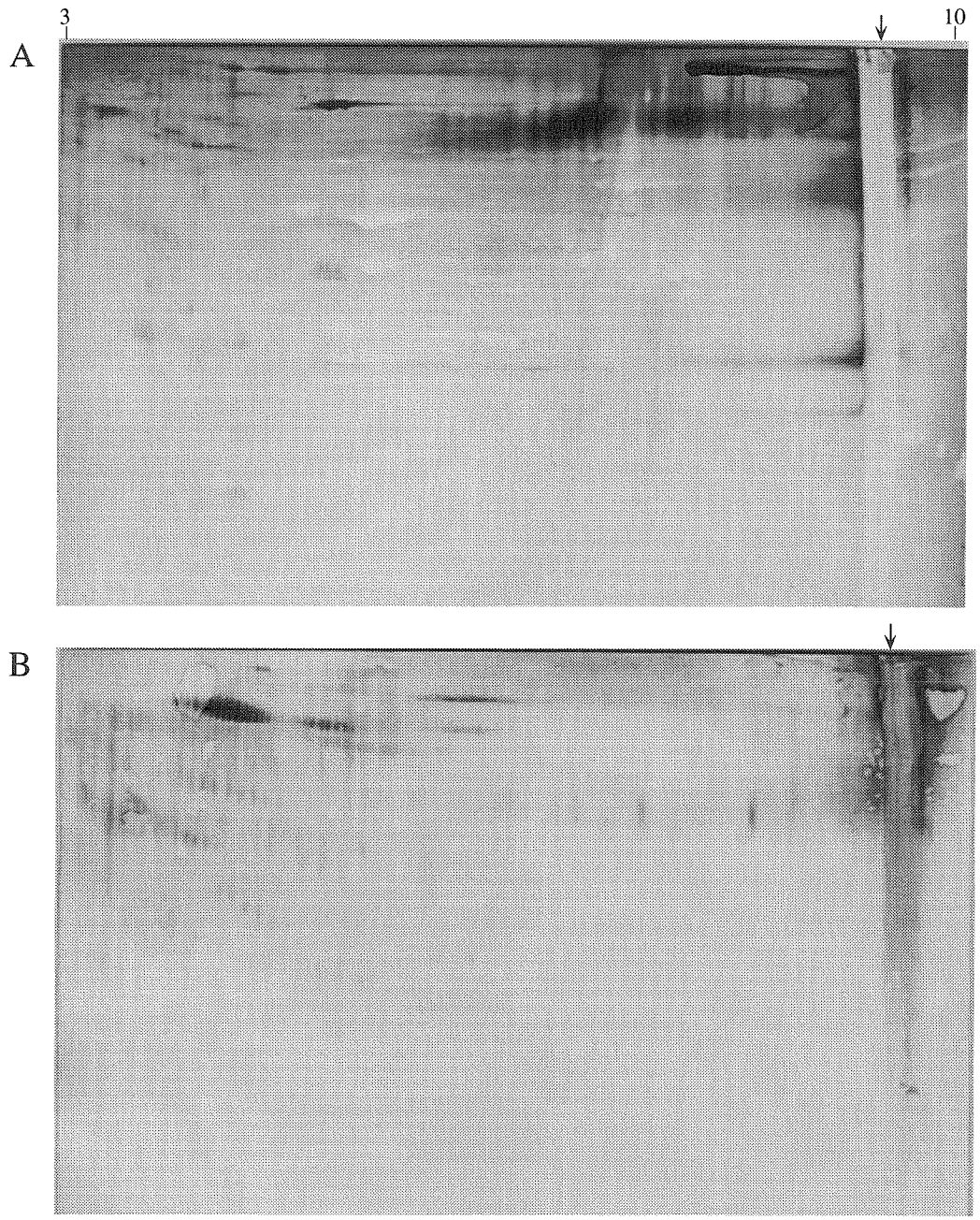


**Figure 4.1.** LEC10 cell extracts separated by 2-DE and glycoproteins detected with the periodate-based general glycoprotein stain (A) or E-PHA-AP (B). The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

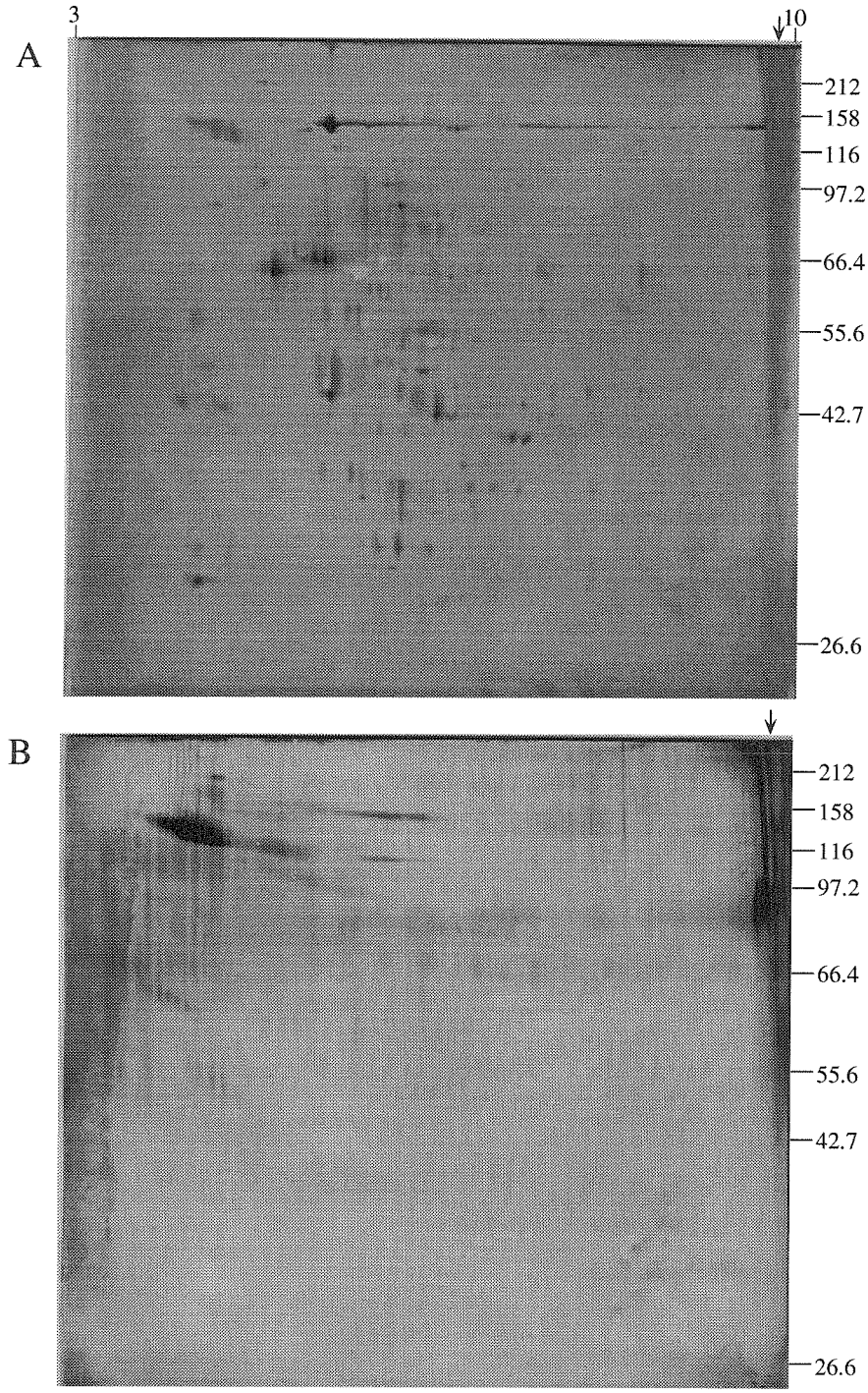


**Figure 4.2.** LEC10 cell extracts separated by 2-DE and glycoproteins detected with ConA-AP (A) or E-PHA-AP (B). Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

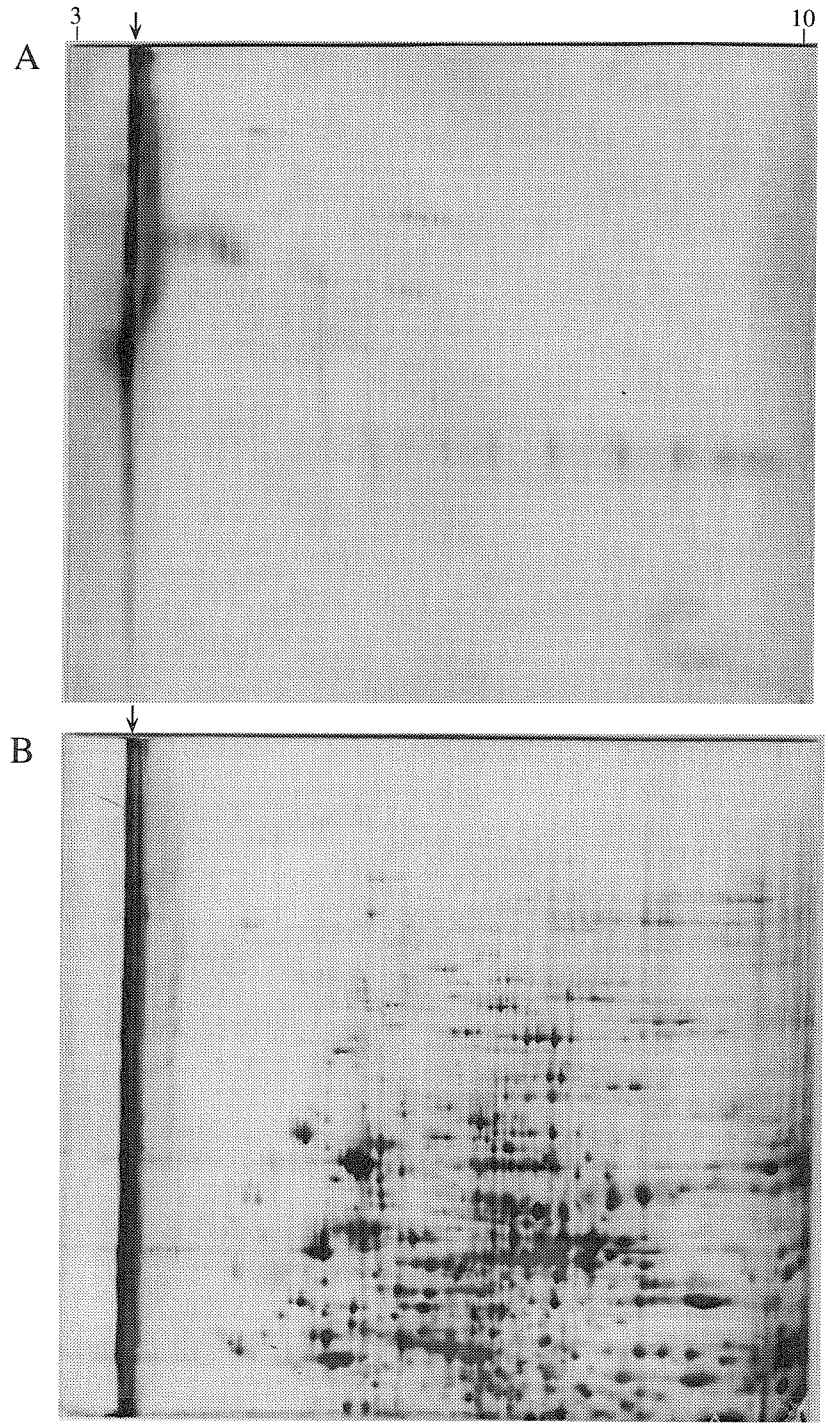


**Figure 4.3.** LEC10 cell extracts separated by IEF and SDS-PAGE on a 7% T gel and detected with E-PHA-AP (A) and colloidal gold (B). Note the absence of gold-stained proteins which correspond with the E-PHA-stained proteins. The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

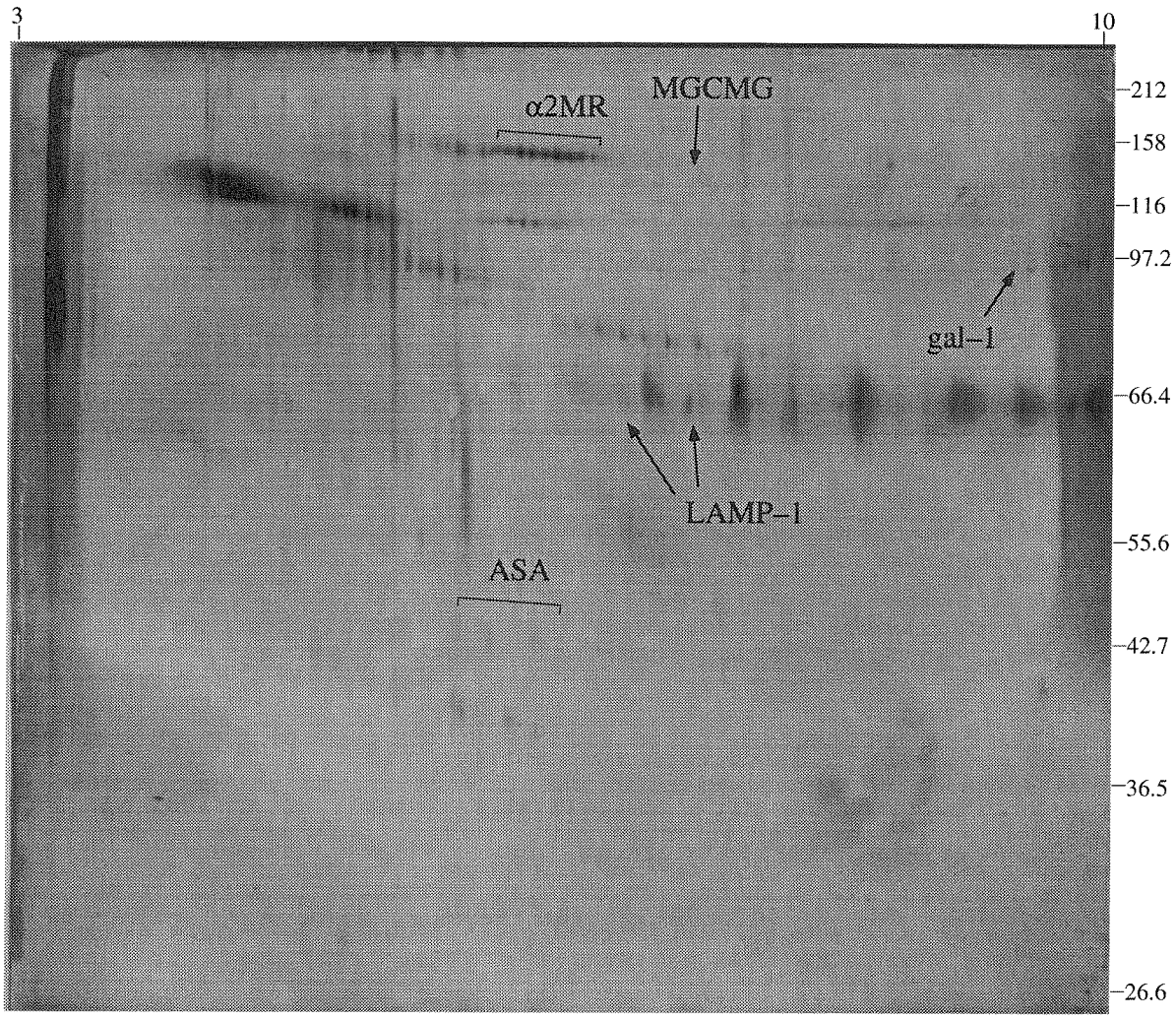


**Figure 4.4.** Proteins which were immunoreactive with antibodies to glycoproteins. Text corresponding to arrows and brackets indicates what the immunoreactive antibody was raised against. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top.

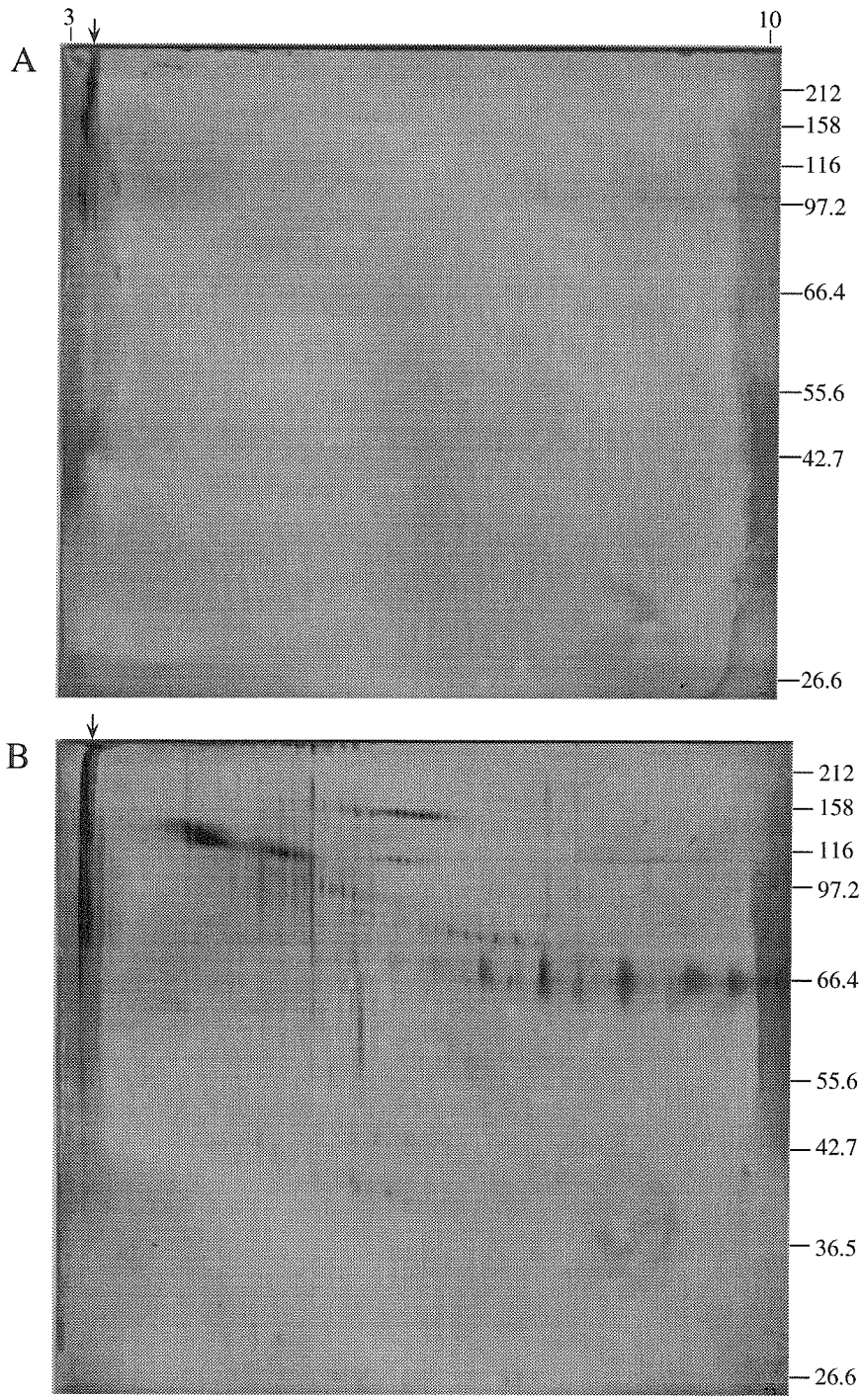


**Figure 4.5.** E-PHA-binding patterns of Pro-5 and LEC10 cell extracts. The extract of $2.9 \times 10^6$ cells was separated by 2-DE and detected with E-PHA-AP. (A) Pro-5 cells, (B) LEC10 cells. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

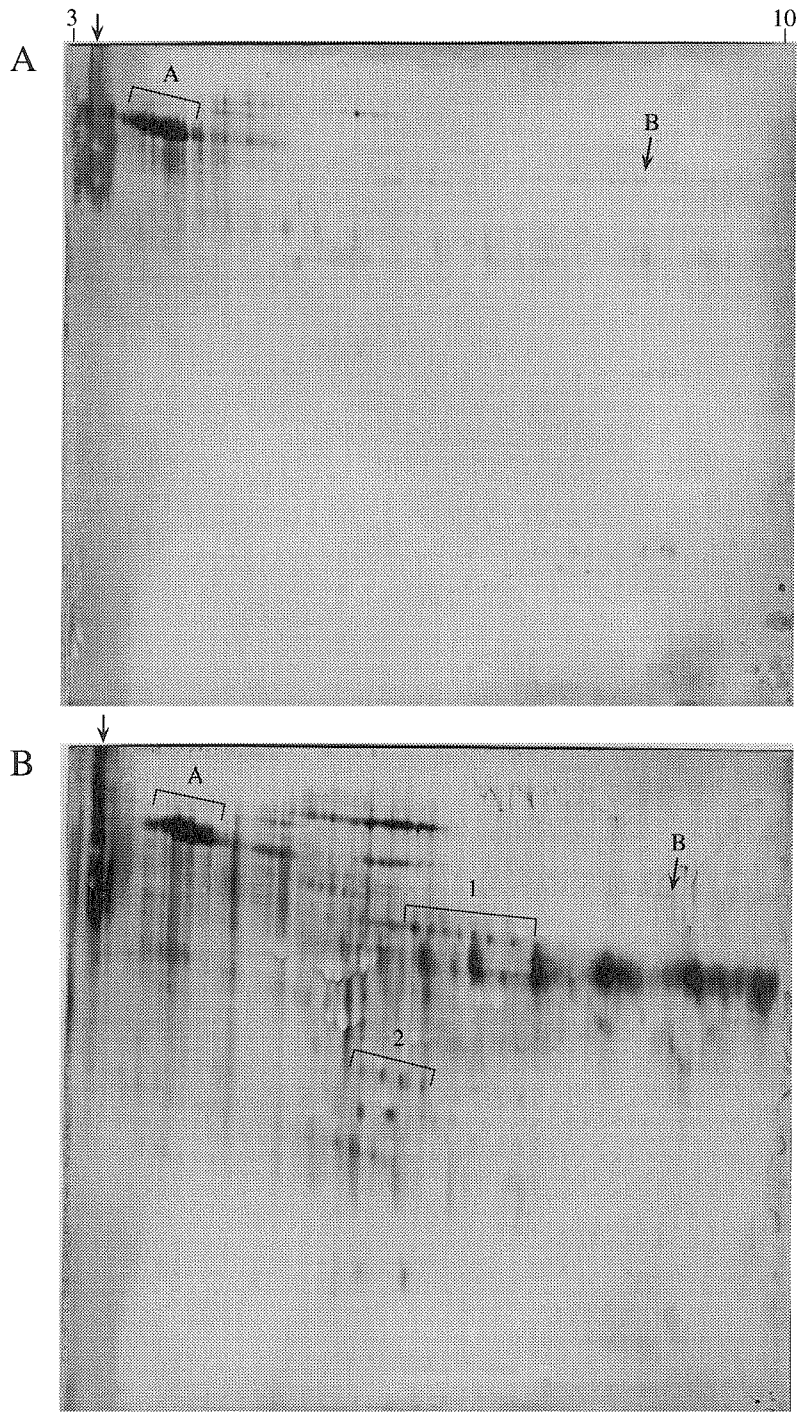


**Figure 4.6.** E-PHA-binding patterns of Pro-5 and LEC10 cell extracts. The extract of $1.16 \times 10^7$ cells was loaded serially onto IPG strips as described in the text, separated by 2-DE, and detected with E-PHA-AP. (A) Pro-5 cells, (B) LEC10 cells. Note the shift in the position of chain "A" relative to the loading site. The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

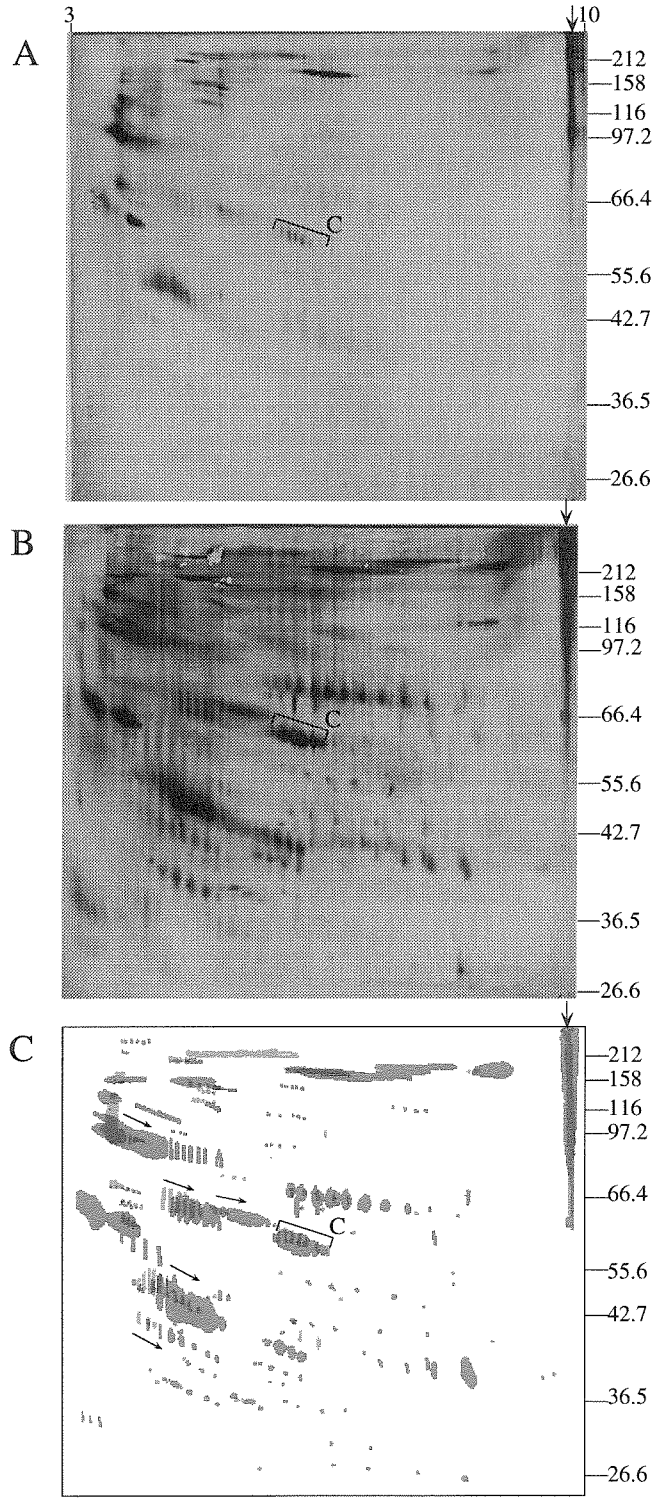


**Figure 4.7.** E-PHA-binding patterns of Pro-5 and LEC10 concentrated supernatants. Supernatant was harvested, concentrated, separated by 2-DE, and detected with E-PHA-AP. (A) Pro-5 cells, (B) LEC10 cells. (C) The E-PHA-binding patterns of the two samples superimposed, with the Pro-5 supernatant pattern (A) in blue and the LEC10 supernatant pattern (B) in red. Note the shift in binding pattern of the five chains highlighted by arrows, and the absence of a shift in binding pattern of the chain marked "C." Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

# Chapter 5

# Environmental Conditions and Their Influence on the Expression of a Genetic Manipulation of the Glycosylation Pathway

## Influence of Cell Culture Environment on Glycosylation

Many of the protein therapeutics currently being researched and produced are glycoproteins. As a greater understanding of the possible influences of glycosylation has emerged (as reviewed in Chapter 1), significant attention has been focused on understanding the causes and effects of glycosylation with regard to protein therapeutics and clinical applications, with special focus on ensuring the safety, efficacy, and reproducibility of protein products which will be administered to humans. Many protein therapeutics are developed initially at a very small scale under a certain set of culture conditions. As the product proceeds through clinical trials for safety and efficacy, work begins to scale up the manufacturing of the product of interest, which typically involves significant alterations in culture conditions. These modifications can involve, among other things, changing from batch to continuous culture, from serum supplemented media to serum-free media, or from adherent cell culture to suspension cell culture.

It has become increasingly clear that the cell culture environment can have significant effects on protein glycosylation [46, 47, reviewed in 79]. Studies have documented that the method of culture affects the glycosylation of proteins; an extremely thorough study demonstrated significant differences in both clinical characteristics and oligosaccharide structures among IgMs produced in a variety of culture vessels, ranging from mouse ascites to airlift fermentors [98]. Another study compared IgG produced in ascites with IgG produced in spinner flasks with or without serum. IgG made in serum-free media had more sialic acid than IgG made in the presence of serum, and the level of sialic acid on IgG produced in ascites was too low to be detected [131]. A comparison of recombinant human tissue kallikrein produced in CHO cells in either microcarrier culture or suspension culture revealed that the kallikrein from microcarriers had less sialylated oligosaccharides and a lower proportion of more complex (higher branched) oligosaccharides [178].

Other research has demonstrated changes in glycosylation within a single culture vessel as a function of time. Multiple studies with interferon-gamma (IFN-$\gamma$) have shown that the proportion of IFN-$\gamma$ which is glycosylated at both potential glycosylation sites decreases over time in a batch culture [23, 27, 62]; these finding have been linked to the physiological state of the cell rather than glucose limitations or degradation of secreted products. Other studies have shown that cells which have reached confluency produce a higher proportion of lower-molecular weight oligosaccharides (such as biantennary)

compared to cells which are growing exponentially [54, 122, 145]. A study utilizing a chemostat for steady-state culture production of IFN-γ found that less IFN-γ was glycosylated at both sites when it was produced under glucose-limited steady state than when glucose was present in excess, and that the proportion of fully glycosylated IFN-γ was significantly lower at higher steady-state cell concentrations [63]. Based on a consideration of many results, the authors suggested that the glycosylation was probably influenced by factors other than simply the availability of glucose and that the glucose level and other culture conditions may have exerted an influence on glycosylation by affecting cell physiology.

Another major component of the cell culture environment which can affect glycosylation is the culture medium itself. Of particular importance is the observation that the presence or absence of serum can have significant effects on the glycosylation of proteins [98, 102, 131]. The amount of glucose in the medium can also affect the glycosylation [166, 172], as can additives such as butyrate [155], interleukin-6 [111], and various lipids [23]. Other, more general extracellular conditions, such as extracellular pH and extracellular phosphate levels, can also affect the glycosylation of proteins [100]. The temperature of the culture medium has also been shown to exert significant effects on glycosyltransferase expression, and it has been proposed that enhanced glycosylation may be associated with the development of thermotolerance [66, 67].

Considering the number of culture variables that can affect the glycosylation of a protein of interest, it is essential that these possible effects be taken into account as one engineers the glycosylation of a protein of interest which will be produced on a large scale. Any intended manipulation of a protein's glycosylation might be met with immediate lack of success due to an over-riding environmental condition, or the intended manipulation might work under the initial testing conditions and not under later production conditions. Therefore, it was important in light of the overall goal of this work, to begin at least preliminary investigations into the interplay of genetic and environmental controls of glycosylation in the specific example of the cell lines of this work.

Influence of Cell State on Oligosaccharide Branching

In many large scale culture conditions, cells are maintained at confluent densities for extended periods of time. As mentioned above, cells which have reached confluency tend to have lower molecular weight oligosaccharides, most likely due to a decrease in complexity and branching. One report documented a decrease in GlcNAc-T V, a branching enzyme, concomitant with an increase in biantennary oligosaccharides [54]. In the case of the LEC10 mutant which has been previously discussed in this report, the alteration in the glycosyltransferase complement of the cell is the novel expression of GlcNAc-T III, also a branching enzyme. Thus it is possible that the expression of GlcNAc-T III would be reduced by environmental conditions similar to those which have been shown to reduce the expression of another branching enzyme. One might suppose

that such an environmental effect would be mediated through transcriptional controls, and one might further assume that if a mutant cell line is characterized by altered expression of a glycosyltransferase, the normal transcriptional controls over that enzyme have been abolished. However, there are many plausible scenarios in which the environmental effect could still control the mutant expressers, either by exerting transcriptional control at a location which is not mutated, or by exerting control over the translation or activity of the branching enzymes in a more general way. If the environmental effect is a general effect that can influence all branching enzymes at a level other than through a promoter, then this mutant will not be useful in generating the desired glycoforms if the cells are cultured under sustained, high density conditions. Thus, it was of particular interest to determine if the expression of the bisecting N-acetylglucosamine on glycoproteins made in the LEC10 cell could be hindered or abolished by sustained or high density culture.

Methods for Analyzing the Influence of the Environment on Glycosylation

Published investigations into the effects of environmental conditions on cellular glycosylation have for the most part explored changes in the glycosylation of a purified protein. Some investigations have included detailed structural analyses of released and fractionated oligosaccharides using chromatography columns and/or sequential exoglycosidase digestions [98, 131, 178]; while the detailed structural characterization of released oligosaccharides from a purified protein is certainly the method of choice when one is interested in analyzing only in a single protein, the use of such procedures may be infeasible because of their cost in time and money. In other studies, purified proteins have been analyzed by using 1-D SDS-PAGE to reveal differences in apparent molecular weight between samples or changes in apparent molecular weight following digestions with glycosidases [23, 27, 38, 62, 63, 100, 119, 122]. Analysis of molecular weight shifts by SDS-PAGE is convenient in that it allows simple, rapid comparisons of multiple samples, but it is extremely crude and limited in the information it supplies. Still other studies have used 1-D isoelectric focusing to analyze and compare the constituent isoforms of purified glycoproteins made under different conditions [98, 178].

However, as previously mentioned, any method which analyzes changes in a single purified protein will not allow for the evaluation of changes in the other proteins produced in the same cell. Because the research of this thesis is concerned with studying the interaction of a genetic glycosyltransferase manipulation with environmental manipulations in a global sense in order to enable a better understanding of what the effects of such interactions might be on a wide variety of glycoproteins, 2-D electrophoresis in combination with lectin binding was selected as the method of analysis. This method enables one to determine if the environmental conditions can override the effects of a genetic manipulation on the oligosaccharide structure distributions of all, some, or none of the cellular glycoproteins, thereby enabling one to better predict if a particular protein of interest will likely be under the influence of the environment more than the genetic condition of the cell.

# Methods

*Reagents:* Most reagents used in electrophoresis were electrophoresis grade and were purchased from Sigma (glycerol, NP-40, iodoacetamide) or BioRad (sodium dodecyl sulfate (SDS), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea, glycine, BioLyte carrier ampholytes, dithiothreitol (DTT), piperazine diacrylamide (PDA), acrylamide, β-mercaptoethanol (BME), N,N,N',N',-tetramethylethylenediamine (TEMED), ammonium persulphate). Dimethylformamide (DMF) was from EM.

*Cell culture:* Pro-5 (ATCC CRL 1781) and LEC10 Chinese hamster ovary cells were grown in adherent monolayer culture in α-MEM (GIBCO) supplemented with 5% dialyzed fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO) in a humidified incubator with 5% $CO_2$. They were routinely subcultured at a 1:10 dilution every 2 to 3 days, before reaching over-confluency. The LEC10 cells were generously provided by Professor Pamela Stanley.

*Extraction of cellular proteins:* 100 mm dishes were seeded as described in the text, and cells were harvested after the length of time given in the text. To harvest the cells, plates were washed once with warm PBS, and then 3 mL of Sigma (non-enzymatic) Cell Dissociation Reagent in PBS was added to each plate. Plates were incubated at 37°C for 10 minutes, after which they were tapped on the side to free the cells from the plate. The cells were pipetted off the plate into a 50 mL centrifuge tube, and the plate was washed with 2 mL of warm PBS, which was added to the cell suspension. The tube with the cells was kept on ice for the duration of the processing. The cells were spun at 1000 rpm for 10 minutes in a Beckman TJ 6 centrifuge at 4°C. The supernatant was removed and the cells were washed 2 more times. After the final spin, cells were resuspended at $5.8 \times 10^7$ cells/mL in 100 mM Tris pH 6.8, 2% BME, 25% DMF. Cells were sonicated for 2 minutes and then heated to 100°C for 5 minutes. Extracts were frozen at this point.

*Sample preparation:* The method of sample preparation was as follows: to 125 µL of either cell extract or concentrated supernatant was added 5 µL of 0.1% bromophenol blue, 7.5 µL of 40% BioLyte 3-10 carrier ampholytes, 15.0 µL of 20% CHAPS, and 0.14 g urea (to yield a final concentration of about 9.5 M urea in about 250 µL); this particular quantity of sample was used to load two IPG strips, and sample preparation was adjusted proportionately up or down to load a different number of IPG strips.

*Isoelectric focusing:* Immobiline DryStrips, pH 3-10L, 18 cm, were used. The strips were rehydrated in 8 M urea, 0.5% NP-40, 1.5% CHAPS, 0.2% BioLyte 3-10 carrier ampholytes, 0.2% DTT, 10% DMF. Electrode strips were soaked in either 0.05% NaOH or 0.0595% phosphoric acid. After placing the IPG strips in the Immobiline DryStrip tray, mineral oil was added to the tray to a depth which covered the strips but not the sample cups. Samples were loaded at the extreme cathodic or anodic end as indicated. After the sample was added to the cup, mineral oil was added to completely cover the sample cups.

The program for isoelectric focusing was 2 hours at 360 V (20 V/cm), 2 hours at 1440 V (80 V/cm), and 35-40 hours at 2970 V (165 V/cm), all at a controlled temperature of 25°C.

*IPG strip equilibration:* Strips were incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2% w/v DTT, 50 mM Tris pH 6.8 on a shaking platform for 10 minutes. They were rinsed briefly with water and then incubated in 30% w/v glycerol, 2% w/v SDS, 6 M urea, 2.5% w/v iodoacetamide, 50 mM Tris pH 6.8, and a dash of bromophenol blue on a shaking platform for 5 minutes. Each strip was touched along its edge briefly on wet filter paper before being trimmed to fit onto the SDS-PAGE gel; the gel was trimmed approximately 1 cm on the end at which it was loaded and typically about 1.5 cm on the opposite end.

*Second dimension SDS-PAGE:* For the second dimension, 10% T polyacrylamide gels crosslinked with PDA of dimensions 19 cm × 16 cm × 1.5 mm were used. The running buffer was 28.8 g/L glycine, 6 g/L Tris base, 1 g/L SDS. Up to six gels were run in parallel using the BioRad Protean II Multicell. 5 µL of broad range molecular weight standards (New England BioLabs) were loaded between the spacer and the basic end of the IPG strip. Gels were run at 4°C at 40 mA per gel for approximately 5 hours.

*Transfer of proteins onto a solid support:* The Pharmacia Multiphor II NovaBlot semi-dry blotting unit was used for blotting. Proteins were blotted onto Immobilon P membranes (Millipore) wetted in 100% MeOH and equilibrated in blotting buffer (39 mM glycine, 48 mM Tris base, 20% MeOH). Gels were transferred from the glass plates into water briefly, after which they were stacked in units (anode to cathode) of filter paper, membrane, and gel, with two pieces of Whatman 3 mm chromatography paper on both sides of the stack. Gels were blotted for 6 hours at 200 mA. After blotting, the membranes were either processed immediately as described below, or transferred to water until needed.

*Detection of E-PHA-binding glycoproteins:* This protocol for the detection of E-PHA-binding glycoproteins was based on the "Applications of digoxigenin-labeled lectins in glycoconjugate analysis" technical sheet from Boehringer Mannheim. Membranes (approx. 15 cm × 16 cm) with bound proteins were rinsed in 100 mL TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 75 mL 0.5% blocking reagent (Boehringer Mannheim) in TBS for 1 hour on a rocker platform. This was followed by two ten-minute rinses in 100 mL TBS and one ten-minute rinse in buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, in TBS, pH 7.5). The blots were then incubated in 35 mL of 4 µg/mL E-PHA-alkaline phosphatase (E-Y Labs) in buffer 1 for one hour on a rocker platform. This was followed by one ten-minute rinse in 150 mL TBS on a rocker platform and two ten-minute rinses in TBS on a shaking platform; the blots were switched to clean trays before the final rinse. The developing solution was 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5 with 187.5 µg/mL 5-bromo-4 chloro-3-indolyl-phosphate (X-phosphate) and 500 µg/mL 4-nitro blue tetrazolium chloride (NBT); 40 mL of developing solution was

used per membrane. The membranes were incubated with developing solution without shaking for approximately 1 hour, 45 minutes, after which they were rinsed thoroughly with water and dried on filter paper.

# Results

## E-PHA Binding Patterns of Cells Harvested After Different Times in Culture

To study the effect of increasing time in culture on the E-PHA-binding patterns of LEC10 cell extracts, 100 mm dishes were inoculated with $2.75 \times 10^5$ LEC10 cells and harvested after 24 hours ("Day 1"), 48 hours ("Day 2"), 72 hours ("Day 3"), and 96 hours ("Day 4"). The growth curve of the cells is shown in Figure 5.1; the cells reached confluency at around 72 hours. Separation and lectin probing of the cell extracts was conducted as described in the Methods section of this chapter and the results are shown in Figure 5.2. The E-PHA binding patterns of the four samples were very similar; this indicated that sustained culture time and confluency did not significantly or globally alter the oligosaccharide distributions of the E-PHA binding proteins.

In the context of this lack of global change, the blots were also evaluated for protein-specific changes in E-PHA binding. Inspection of the mid-molecular weight, basic region of the gels revealed a chain which increased in both the number of spots and E-PHA binding per spot with increasing culture time; this chain is marked by arrows on Figure 5.2. As a point of reference, one can note that the more prominent chain in this region of the gel, just above the chain of interest, bound similar quantities of E-PHA in a similar pattern in all four samples.

Another chain which showed altered E-PHA-binding over the course of the experiment was found in the higher molecular weight, acidic region of the gel and is marked by a bracket in Figure 5.2. This chain increases in staining intensity, while staying roughly the same shape, from day 1 to day 3, and then is barely visible on day 4. This chain is particularly interesting in that it was consistently one of the most prominent chains in the LEC10 cell extracts analyzed in Chapter 4 (see Figure 4.5B), and yet, even on day 3, at its highest level of E-PHA binding in this experiment, it is not stained as darkly as in Figure 4.5B. Many other chains do not show a significant difference in E-PHA binding either within this experiment or compared to LEC10 cell extract shown in Figure 4.5B, which suggests that the decrease in E-PHA binding in this region is specific and not the result of a general effect such as a lower protein load. It should be pointed out that for the experiments in Chapter 4, 100 mm dishes were inoculated with $1.5 \times 10^6$ cells and harvested 24 hours later; these conditions were not replicated by any sample in this experiment.

The changes in the E-PHA binding pattern of these two chains (marked by arrows and a bracket on Figure 5.2) could represent an changes in the amount of peptide backbone in the context of a constant level of GlcNAc-T III modification per amount of peptide backbone, or it could represent an changes in the level of GlcNAc-T III modification per amount of peptide backbone in the context of a constant level of the peptide backbone (or a combination of the two). Regarding the latter possibility, since the overall E-PHA-staining

patterns of the four samples is very similar, it is very unlikely that the level of GlcNAc-T III has changed. Therefore, if the level of GlcNAc-T III modification per amount of peptide backbone were changing, it would likely be the result of a protein-specific change in level of modification per GlcNAc-T III molecule in the presence of an unchanged level of GlcNAc-T III activity, rather than a protein-specific response to a change in the GlcNAc-T III activity. Considering the former possibility, if the amount of peptide backbone were changing, the change in the E-PHA binding pattern could just be the result of a proportional increase in the quantities of the various glycoforms, with the two most basic spots of the chain marked by arrows appearing as the proportional increase brought their quantities to detectable levels. Unfortunately, the most conclusive way to distinguish between the two possible explanations would be to have antibodies to these proteins, which are as yet unavailable; quantitative immunostaining could be used to determine if the expression of a polypeptide backbone was changing. It is likely in these instances that the level of peptide backbone expression *is* changing, as it would be difficult to propose a mechanism by which a protein backbone, as a result of culture conditions, could become a better substrate for a fixed amount of glycosyltransferase.

E-PHA Binding Patterns of Cells Inoculated At Different Densities

A second approach to studying the effect of cell density on E-PHA binding patterns was also undertaken; other researchers have shown that plating cells at different densities and harvesting them after the same time in culture can give results which are different from harvesting cells which have grown to different densities after different times in culture [122]. Cells were plated out at densities equal to the cell densities on days 1, 2, and 3 of the experiment just described ($8.25 \times 10^5$, $2.2 \times 10^6$, and $5.3 \times 10^6$ cells per 100 mm dish, respectively) and harvested 24 hours later, at approximately the same density as the cells harvested on days 2, 3, and 4 of the experiment just described. Samples will be referred to as "Day 2," "Day 3," or "Day 4" based on which cells they correspond to from the previous experiment in terms of density at harvest and 24 hours before harvest; of note is the fact that "Day 3" and "Day 4" cells were harvested at the same density, the difference being that "Day 4" cells were inoculated at a confluent density and had very little room for growth over the 24 hours.

Overall, the E-PHA binding pattern was roughly the same in the three extracts, indicating that globally, the oligosaccharide distributions of E-PHA binding proteins were not being influenced in any significant way by differences in cell density over a period of 24 hours (data not shown). A protein-specific difference in E-PHA staining as a result of differences in cell density was seen in one of the chains which was considered at length in the previous experiment; Figure 5.3 focuses on the region which contains this chain, and the chain of interest is marked with arrows. One can see that this chain bound more E-PHA in the cell samples harvested at "Day 3" and "Day 4" densities (panels B and C) than in the cell sample harvested at "Day 2" density (panel A). It is also of note that on all three blots there is a chain of small, round, closely spaced spots (marked by a bracket on Figure

5.3) which had not been seen on any of the blots from the previous experiment (Figure 5.2). However, this chain regularly appeared on blots of cells harvested after 24 hours as part of other experiments (see chain 1 on Figure 4.5B).

Influence of Condition of Media on E-PHA Binding Patterns

To investigate the influence on E-PHA binding patterns of glycosidases which might collect in the supernatant with prolonged culture time, or the influence of any other agents which might change in concentration with increased culture time, an experiment was conducted in which cells which had been in culture only a short time were exposed for 24 hours to media from cells which had been in culture a longer time, and cells which had been in culture a long time were exposed for 24 hours to fresh media. Specifically, 100 mm dishes were inoculated with $2.85 \times 10^5$ cells on day 0 and again on day 2. On day 3, the conditioned media from the cells which had been in culture since day 0 (72 hours) was transferred to cells which had been in culture only 24 hours; fresh media was added to the cells which had had their media removed. As controls, additional plates inoculated on day 0 and day 2 were maintained with no media manipulation. On day 4, all of the cells were harvested; thus, the cell extract samples included, as controls, cells which were identical to "Day 2" and "Day 4" cells of the first experiment of this chapter (Figure 5.2), and, as experiments, cells similar to "Day 2" cells, but having been cultured in very conditioned media, and cells which were similar to "Day 4" cells, but having been cultured in fresh media for the last 24 hours of culture.

The resulting 2-D E-PHA-stained blots are shown in Figure 5.4. Again, no global change in the E-PHA binding pattern was noticeable, indicating that the condition of the medium, fresh or significantly used, did not exert a global effect on the oligosaccharide distributions of E-PHA binding proteins. Returning again to the chain on the basic side of the gel, marked by arrows, one can see that the intensity of each spot in the chain appears to be similar across the four blots; however, both types of "Day 2" extracts show only two obvious members of the chain, while both types of "Day 4" extracts show three obvious members of the chain, with the additional spot being located at the basic end. Unfortunately, it is difficult to integrate this data with the data from the other experiments because the pattern of this chain in the control "Day 4" sample has only three of the six spots which were seen in the "Day 4" samples of the experiment shown in Figure 5.2 and two other experiments; the fact that this control did not reproduce the results of the other experiments makes it difficult to discern if the patterns seen in the other three samples have also been affected by the same unknown alteration in the experiment.

# Discussion

These results show that the E-PHA binding pattern of 2-D separated LEC10 cell extracts is not altered in a global way by sustained or high density culture. This suggests that the expression of GlcNAc-T IIII and the presence of bisecting N-acetylglucosamines on glycoproteins made in LEC10 cells is not globally altered by conditions similar to those which have been shown in other research to reduce the attachment of N-acetylglucosamine branches to the core structures of oligosaccharides. In more general terms, these experiments demonstrated that the distribution of oligosaccharide structures resulting from this particular genetic alteration of glycosyltransferase expression was not affected in any significant or global way by the additional changes in environmental conditions.

While the overall pattern of E-PHA binding was not altered in a global way by sustained or high density cell culture, some protein-specific changes in E-PHA binding patterns were observed. The fact that these protein-specific changes were observed against a background of glycoproteins which did not show any change suggests that these protein-specific changes were the result of changes in the expression of polypeptide backbones rather than protein-specific responses to a change in the level of GlcNAc-T III.

This experiment explored only one area of environmental manipulation. There are many other environmental conditions which would be commonly experienced only in large scale culture. If it were desired to use a cell line expressing a genetically modified glycosylation pathway for the large scale production of a particular glycoform distribution of a glycoprotein product, it would be prudent to ensure that the conditions of large scale culture do not significantly influence the glycosylation pathway in a manner which is not in line with the desired glycoform distribution. While oligosaccharide characterization of the purified glycoprotein product of interest would certainly be one facet of this type of evaluation, the use of 2-D electrophoresis in combination with lectin probing as described in this chapter would provide a different level of useful information, that is, information about how the rest of the glycoproteins being produced by the cell are responding to the combination of genetic and environmental manipulations, thus allowing one to discern if changes which are occurring in the protein of interest are protein-specific.

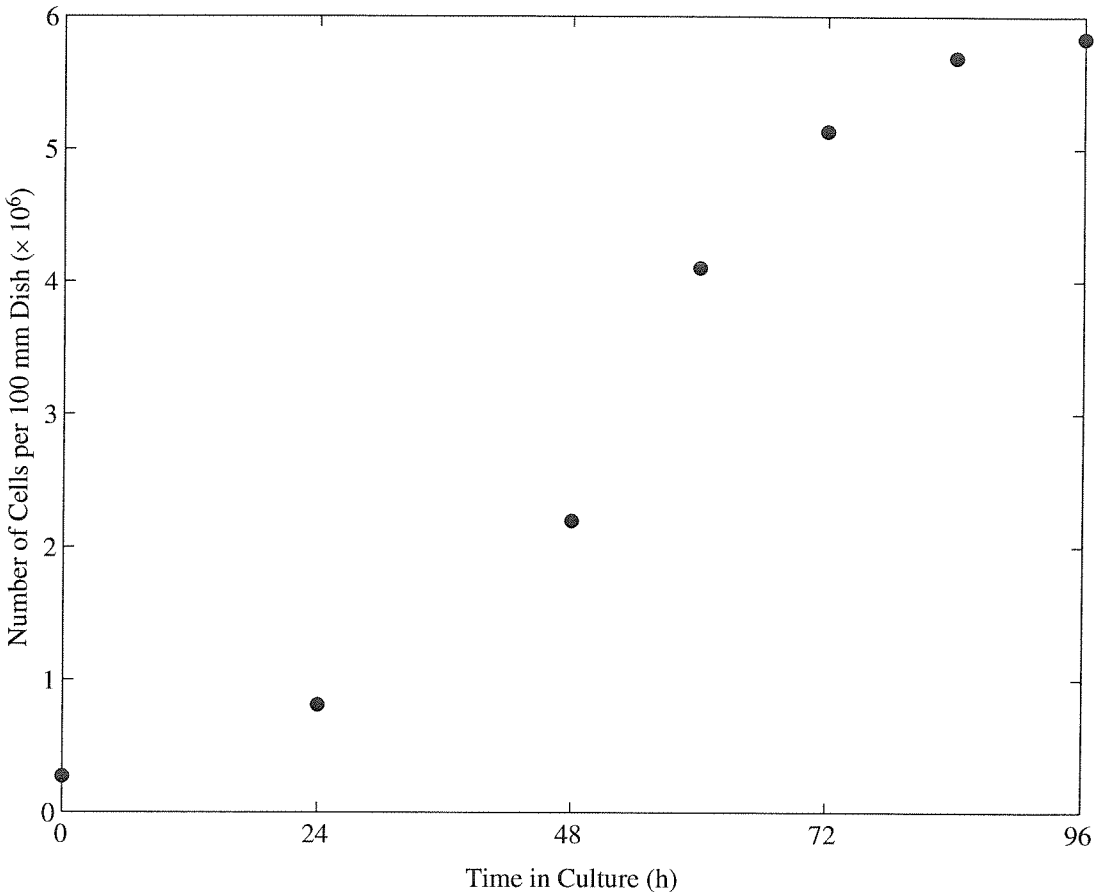


**Figure 5.1.** Cell growth curve for LEC10 cells which were used in experiments to determine the effect of days in culture on E-PHA binding patterns. 100 mm dishes were inoculated with $2.75 \times 10^5$ LEC10 cells at time 0. Note that the cells reach stationary phase by day 4.

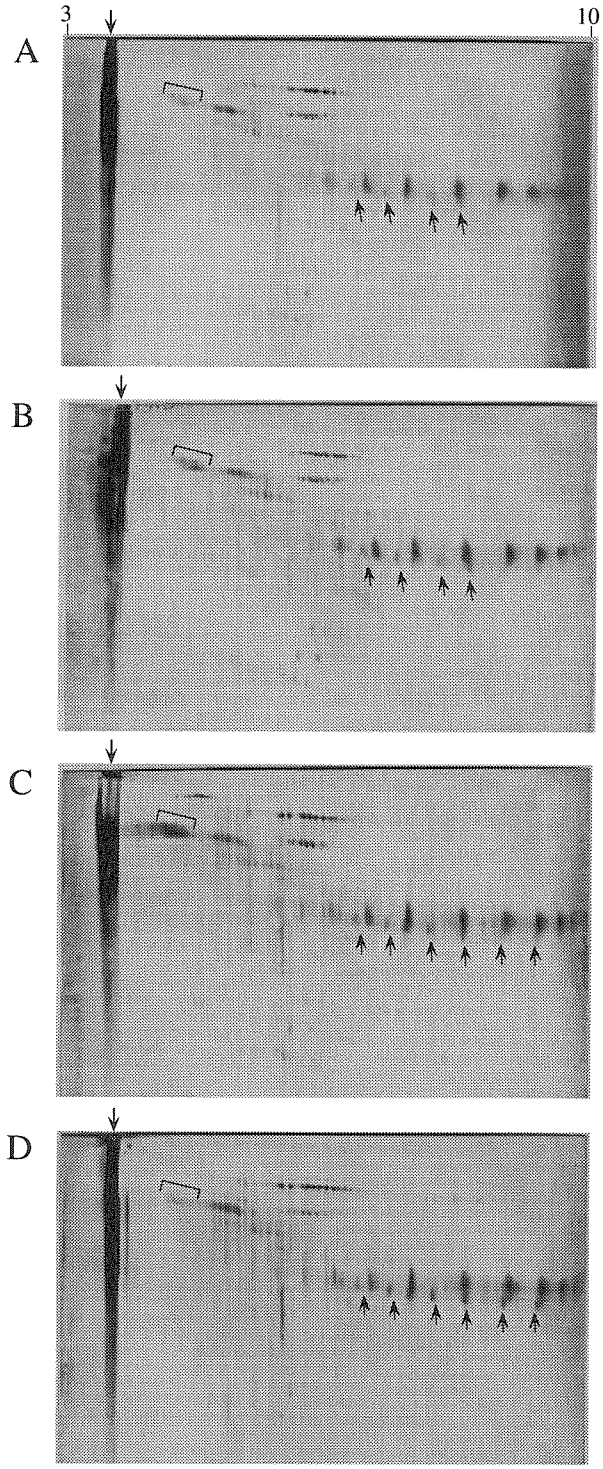


**Figure 5.2.** Effect of number of days in culture on E-PHA-binding patterns of LEC10 cell extracts. 100 mm dishes were inoculated with $2.75 \times 10^5$ LEC10 cells and cells were harvested after 24 hours (A), 48 hours (B), 72 hours (C), and 96 hours (D). Cell extracts were separated by 2-DE and detected with E-PHA-AP. Note that most of the E-PHA-binding chains do not change in pattern or staining intensity with increasing culture time. Note also the increasing length and staining intensity of the chain marked by arrows with increasing time in culture; further note the changing staining intensity of the chain marked by a bracket (compare with Figure 4.5B). The orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the loading site.

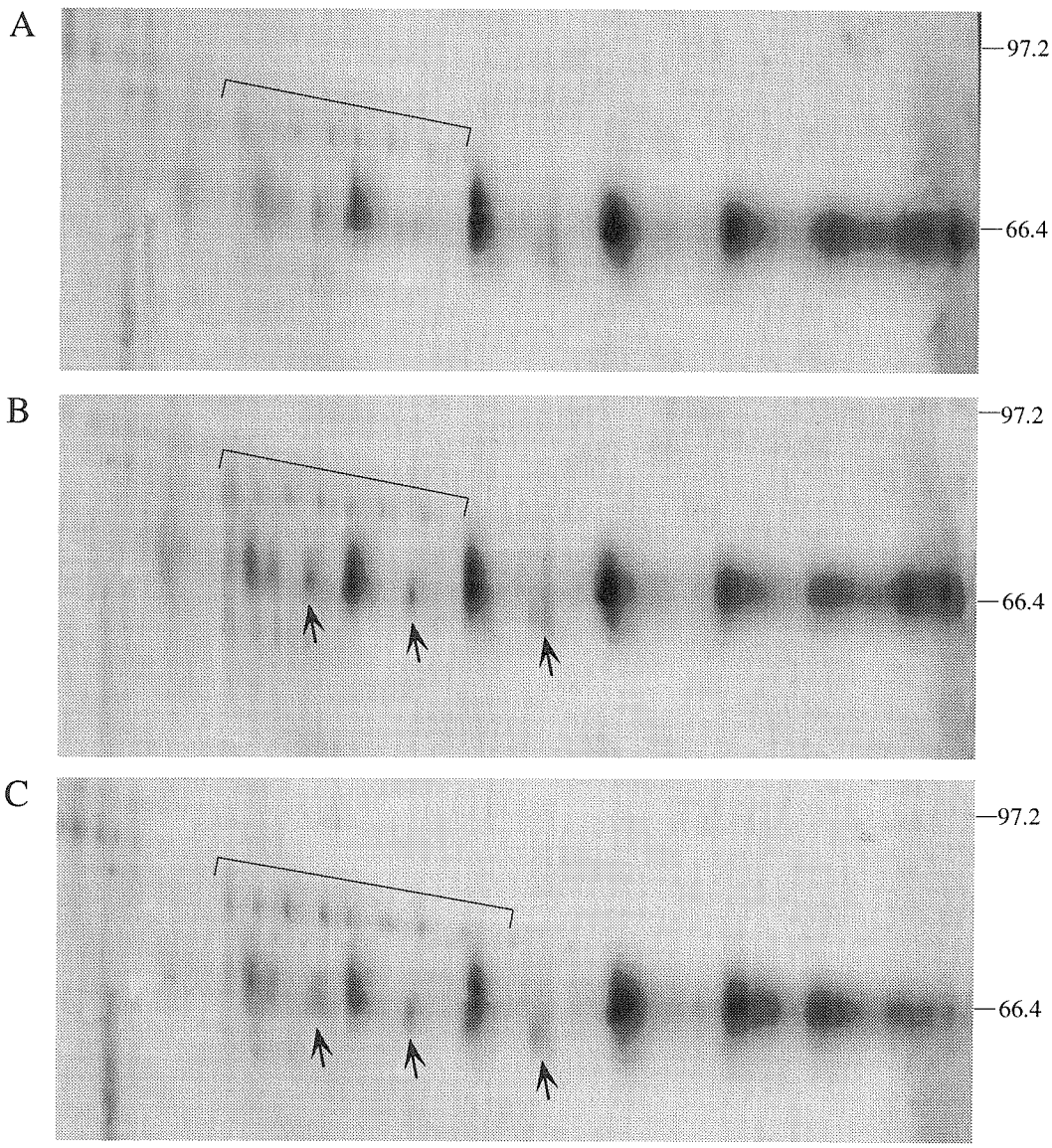


**Figure 5.3.** Effect of culture density on E-PHA-binding patterns of LEC10 cell extracts. See text for full explanation. 100 mm dishes were inoculated with (A) $8.25 \times 10^5$ cells ("Day 2"), (B) $2.2 \times 10^6$ cells ("Day 3"), and (C) $5.3 \times 10^6$ cells ("Day 4"), and harvested 24 hours later. Cell extracts were separated by 2-DE and detected with E-PHA-AP. Note that the prominent chain in this region does not change in pattern or staining intensity as a function of cell density. Also note the similarity of the chain marked by arrows in panels (B) and (C), and the difference in staining intensity and pattern of this chain in panel (A); this chain is the same chain which is marked by arrows in Figure 5.2. Also note the presence of a protein chain, marked by a bracket, that is not discernable in the blots shown in Figure 5.2. Positions of MW markers (in kD) are shown on the right.

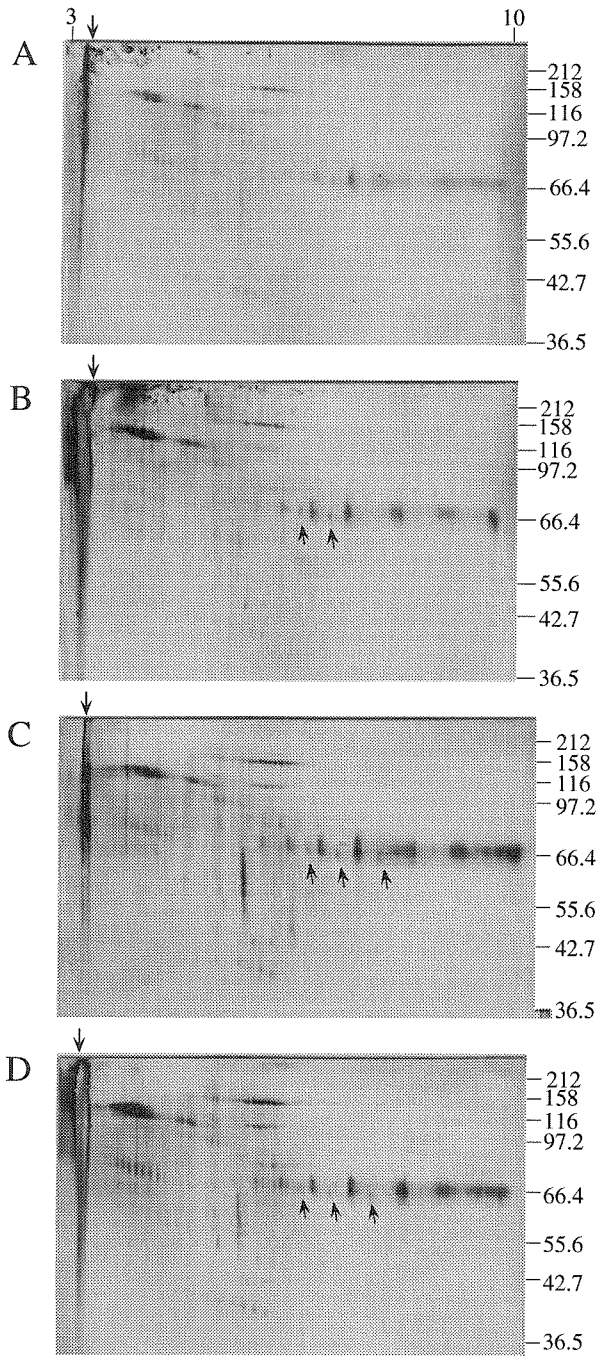


**Figure 5.4.** Effect of media condition and days in culture on E-PHA-binding patterns of cell extracts. See text for full explanation. 100 mm dishes were inoculated with $2.75 \times 10^5$ LEC10 cells on day 0 and day 2. On day 3, the conditioned media of cells inoculated on day 0 was transferred to cells inoculated on day 2; fresh media was used to replace the conditioned media which was transferred. Cells were harvested on day 4, and cell extracts were separated by 2-DE and detected with E-PHA-AP. (A) and (B) Cells inoculated on day 2; (A) no media change, (B) received media from cells in culture for three days. (C) and (D) Cells inoculated on day 0; (C) no media change, (D) media removed and replaced with fresh media. Note: the chain marked by arrows stains to similar levels in panels (B), (C), and (D), but in panels (C) and (D) there is an additional spot at the basic end of the chain. Positions of MW markers (in kD) are shown on the right and the orientation of the pH gradient is shown at the top; the arrow at the top of each gel indicates the sample loading site.

# Chapter 6

# EPILOGUE

The research of this report has clearly demonstrated the feasibility of manipulating the glycosylation of cellular and heterologous glycoproteins of interest by the genetic alteration of glycosyltransferase expression. This work has explored two approaches to altering oligosaccharide structure by the genetic manipulation of glycosyltransferase expression: the use of a cell line expressing a characterized alteration in glycosyltransferase expression (a glycosylation "mutant") and the introduction into a host genome of a cloned glycosyltransferase gene under the control of an inducible promoter. In the latter case, the oligosaccharide structures of a specific heterologous protein were shown to be altered by the inducible expression of a transfected glycosyltransferase. This research has also put forth a model, and the associated techniques, for evaluating the influence of the various factors, such as protein-specific limitations and environmental effects, which could affect the outcome of a genetic manipulation of glycosyltransferase expression.

What does the future of glycosylation engineering look like? As was touched on briefly at the end of Chapter 3, armed with a host of cloned glycosyltransferases, a battery of promoter options, a hardy cell line, and a good strategy, the possibilities seem unlimited. As an example of how techniques to manipulate glycosylation through the genetic alteration of glycosyltransferase expression might be used in the not too distant future, Figure 6.1 depicts a possible scenario for engineering the glycosylation of a recombinant glycoprotein produced on a large scale.

After the glycoprotein product was successfully expressed in a recombinant host cell line, the first round of glycosylation analysis and alteration would be focused on finding the most effective glycoform for the application. If one was utilizing a cell line which had different glycosyltransferase genes under the control of different inducible promoters, one could envision generating, in a series of experiments, a wide spectrum of oligosaccharide structures and glycoforms, each of which could be analyzed independently to evaluate its biochemical properties. If such a cell line were not available, one could analyze the effectiveness of glycoforms produced by an unmodified glycosylation pathway, and the results could guide one to remove, add, or modulate the activity of a single enzyme. The resulting glycoforms could be analyzed, and the results could suggest the removal, addition, or modulation of another enzyme. By alternating manipulation and evaluation, one could determine the most effective glycoform for the application.

Having found an optimal glycoform of the recombinant glycoprotein, a second goal would be to manipulate the glycosyltransferase expression of the host cell line such that it was optimized for producing the desired glycoform. A comparison of the current glycoform distribution with the desired distribution would likely point to key glycosyltransferase

activities which could be removed, added, or modulated to alter the glycoform distribution. In evaluating the response of the glycoform distribution to changes in glycosyltransferase expression, it would be important to also evaluate the response of other cellular glycoproteins; this would reveal if the response of the glycoprotein of interest was being influenced by protein-specific effects, which could be mistaken for a global problem in the genetic manipulation. Global analysis of glycoprotein response could be accomplished by 2-DE in conjunction with lectin probing, as demonstrated in this report. Each analysis would likely suggest a new alteration of glycosyltransferase expression which would shift the glycoform distribution toward the target distribution. A series of alterations and analyses would likely lead to the development of a cell line which was optimized for producing the desired glycoform of the glycoprotein of interest.

Having optimized the glycoform distribution of the glycoprotein of interest when produced in small scale culture, the third goal would be to retain the optimized glycoform distribution when the glycoprotein was produced under large scale bioprocess conditions. Initial analysis of the glycoprotein made in large scale might reveal that environmental factors influence its glycoform distribution, and such an analysis would likely highlight glycosyltransferases which would need to be further manipulated to return the distribution to the target. This initial analysis, and all subsequent analyses of environmental effects and genetic manipulations, should include evaluation of the response of other host cell glycoproteins to ensure that limitations or responses which are specific to the protein of interest are considered in subsequent manipulations. After conducting additional manipulations and evaluations as necessary, the result would be a cell line which is optimized for production of the desired glycoform of the glycoprotein of interest under large scale production conditions, and a recombinant glycoprotein that is as effective as possible.

This is only one possible way to engineer the glycosylation of a protein of interest using the approaches and techniques described in this work. This work is not in any sense the consummation of a discipline; it is more like a seed which has just begun to put down roots in fertile but uncolonized ground. In one sense, it possesses unknown potentials of growth and purpose within itself; in another sense, it is one of the initial building blocks of a whole new field, a small tree which has shown in a small way what is possible, a tree which will someday probably be lost amidst the forest that will follow.

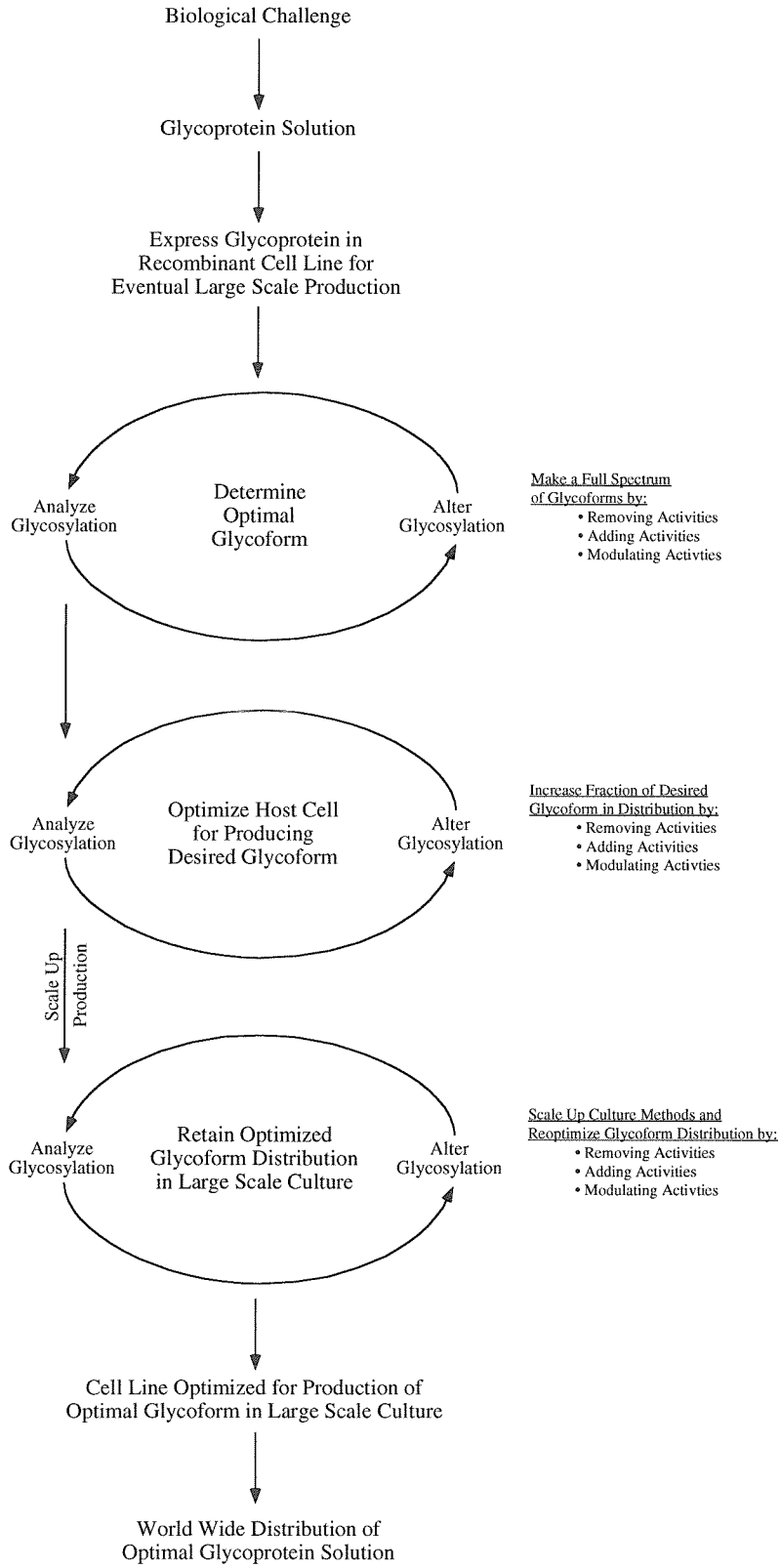


**Figure 6.1.** Possible scenario for the engineering of the glycosylation of a glycoprotein produced in large scale.

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