

PROTEIN N-GLYCOSYLATION IN THE BACULOVIRUS–INSECT CELL EXPRESSION SYSTEM AND ENGINEERING OF INSECT CELLS TO PRODUCE “MAMMALIANIZED” RECOMBINANT GLYCOPROTEINS

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ABSTRACT

Baculovirus expression vectors are frequently used to express glycoproteins, a subclass of proteins that includes many products with therapeutic value. The insect cells that serve as hosts for baculovirus vector infection are capable of transferring oligosaccharide side chains (glycans) to the same sites in recombinant proteins as those that are used for native protein *N*-glycosylation in mammalian cells. However, while mammalian cells produce compositionally more complex *N*-glycans containing terminal sialic acids, insect cells mostly produce simpler *N*-glycans with terminal mannose residues. This structural difference between insect and mammalian *N*-glycans compromises the *in vivo* bioactivity of glycoproteins and can potentially induce allergenic reactions in humans. These features obviously compromise

the biomedical value of recombinant glycoproteins produced in the baculovirus expression vector system. Thus, much effort has been expended to characterize the potential and limits of *N*-glycosylation in insect cell systems. Discoveries from this research have led to the engineering of insect *N*-glycosylation pathways for assembly of mammalian-style glycans on baculovirus-expressed glycoproteins. This chapter summarizes our knowledge of insect *N*-glycosylation pathways and describes efforts to engineer baculovirus vectors and insect cell lines to overcome the limits of insect cell glycosylation. In addition, we consider other possible strategies for improving glycosylation in insect cells.

I. INSECT PROTEIN *N*-GLYCOSYLATION AND ITS IMPORTANCE

A. *Significance of N-Glycosylation for Functional Glycoprotein Production*

One of the most common eukaryotic posttranslational protein modifications is *N*-glycosylation, which involves the addition of oligosaccharides on asparagine side chains and their subsequent processing. Many proteins expressed in baculovirus–insect cell and other expression systems are glycoproteins, which include many proteins of medical and veterinary therapeutic and diagnostic significance, such as hormones, receptors, and antibodies. The addition and processing of *N*-linked oligosaccharides (referred to as *N*-glycans) play an essential role in the folding and quality control of most membrane-associated and secreted glycoproteins (Helenius and Aebi, 2001). The presence of *N*-glycans can influence and stabilize protein structure and protect against proteolysis (Imperiali and O'Connor, 1999; Lis and Sharon, 1993; Wyss and Wagner, 1996). *N*-Glycans also can play an important role in determining the specificity of protein–protein interactions involved in a wide variety of processes, including clearance of glycoproteins from mammalian circulatory systems, intracellular trafficking of enzymes, cell–cell interactions, signal transduction, and antigen recognition (Lis and Sharon, 1993; Opdenakker *et al.*, 1993; Rudd *et al.*, 2001; Varki, 1993). In addition, enzyme activity can be influenced by glycosylation. For example, the activities of plasminogen and tissue plasminogen activator are altered by glycosylation at specific sites, while the activity of RNase A is inversely proportional to the size of its *N*-glycans (Rudd *et al.*, 1995). Another example is the loss of membrane fusion activity in paramyxovirus fusion (F) proteins, which occurs when specific *N*-glycosylation sites are obliterated by

mutagenesis of the asparagine codon (McGinnes *et al.*, 2001; Segawa *et al.*, 2000).

Because of the role that *N*-glycosylation plays in protein folding, the efficiency of *N*-glycosylation in insect cells potentially can affect the yield of secreted, baculovirus-expressed glycoprotein. In addition, the structural characteristics of insect *N*-glycans can affect the utility of recombinant glycoproteins produced with baculovirus expression vectors. Glycosylation is a very important factor controlling the *in vivo* behavior and activity of candidate therapeutics. Thus, the value of baculovirus vector-derived glycoproteins for therapeutic use in humans depends on the ability of the host-insect cells to efficiently and accurately synthesize mammalian-like *N*-glycans. Hence, much effort has been dedicated to elucidating the characteristics of insect *N*-glycosylation, and to engineering both cell lines and baculoviral vectors to modify *N*-glycosylation pathways in the virus–host system. This chapter describes what we know about *N*-glycosylation of both endogenous proteins and recombinant, baculovirus-expressed proteins produced in insect cells. We also summarize the results of efforts to engineer the insect *N*-glycosylation pathway to produce mammalian-type glycans and discuss possible approaches that might be used to further improve protein glycosylation in insect cells. Readers are referred to other reviews focusing on insect *N*-glycosylation and the engineering of insect protein *N*-glycosylation pathways (Jarvis, 2003; Marchal *et al.*, 2001; Tomiya *et al.*, 2003a, 2004).

B. The Insect N-Glycosylation Pathway

The assembly and processing of *N*-glycans in mammalian cells have been extensively characterized, but we know much less about these same pathways in insects. Much of what is known about insect protein *N*-glycosylation was derived from studies conducted with material from insect cell lines. The sequence of the *Drosophila melanogaster* genome (Adams *et al.*, 2000) has further expanded our knowledge of insect *N*-glycosylation by facilitating the identification and characterization of additional mammalian *N*-glycosylation enzyme homologues. The recently reported genome sequences of other insects, such as the mosquito *Anopheles gambiae* (Holt *et al.*, 2002) and the silkworm *Bombyx mori* (Xia *et al.*, 2004), promise to lead to an even more complete and detailed portrayal of the *N*-glycan biosynthetic capacity of insects.

The initial events of protein *N*-glycosylation are conserved among vertebrates, invertebrates, plants, and fungi (Kornfeld and Kornfeld, 1985). The first step in the pathway is a cotranslational process that

takes place in the endoplasmic reticulum (ER). A branched oligosaccharide is transferred *en bloc* from a lipid (dolichyl pyrophosphate)-linked precursor to the side chain amide of an Asn residue in the consensus sequence Asn-Xaa-Ser/Thr (where Xaa is any amino acid other than proline) (Burda and Aebi, 1999). The shorthand formula for this oligosaccharide is Glc₃Man₉GlcNAc₂, where Glc is glucose, Man is mannose, GlcNAc is *N*-acetylglucosamine, and the subscripts refer to the numbers of each residue in the oligosaccharide (Fig. 1). Lipid-linked oligosaccharides with the same structure have been identified in cell lines derived from the dipterans *Aedes albopictus* (Hsieh and Robbins, 1984) and *D. melanogaster* (Parker *et al.*, 1991; Sagami and Lennarz, 1987) and from the lepidopteran *Spodoptera frugiperda* (Marchal *et al.*, 1999).

After being transferred to asparagine, the core oligosaccharide is trimmed by a series of glycosidases in the ER and Golgi apparatus (Trombetta, 2003) (Fig. 1). The first trimming reaction removes the terminal glucose and is catalyzed by α -glucosidase I. α -Glucosidase II then removes the next two terminal glucose residues, yielding Man₉GlcNAc₂. Although genes demonstrated to encode processing ER α -glucosidases have not been identified in any insect system, studies using a glucosidase inhibitor indicate that they are present in lepidopteran cells (Davis *et al.*, 1993; Jarvis and Summers, 1989; Marchal *et al.*, 1999).

The removal of terminal glucose is followed by the removal of four mannose residues by class I α -mannosidases to produce Man₅GlcNAc₂ (Fig. 1). If perfectly analogous to higher eukaryotes, this process would begin in the ER and continue through the action of several additional mannosidases in the Golgi apparatus. A class I α -mannosidase gene, *mas-1*, has been identified in *D. melanogaster* and characterized (Kerscher *et al.*, 1995). The *mas-1* gene does not seem to be strictly required for *N*-glycan processing in the fruit fly, suggesting that a redundant function or alternative pathway for mannose trimming exists in *Drosophila* (Roberts *et al.*, 1998). A class I α -mannosidase enzyme and gene have been isolated from *S. frugiperda* cells and characterized. With the exception of a difference in the precise sequence of mannose trimming, this Golgi enzyme appears to be orthologous to one or more of the class I Golgi α -mannosidases of higher eukaryotes (Kawar and Jarvis, 2001; Kawar *et al.*, 1997, 2000; Ren *et al.*, 1995).

Following trimming of the α -1,2-linked mannose residues, *N*-acetylglucosamine is added to the lower (α -1,3) branch of the remaining structure by *N*-acetylglucosaminyltransferase I (GlcNAcT-I) to produce GlcNAcMan₅GlcNAc₂ (Fig. 1). A *D. melanogaster* gene encoding an active GlcNAcT-I enzyme has been identified and characterized (Sarkar and Schachter, 2001). GlcNAcT-I activity has also been

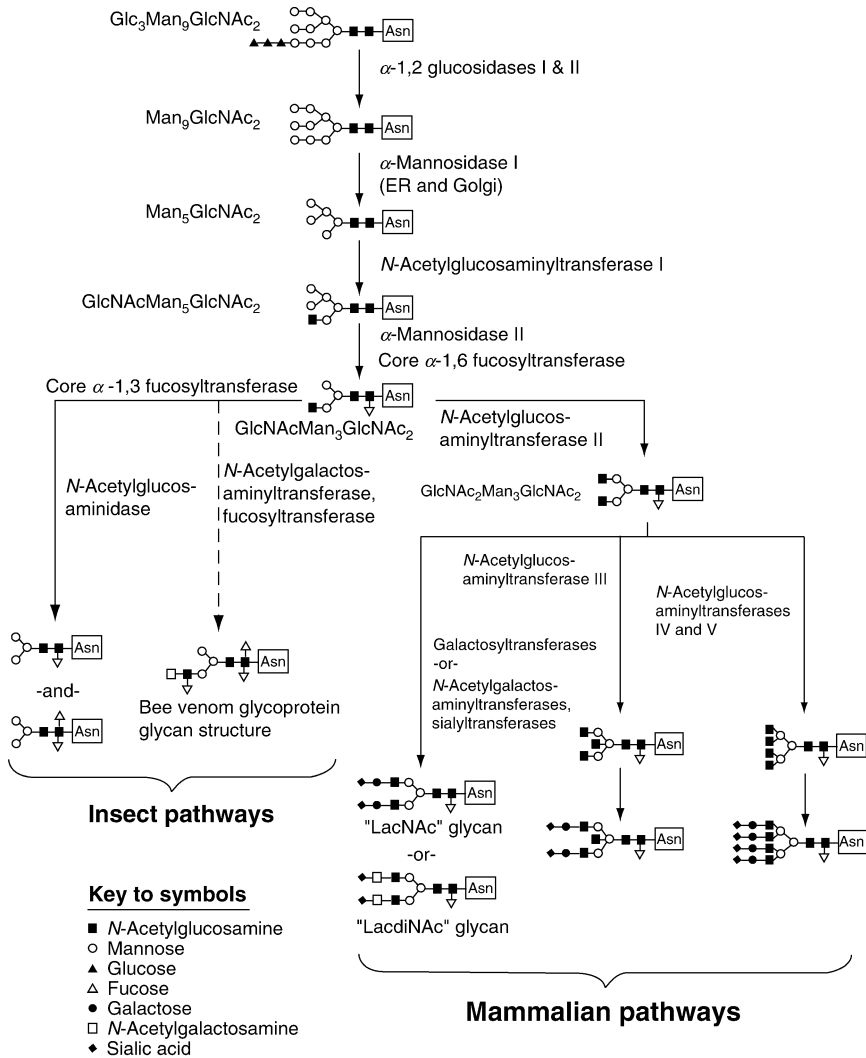


FIG 1. Protein *N*-glycosylation pathways in insects and mammals, showing structures and symbolic descriptions of the *N*-glycan-processing intermediates and indicating the enzymes involved at each step. Each monosaccharide is represented by its standard symbol, as defined in "Essentials of Glycobiology" (Varki *et al.*, 1999). The major product of the insect *N*-glycan-processing pathway, classified as a paucimannose structure, is presented alongside the most complex insect *N*-glycan confirmed by mass spectrometry. Mammalian disialylated, biantennary *N*-glycans are presented alongside biantennary structures bisected with *N*-acetylglucosamine and sialylated tri- and tetraantennary structures.

detected in cell lines derived from the lepidopterans *S. frugiperda*, *B. mori*, *Mamestra brassicae*, and *Estigmene acrea* (Altmann *et al.*, 1993; Velardo *et al.*, 1993; Wagner *et al.*, 1996a).

The class II Golgi α -1,2-mannosidase subsequently trims the α -1,3- and α -1,6-linked mannose residues from the upper (α -1,6) branch of the biantennary glycan intermediate, thereby converting GlcNAcMan₅GlcNAc₂ to GlcNAcMan₃GlcNAc₂ (Fig. 1). A gene encoding this enzyme has been identified in the genome of *D. melanogaster* (Foster *et al.*, 1995; Rabouille *et al.*, 1999) and an enzyme with this same hydrolytic activity and substrate specificity has been identified in *S. frugiperda* Sf9 lysates and cell membranes (Ren *et al.*, 1997; Wagner *et al.*, 1996a). It has also been shown that *S. frugiperda* encodes a separate class II α -mannosidase, which cannot remove mannose residues from GlcNAcMan₅GlcNAc₂ but removes terminal α -1,3- and α -1,6-linked mannose residues from *N*-glycans lacking a terminal *N*-acetylglucosamine residue instead (Jarvis *et al.*, 1997; Kavar *et al.*, 2001). To date, this is the only clear example of a cloned gene encoding the so-called α -mannosidase III activity that was described in knockout mice lacking the classic Golgi class II α -1,2-mannosidase gene (Chui *et al.*, 1997).

At this stage of the *N*-glycan processing pathway, mammalian cells can produce a wide variety of complex, branched oligosaccharide side chains by elongating both branches of GlcNAcMan₃GlcNAc₂. The first step in this process involves the transfer of an *N*-acetylglucosamine to the upper (α -1,6) branch by *N*-acetylglucosaminyltransferase II (GlcNAcT-II) to make GlcNAc₂Man₃GlcNAc₂ (Fig. 1). A low level of GlcNAcT-II activity has been detected in cell lines from three different lepidopteran species (Altmann *et al.*, 1993), and a *Drosophila* gene (*Mgat2*) with sequence similarity to GlcNAcT-II has been identified, although the activity of the encoded gene product was not characterized (Tsitolou and Grammenoudi, 2003).

The branches on mammalian *N*-glycans are often elongated by the transfer of a single galactose to the terminal GlcNAc residues by β -1,4-galactosyltransferase, producing a lactosamine (LacNAc) disaccharide unit (Fig. 1). Alternatively, *N*-acetylgalactosamine may be added to GlcNAc, producing a LacdiNAc disaccharide unit (Fig. 1). β -1,4-Galactosyltransferase activity has been detected at low levels in *Trichoplusia ni* Tn-5B1-4 (High FiveTM) cells and also in *Danaus plexippus* DpN1 cells but not in *S. frugiperda* Sf9 or *Pseudaletia unipuncta* A7S cells (Abdul-Rahman *et al.*, 2002; Palomares *et al.*, 2003; van Die *et al.*, 1996). Genes with significant sequence similarity to mammalian β -1,4-galactosyltransferases have been identified in *D. melanogaster* and in

the lepidopteran *T. ni*, but in both cases the encoded enzymes preferentially transferred *N*-acetylgalactosamine (GalNAc) to GlcNAc (Haines and Irvine, 2005; Vadaie and Jarvis, 2004). This result is consistent with an earlier observation of *N*-acetylgalactosaminyltransferase activity in cell lines derived from *T. ni*, *S. frugiperda*, and *M. brassicae* (van Die *et al.*, 1996). An *N*-acetylgalactosaminyltransferase activity would produce the LacdiNAc disaccharide unit, which is found on honeybee venom phospholipase and hyaluronidase (Fig. 1).

Many mature mammalian *N*-glycans terminate in sialic acid residues, which are added to galactose residues by α -2,3- and α -2,6-sialyltransferases (Fig. 1). Sialyltransferase activity has not been detected by enzymatic assay in any lepidopteran cell line examined (Hollister and Jarvis, 2001; Hooker *et al.*, 1999; Joshi *et al.*, 2001), but an α -2,6-sialyltransferase homologue was identified in the *D. melanogaster* genome (Koles *et al.*, 2004). The encoded enzyme preferentially sialylated terminal LacdiNAc units in both free oligosaccharide and *N*-glycoprotein substrates (Koles *et al.*, 2004).

The studies cited above, with insects from two different orders, suggest that insects have the potential to form complex *N*-glycans similar in structure to those found on mammalian glycoproteins. However, analyses of native insect *N*-glycans from dipterans and lepidopterans have revealed that most *N*-glycans found in these organisms have simple oligomannose (Man₅₋₉GlcNAc₂) or paucimannose (Man₁₋₃GlcNAc₂) structures containing no galactose or sialic acids (Fig. 1) (Butters and Hughes, 1981; Fabini *et al.*, 2001; Kim *et al.*, 2003; Kubelka *et al.*, 1994; Park *et al.*, 1999; Williams *et al.*, 1991). The most complex endogenous insect *N*-glycan unambiguously identified by direct structural analysis by mass spectrometry has a hybrid structure in which the upper branch terminates with mannose and the lower branch terminates with LacdiNAc (Fig. 1). This structure is a minor *N*-glycan, which occupies a subpopulation of the glycosylation sites in honeybee venom phospholipase A2 (Kubelka *et al.*, 1993) and hyaluronidase (Kubelka *et al.*, 1995). In addition, other, more unusual *N*-glycan structures have been described in insects. The *N*-glycans on apolipoprotein isolated from the locust *Locusta migratoria* were found to terminate in GlcNAc on both branches and were modified on the lower-branch terminal GlcNAc and the upper-branch subterminal mannose residues with 2-aminoethylphosphonate (Hard *et al.*, 1993). A minority of the *N*-glycans on *Manduca sexta* aminopeptidase N consist of highly fucosylated structures with fucose attached to both GlcNAc residues in the chitobiose core and also to terminal GlcNAc residues in the antennae (Stephens *et al.*, 2004).

If insects have the potential to form complex *N*-glycans, why do oligomannose and paucimannose structures predominate? One possible reason is that the expression of key glycosyltransferases is restricted to specific tissues and developmental stages in insects. The *D. melanogaster* *N*-acetylgalactosaminyl- and *N*-acetylglucosaminyl-transferase genes were found to be transcribed ubiquitously throughout development, but in embryos and larvae GlcNAcT-II transcripts were restricted primarily to neural tissue and eye imaginal discs (Haines and Irvine, 2005; Tsitolou and Grammenoudi, 2003). Expression of the *D. melanogaster* α -2,6-sialyltransferase also was restricted to a subset of central nervous system cells in embryos (Koles *et al.*, 2004). Thus, it is possible that many of the genes encoding enzymes involved in producing complex *N*-glycans are simply inactive in many insect tissues or in many of the insect cell lines that have been used to study insect protein *N*-glycosylation.

In addition, several studies have identified intracellular and extracellular sialidase, β -galactosidase, β -*N*-acetylgalactosaminidase, and β -*N*-acetylglucosaminidase activities (Joosten and Shuler, 2003a; Licari *et al.*, 1993; Sommer and Spindler, 1991; van Die *et al.*, 1996; Wagner *et al.*, 1996a), which could either prevent the formation of complex *N*-glycans or degrade them to paucimannose structures prior to analysis.

There is also a membrane-bound β -*N*-acetylglucosaminidase activity in *D. melanogaster* and lepidopteran insect cell lines (Altmann *et al.*, 1995). This appears to be a branch specific, processing enzyme activity, which removes the terminal *N*-acetylglucosamine residue from the lower (α -1,3) branch of *N*-glycans in the final processing step leading to the production of paucimannose end products (Fig. 1). The presence of terminal *N*-acetylglucosamine on influenza virus *N*-glycans produced by *E. acreea* EaA cells, but not *S. frugiperda* Sf9 cells, was attributed to the absence of this putative processing *N*-acetylglucosaminidase activity in the former (Wagner *et al.*, 1996a). Removal of the lower-branch *N*-acetylglucosamine not only blocks elongation of the lower branch but might also prevent elongation of the upper branch, as mammalian GlcNAcT-II requires terminal *N*-acetylglucosamine on the lower branch for activity (Bendiak and Schachter, 1987).

Finally, the failure to detect terminal sialic acids in endogenous insect *N*-glycans could also reflect the absence of a conventional donor substrate for sialylation, which is the nucleotide sugar, CMP-sialic acid. In vertebrates, the pathway for the production of CMP-sialic acid (Angata and Varki, 2002) begins with the conversion of UDP-GlcNAc to *N*-acetylmannosamine, or ManNAc, which is then phosphorylated to produce ManNAc-6-phosphate (Fig. 2). These reactions are catalyzed

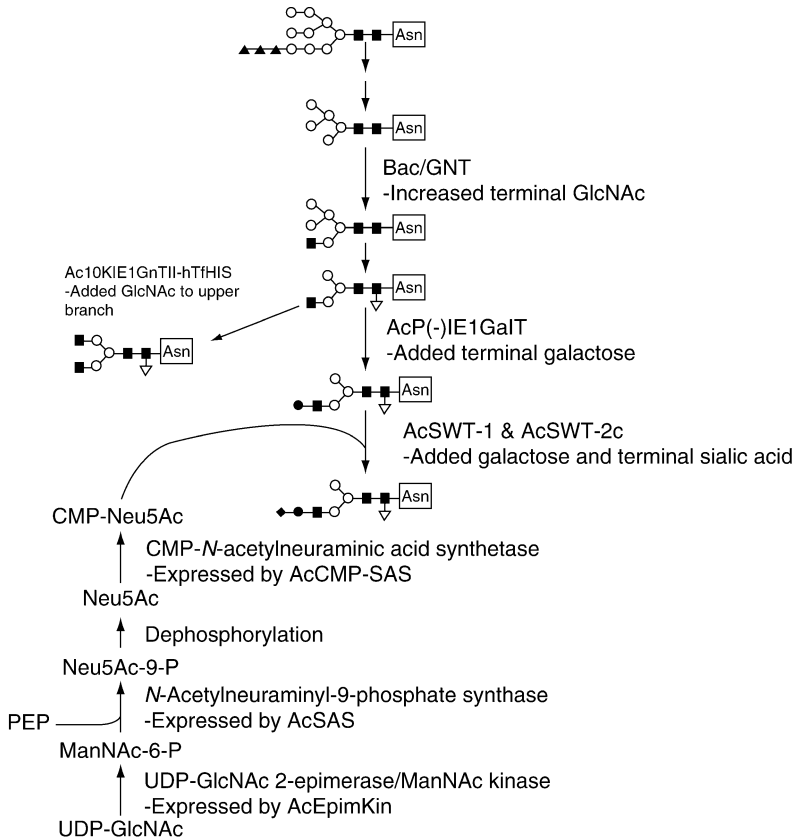


FIG 2. Structures of the *N*-glycans produced in insect cells infected with recombinant baculoviruses expressing mammalian glycosyltransferases. The pathway for synthesis of the sialylation donor substrate CMP-sialic acid (CMP-Neu5Ac) also is shown, and the names of the recombinant baculoviruses encoding mammalian enzymes that participate in this pathway are indicated. The standard monosaccharide symbols used in this figure are defined in the key shown in Fig. 1.

by the bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase. ManNAc-6-phosphate is then condensed with phosphoenolpyruvate by *N*-acetylneuraminy-9-phosphate synthase to produce *N*-acetylneuraminy-9-phosphate, which is then dephosphorylated to produce *N*-acetylneuraminic acid (Neu5Ac), the form of sialic acid most commonly found in *N*-glycans. Finally, Neu5Ac is conjugated to cytosine monophosphate by CMP-*N*-acetylneuraminic acid synthetase to produce CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac). Although

free sialic acid has been unambiguously identified in *D. melanogaster*, in the cicada *Philaenus spumarius* (Malykh *et al.*, 2000; Roth *et al.*, 1992), and at low levels in Sf9 cells (Lawrence *et al.*, 2000), the levels of CMP-sialic acid were found to be negligible in lepidopteran cell lines (Hooker *et al.*, 1999; Tomiya *et al.*, 2001). A gene encoding a sialic acid phosphate synthase homologue has been identified in *D. melanogaster* (Kim *et al.*, 2002). This gene was transcribed at every developmental stage, and the encoded product was able to synthesize sialic acid phosphate from *N*-acetylmannosamine-6-phosphate and phosphoenolpyruvate. These same authors also performed a homology search and found a CMP-sialic acid synthetase homologue in the fly genome, suggesting that dipterans encode at least some of the enzymes involved in CMP-sialic acid biosynthesis.

C. N-Glycosylation of Proteins Produced with Baculovirus Expression Vectors

The preponderance of studies on the glycans of recombinant *N*-glycoproteins produced in lepidopteran cell lines with baculovirus expression vectors report mostly oligomannose or paucimannose structures (e.g., Ding *et al.*, 2003; Grabenhorst *et al.*, 1993; Hollister *et al.*, 2002; Kuroda *et al.*, 1990; Lopez *et al.*, 1997; Manneberg *et al.*, 1994; Takahashi *et al.*, 1999; Wendeler *et al.*, 2003), with a minority of *N*-glycans (<10%) containing terminal GlcNAc on one branch in some studies (Ailor *et al.*, 2000; Choi *et al.*, 2003). This trend also has been observed with recombinant glycoproteins produced in baculovirus-infected lepidopteran larvae (Hogeland and Deinzer, 1994; Kulakosky *et al.*, 1998). One study reported that a majority of the *N*-glycans assembled in *E. acrea* EaA cells contained terminal GlcNAc (Wagner *et al.*, 1996a). Yet other studies have reported the assembly of complex *N*-glycans containing terminal galactose or sialic acid residues on recombinant glycoproteins expressed in a variety of lepidopteran cell lines (Davidson and Castellino, 1991a,b, 1993; Davidson *et al.*, 1990; Hsu *et al.*, 1997; Joosten and Shuler, 2003a,b; Joosten *et al.*, 2003; Joshi *et al.*, 2000, 2001; Ogonah *et al.*, 1996; Pajot-Augy *et al.*, 1999; Palomares *et al.*, 2003; Rudd *et al.*, 2000; Watanabe *et al.*, 2001). In two of these studies, the presence of galactose was confirmed by direct compositional analysis with mass spectrometry (Ogonah *et al.*, 1996; Rudd *et al.*, 2000). In both cases, the authors used matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to verify that a minority of the *N*-glycans on glycoproteins expressed in *E. acrea* Ea4 (Ogonah *et al.*, 1996) and *T. ni*

Tn-5B1-4 cells (Rudd *et al.*, 2000) contained terminal galactose on a single branch. To date, terminal sialylation of recombinant glycoproteins synthesized in insect cells has not been confirmed by mass spectrometry (Marchal *et al.*, 2001).

Although it is generally the case that recombinant proteins synthesized in insect cells bear *N*-glycans at the sites where they occur in the natural products, one might expect *N*-glycan assembly and processing to be less efficient in cells infected with a baculovirus expression vector. The steady state levels of transcripts derived from host cell genes decrease as viral infection progresses (Nobiron *et al.*, 2003; Ooi and Miller, 1988) and this general effect might extend to the expression of host genes encoding glycosylation pathway functions. In addition, the *N*-glycosylation capabilities of the host might simply be overwhelmed by the massive amounts of recombinant protein produced by a baculovirus expression vector, especially one that uses the highly active polyhedrin promoter to drive foreign gene expression. In their assessment of these issues, Kretzschmar *et al.* (1994) reported no discernible difference in the structures of the *N*-glycans of total glycoproteins isolated from mock- and baculovirus-infected *S. frugiperda* cells. Similarly, Joosten and Shuler (2003a) reported no significant differences among the profiles of secreted alkaline phosphatase *N*-glycans harvested at 48, 72, and 96 h postinfection. However, in a study of baculovirus-mediated expression of HIV envelope proteins, Murphy *et al.* (1990) found that the proportion of nonglycosylated protein increased as infection progressed from 24 to 48 h postinfection. Baculovirus-mediated expression of human thyrotropin receptor under the control of the *p6.9* promoter, which is activated earlier than the polyhedrin promoter, produced a more extensively glycosylated form of the receptor than conventional baculovirus-mediated expression under the control of the polyhedrin promoter (Chazenbalk and Rapoport, 1995). A similar effect on the extent of lutropin receptor glycosylation was observed with baculoviruses that expressed the porcine lutropin receptor under the control of either the polyhedrin or *p10* promoters, perhaps reflecting the fact that the latter is activated a few hours earlier and mediates a weaker level of expression than the polyhedrin promoter (Pajot-Augy *et al.*, 1999; Roelvink *et al.*, 1992). Thus, the results of these studies indicate that, for at least some recombinant products, the quality and extent of protein *N*-glycosylation can be affected by baculoviral infection, with glycosylation efficiency decreasing as the infection proceeds. This conclusion is consistent with the original observation that baculovirus infection has an adverse effect on secretory pathway function (Jarvis and Summers, 1989). It also is consistent

with the general idea that baculovirus infection represses host gene expression (Nobiron *et al.*, 2003; Ooi and Miller, 1988) and with the pathway-specific observation that host *N*-acetylgalactosaminyltransferase activity decreases with time of baculovirus infection (van Die *et al.*, 1996). On the other hand, this conclusion is inconsistent with results indicating that *N*-glycan-processing activities, including both GlcNAc-TI (Velardo *et al.*, 1993) and α -mannosidase (Davidson *et al.*, 1991) activities, are induced at later times after baculovirus infection.

The general inability of established lepidopteran cell lines to produce terminally sialylated *N*-glycans is problematic for the production of efficacious therapeutic glycoproteins. Terminal sialic acids protect glycoproteins from being cleared from the bloodstream by various mammalian lectins (Ashwell and Harford, 1982). Thus, it was not surprising to find that glycoproteins produced with a baculovirus expression vector were rapidly cleared from the bloodstream after being injected into rodents (Kurschat *et al.*, 1995; Sareneva *et al.*, 1993). A comparison of the clearance rates of the glycopeptide hormone thyrotropin produced in either baculovirus-infected insect cells or DNA-transfected Chinese hamster ovary (CHO) cells revealed that only 0.1% of the former product, as compared to >10% of the latter, remained in the bloodstream 2 h after injection (Grossmann *et al.*, 1997). Because prolonged residence time in the plasma is necessary for therapeutic efficacy, the rapid clearance of nonsialylated products is a significant impediment to the commercialization of baculovirus vector-produced therapeutic glycoproteins.

Another impediment is the presence of a fucose residue linked to the asparagine-bound GlcNAc residue in the *N*-glycan chitobiose core produced by some lepidopteran insect cells. Two main types of core fucosylation actually occur in insects: (1) monofucosylation with an α -1,6 linkage, which is also found in mammalian *N*-glycans, and (2) difucosylation with α -1,6 and α -1,3 linkages, which is not found in mammalian *N*-glycans (Staudacher *et al.*, 1999) (Fig. 1). Core difucosylation requires two separate enzymes that transfer first the α -1,6- and then the α -1,3-linked fucose residues to the chitobiose core (Staudacher and Marz, 1998). The presence of core α -1,3-linked fucose is a potential problem for the use of baculovirus vector-generated glycoproteins for therapeutic purposes because it represents an allergenic carbohydrate epitope in mammalian species, one that plays a significant role in immunogenic responses to plant and invertebrate glycoproteins (Prenner *et al.*, 1992; Tretter *et al.*, 1993; Wilson *et al.*, 1998). However, it is important to recognize that the extent to which core difucosylation occurs varies among different insect cell lines and, in some cases, it

is actually a relatively infrequent modification of endogenous insect *N*-glycans. The molar percentage of native insect *N*-glycans with core α -1,3-fucose ranges from 2.5% for a *B. mori* cell line (BmN) to 30% for an *M. brassicae* cell line (Mb-0503) (Kubelka *et al.*, 1994). The *S. frugiperda* Sf9 cell line has no detectable α -1,3-fucosyltransferase activity (Staudacher *et al.*, 1992), but a subsequent study revealed that these cells contain very small subpopulations of *N*-glycans with either difucosylated or α -1,3-monofucosylated cores (Kubelka *et al.*, 1994). The presence of core α -1,3 fucosylation, as assessed by staining with anti-horseradish peroxidase polyclonal antibodies, was restricted to neurons in grasshopper and *D. melanogaster* embryos (Fabini *et al.*, 2001; Snow *et al.*, 1987). A gene encoding an enzymatically active core α -1,3-fucosyltransferase has been identified in the *D. melanogaster* genome (Fabini *et al.*, 2001). However, in adult *D. melanogaster*, *N*-glycans with a difucosylated core GlcNAc represent only 0.8% of total, as assessed by MALDI-TOF (Fabini *et al.*, 2001). No core difucosylated *N*-glycans were detected in hemolymph glycoproteins isolated from larvae of *Antheraea pernyi*, the Chinese oak silkworm (Kim *et al.*, 2003). Core difucosylation of recombinant glycoproteins is also highly variable. Approximately 12–23% of the *N*-glycans on baculovirus-expressed recombinant glycoproteins isolated from *T. ni* Tn-5B1-4 cells carried core α -1,3-fucose (Hsu *et al.*, 1997; Takahashi *et al.*, 1999). On the other hand, there was no detectable core α -1,3 fucosylation of latent TGF- β -binding protein-1 expressed in Sf9 cells (Rudd *et al.*, 2000), human interferon gamma expressed in *E. acrea* Ea4 cells (Ogonah *et al.*, 1996), or human transferrin expressed in *Lymantria dispar* Ld652Y cells (Choi *et al.*, 2003). Thus, the extent to which core difucosylation will be a problem associated with the use of baculovirus–insect cell expression systems will depend strongly on the nature of the host and the nature of the recombinant glycoprotein being produced.

II. MODIFICATION OF LEPIDOPTERAN *N*-GLYCOSYLATION PATHWAYS FOR IMPROVED PROCESSING AND FUNCTION OF GLYCOPROTEINS PRODUCED WITH BACULOVIRUS EXPRESSION VECTORS

A. *Baculovirus* Expression of Mammalian *N*-Glycosylation Pathway Enzymes

Much effort has been directed toward modifying protein *N*-glycosylation pathways in lepidopteran cells in order to obtain baculovirus vector-expressed glycoproteins bearing mammalian-style, complex,

terminally sialylated *N*-glycans. This effort has involved introducing genes encoding functions that are either missing or present at sub-optimal levels in lepidopteran host cells but required for complex *N*-glycan assembly.

One approach to providing these missing processing activities has entailed inserting the desired *N*-glycan-processing genes into a baculovirus vector so that they can be expressed during infection (Fig. 2). Coexpression of human GlcNAcT-I and fowl plaque virus hemagglutinin in *S. frugiperda* cells using two separate baculoviruses carrying the genes for these proteins resulted in a fourfold increase in the proportion of hemagglutinin *N*-glycans bearing terminal GlcNAc (Wagner *et al.*, 1996b). Expression of a bovine β -1,4-galactosyltransferase gene under the control of *ie1* promoter and *hr5* enhancer sequences with the recombinant baculovirus AcP(-)IE1GalT resulted in the addition of galactose to the *N*-glycans of the viral envelope fusion protein, GP64 (Jarvis and Finn, 1996). This virus also added terminal galactose residues to the *N*-glycans of human transferrin when the latter was coexpressed with a separate baculovirus (Ailor *et al.*, 2000). In this case, 12.6% of the *N*-glycans were terminally galactosylated on the lower (α -1,3) branch. The recombinant baculoviruses AcSWT-1 and AcSWT-2c were designed to express both bovine β -1,4-galactosyltransferase and rat α -2,6-sialyltransferase genes from a bidirectional *ie-1* promoter/*hr5* enhancer element, with the processing genes inserted into different locations in the two recombinant viral genomes (Jarvis *et al.*, 2001). Infections with either of these viruses resulted in the addition of both galactose and sialic acid to the *N*-glycans of the GP64 viral envelope protein. Similar results were obtained when human α 1-antitrypsin was expressed in Ea4 cells infected with a single recombinant baculovirus (BacATgng26) encoding this product together with human GlcNAcT-II, β -1,4-galactosyltransferase, and α -2,6-sialyltransferase under the control of *p10* and polyhedrin promoters (Chang *et al.*, 2003).

Other recombinant baculoviruses have been designed and constructed to express enzymes of the CMP-sialic acid biosynthetic pathway (Fig. 2). Infection of Sf9 cells with the recombinant virus AcSAS, which expresses human *N*-acetylneuraminy-9-phosphate synthase under the control of the polyhedrin promoter, resulted in the production of high levels of Neu5Ac when the cells were incubated in serum-free medium containing the sialic acid precursor, ManNAc (Lawrence *et al.*, 2000). This result indicated that endogenous Sf9 cell enzymes could phosphorylate ManNAc and dephosphorylate *N*-acetylneuraminy-9-phosphate. Coinfection of Sf9 cells with AcSAS and AcCMP-SAS,

which encodes the human CMP-*N*-acetylneuraminic acid synthetase under control of the polyhedrin promoter, resulted in the production of CMP-Neu5Ac in Sf9 cells fed with serum-free medium supplemented with ManNAc (Lawrence *et al.*, 2001). The recombinant virus AcEpimKin, which encodes the UDP-GlcNAc 2-epimerase/ManNAc kinase gene under control of the *polh* promoter (Effertz *et al.*, 1999), induced Neu5Ac production in Sf9 cells coinfecting with AcSAS and cultured without ManNAc supplementation (Viswanathan *et al.*, 2003). Infecting Sf9 cells simultaneously with AcEpimKin, AcSAS, and AcCMP-SAS resulted in the production of CMP-Neu5Ac without ManNAc supplementation, indicating that Sf9 cells can produce CMP-Neu5Ac from endogenous UDP-GlcNAc when mammalian sialic acid synthesis pathway functions are provided in *trans* (Viswanathan *et al.*, 2005).

B. Stable Transformation of Lepidopteran Host Cells with Genes for Mammalian Pathway Enzymes

Another approach that has been used to provide host-insect cells with missing or suboptimal *N*-glycan-processing activities involves stable transformation of cells with processing genes designed to be expressed under the control of a constitutively active promoter. This “metabolic engineering” approach yields new cell lines that are pre-equipped with a mammalian-like glycosylation pathway prior to the time of baculovirus vector infection. It also permits one to use standard transfer vector/recombinant baculovirus production systems and/or preexisting recombinant baculovirus vectors for the expression of glycoproteins with “humanized” *N*-glycans.

This approach was first used to create an Sf9 cell clone that was stably transformed with a bovine β -1,4-galactosyltransferase cDNA positioned under the control of an *ie1* promoter-*hr5* enhancer element (Hollister and Jarvis, 2001). This cell line, called Sf β 4GalT (Fig. 3), had high levels of β -1,4-galactosyltransferase activity. Hence, when these cells were infected with wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) or a conventional baculovirus vector expressing human tissue plasminogen activator (t-PA) under the control of the *polh* promoter, galactose was detected in the *N*-glycans of both GP64 and t-PA by lectin blotting (Hollister and Jarvis, 2001). Further, when a virus expressing rat α -2,6-sialyltransferase under the control of the *ie1* promoter-*hr5* enhancer element (AcP(+))IE1 α 26ST) was used to infect Sf β 4GalT cells, the GP64 produced during infection was found to be both galactosylated and sialylated (Seo *et al.*, 2001).

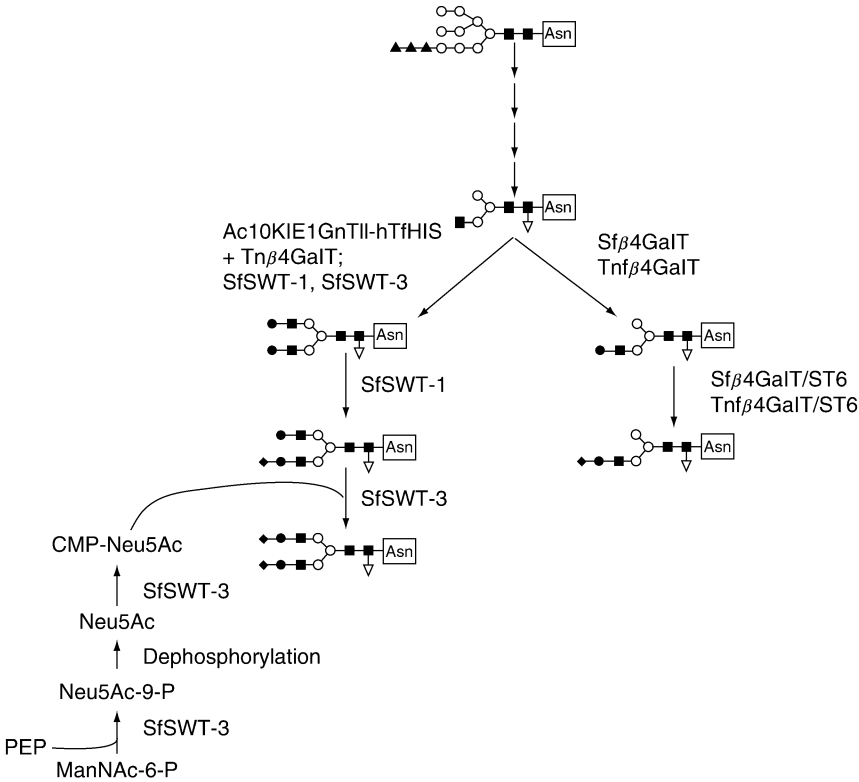


FIG 3. Structures of the *N*-glycans produced by insect cells stably transformed to encode and express mammalian *N*-glycosylation functions. The cell lines capable of producing each intermediate are indicated, as is the SfSWT-3 cell line engineered to express enzymes required for CMP-sialic acid synthesis. The standard monosaccharide symbols used in this figure are defined in the key shown in Fig. 1.

The Sfβ4GalT cell line subsequently was transformed with a rat α-2,6-sialyltransferase cDNA positioned under the control of the *ie1* promoter-*hr5* enhancer element (Hollister and Jarvis, 2001). This cell line, called Sfβ4GalT/ST6 (Fig. 3), had both galactosyltransferase and sialyltransferase activities, and produced sialylated *N*-glycans on both GP64 and an additional model glycoprotein, the *S. frugiperda* class I Golgi α-mannosidase fused to glutathione S-transferase (GST-SfManI).

The *T. ni* cell line Tn-5B1-4 was also transformed with the same constructs to produce cell lines designated Tn5β4GalT, which encoded β-1,4-galactosyltransferase, and Tn5β4GalT/ST6, which encoded both galactosyltransferase and sialyltransferase under the control of the

bidirectional *ie-1* promoter/*hr5* enhancer (Fig. 3; Breitbach and Jarvis, 2001). These cell lines contained the expected glycosyltransferase activities and produced GP64 with galactosylated (Tn5 β 4GalT) or galactosylated and sialylated (Tn5 β 4GalT/ST6) *N*-glycans during infection with wild-type AcMNPV.

Structural analyses of the *N*-glycans produced by insect cells that were either infected or transformed to express the above-mentioned glycosyltransferase genes revealed that they were monoantennary structures in which only the lower (α -1,3) branch was elongated (Ailor *et al.*, 2000; Hollister *et al.*, 2002). Subsequently, it was found that infection of Tn5 β 4GalT cells with a virus (Ac10KIEGnTII-hTfHIS) encoding human GlcNAcT-II resulted in the production of human transferrin with biantennary *N*-glycans that contained terminal galactose on both branches (Tomiya *et al.*, 2003b). This result indicated that both Sf9 and Tn-5B1-4 cells engineered to express galactosyl- and sialyltransferase genes produced monoantennary *N*-glycans because they lacked sufficient levels of GlcNAcT-II, which is needed to initiate elongation of the upper (α -1,6) branch and produce biantennary *N*-glycans. This observation was consistent with the previous finding that GlcNAcT-II activity was present, but only at extremely low levels in three lepidopteran cell lines (Altmann *et al.*, 1993). To produce a cell line that could produce biantennary, complex *N*-glycans, Sf β 4GalT cells were transformed with constructs encoding *N*-acetylglucosaminyltransferase I, *N*-acetylglucosaminyltransferase II, α -2,6-sialyltransferase (ST6GalI), and α -2,3-sialyltransferase (ST3GalIV) cDNAs, all under the control of the *ie1* promoter-*hr5* enhancer element. This cell line, named SfSWT-1 (Fig. 3), was, in fact, able to produce complex, biantennary *N*-glycans on GST-SfManI (Hollister *et al.*, 2002). However, only the α -1,3 branch was sialylated, and the sialic acid was attached with an α -2,6 linkage. This result suggested that ST3GalIV, which transfers sialic acid to galactose in an α -2,3 linkage, was not active in the SWT-1 cell line.

Since Sf9 cells contain very little sialic acid (Lawrence *et al.*, 2000) and no detectable CMP-sialic acid (Hooker *et al.*, 1999; Tomiya *et al.*, 2001), the source of a donor substrate for the sialylation of *N*-glycans assembled in wild-type cells infected with the AcSWT viruses and in the Sf β 4GalT/ST6, Tn5 β 4GalT/ST6, and SfSWT-1 cell lines was unknown. Subsequently, it was found that the *N*-glycans on GST-SfManI were sialylated only when Sf β 4GalT/ST6 cells were cultured in the presence of serum or a purified, sialylated glycoprotein (Hollister *et al.*, 2003). No sialylation was detected when the cells were cultured in serum-free medium. Serum that had been dialyzed with a 50,000-Da cutoff membrane still supported sialylation, suggesting that the molecules

in serum providing the donor substrate source were relatively large. Sf β 4GalT/ST6 cells also produced a very small quantity of sialylated *N*-glycans when cultured in serum-free medium supplemented with Neu5Ac or ManNAc. These results suggested that Sf9 cells have a mechanism for salvaging sialic acids from external sources and, together with the discovery of a gene encoding an active *N*-acetylneuraminic acid synthase and a potential homologue for CMP-Neu5Ac synthetase in *D. melanogaster* (Kim *et al.*, 2002), these results are consistent with the idea that insects have the ability to produce CMP-Neu5Ac.

To produce a cell line that could sialylate *N*-glycoproteins when cultured in serum-free media, SfSWT-1 cells were transformed with mammalian genes encoding Neu5Ac synthase and CMP-Neu5Ac synthetase under the control of the *hr5-ie1* enhancer/promoter element (Aumiller *et al.*, 2003). The resulting cell line, designated SfSWT-3 (Fig. 3), contained CMP-Neu5Ac and was able to produce complex, terminally sialylated *N*-glycans on GST-SfManI when cultured in a serum-free medium supplemented with ManNAc. Like SfSWT-1, SfSWT-3 produced mainly monosialylated, biantennary *N*-glycans, but they also appeared to produce a very minor population of disialylated, biantennary *N*-glycans.

Assuming that the endogenous membrane glycoproteins of Tn β 4GalT/ST6, Sf β 4GalT/ST6, SfSWT-1, and SfSWT-3 cells are terminally sialylated, the surfaces of these cells should be dramatically altered relative to the surfaces of the untransformed parental cell lines (Sf9 and Tn-5B1-4). These structural changes to cell surface glycoconjugates might be expected to inhibit baculovirus virion attachment, penetration, assembly, and/or release. However, all of these transformed cell lines supported wild-type and recombinant AcMNPV infection and replication at levels comparable to those of the untransformed parental cell lines (Hollister and Jarvis, 2001; Hollister *et al.*, 1998, 2002). The transformed cell lines described above also had similar growth properties and morphologies, relative to their untransformed progenitors (Breitbach and Jarvis, 2001). However, SfSWT-1 cells were noticeably smaller than Sf9 and achieved higher final densities in suspension culture than Sf-9 and SfSWT-3 cell lines after an initial period of slower growth (Aumiller *et al.*, 2003). Finally, the GST-S-fManI model glycoprotein was found to be expressed at approximately equal levels in Sf9, SfSWT-1, and SfSWT-3 cells after baculovirus vector infection, although there were some differences in the expression kinetics (Aumiller *et al.*, 2003).

III. OTHER CONSIDERATIONS AND FUTURE IMPROVEMENTS

A. Manipulation of Cell Culture Conditions

Studies with mammalian cells have shown that culture conditions can be manipulated to alter protein *N*-glycosylation profiles (reviewed in [Jenkins *et al.*, 1996](#)). For example, supplementation of CHO cell medium with ManNAc increased the extent of *N*-glycan sialylation ([Gu and Wang, 1998](#)). These types of environmental manipulations can be used to alter the *N*-glycosylation profiles of baculovirus-expressed recombinant proteins as well. The addition of mannosamine to lepidopteran cell culture medium increased the proportion of *N*-glycans with terminal *N*-acetylglucosamine ([Donaldson *et al.*, 1999](#); [Estrada-Mondaca *et al.*, 2005](#)). ManNAc supplementation increased *N*-glycan sialylation in *T. ni* Tn-4h cells ([Joshi *et al.*, 2001](#)). In the same study, culturing Tn-4h cells in a bioreactor that simulated weightlessness and free fall had a similar effect ([Joshi *et al.*, 2001](#)). Supplementation of culture medium with *B. mori* (silkworm) hemolymph increased *N*-glycan sialylation by Tn-4s cells ([Joosten *et al.*, 2003](#)). In contrast to reports on the effects of fetal bovine serum on glycosylation in mammalian cell lines ([Jenkins *et al.*, 1996](#)), the presence or absence of fetal bovine serum had little to no effect on the *N*-glycan profiles of human transferrin expressed in *L. dispar* Ld652Y cells ([Choi *et al.*, 2003](#)).

It is also possible to add exoglycosidase inhibitors to influence the structures of the *N*-glycans found on insect cell-produced glycoproteins. The addition of swainsonine, an α -mannosidase II inhibitor, increased the quantity of terminal *N*-acetylglucosamine on fowl plague hemagglutinin *N*-glycans produced in Sf9 cells ([Wagner *et al.*, 1996a](#)). This effect was explained by the fact that this inhibitor would block the conversion of GlcNAcMan₅GlcNAc₂ to GlcNAcMan₃GlcNAc₂, thereby blocking production of the substrate for the *N*-acetylglucosaminidase activity in these cells ([Wagner *et al.*, 1996a](#)). Supplementation of insect cell medium with an *N*-acetylglucosaminidase inhibitor, 2-acetamido-1,2,5-trideoxy-1,5-imino-D-glucitol, increased the proportion of *N*-glycans with terminal GlcNAc in Sf9 cells ([Wagner *et al.*, 1996a](#)) but failed to significantly alter the *N*-glycan profiles of secreted alkaline phosphatase produced in *T. ni* Tn-4s cells ([Joosten and Shuler, 2003a](#)). The addition of a different *N*-acetylglucosaminidase inhibitor, 2-acetamido-1,2-dideoxynojirimycin, resulted in the appearance of sialylated *N*-glycans in *T. ni* Tn-5B1-4 cells, as assessed by lectin blotting analysis ([Watanabe *et al.*, 2001](#)). The addition of lactose, a competitive inhibitor of β -galactosidase, to infected

Tn-4s cell culture medium did not alter the *N*-glycan profiles of secreted alkaline phosphatase (Joosten and Shuler, 2003a).

B. Eliminating Unwanted Enzymatic Activities

As discussed earlier, the potential for at least some hosts to produce oligomannose or paucimannose *N*-glycans with an allergenic core fucose residue seriously impedes their use to produce recombinant glycoproteins for therapeutic applications. One potential way to address this problem would be to selectively downregulate or eliminate the relevant enzymatic activities. The processing *N*-acetylglucosaminidase and core α -1,3-fucosyltransferase are two potential targets.

Antisense RNA and RNA interference (RNAi) technologies can be used to reduce or eliminate unwanted enzymatic activities in cells (Lee and Roth, 2003). For example, an antisense RNA approach was used successfully to reduce sialidase activity in CHO cell culture supernatants (Ferrari *et al.*, 1998). A sialidase secreted from CHO cells causes a progressive reduction in sialic acid content of glycoproteins produced by CHO cells over time. Thus, Ferrari *et al.* (1998) stably transformed CHO cells with constructs that constitutively produced sialidase antisense RNAs. The transformed CHO clones exhibited reduced sialidase transcript levels, a 60% reduction in extracellular sialidase activity, and a 20–37% increase in sialic acid content of DNase (Ferrari *et al.*, 1998).

While the antisense RNA method rarely leads to complete shutdown of target gene expression, a higher degree of downregulation can be achieved using the RNAi approach (Hannon, 2002). Generally, this approach involves the introduction of double-stranded RNA molecules composed of sense and antisense transcripts derived from the target gene. These transcripts may be introduced by transfection or produced endogenously after stable transformation of cells with a DNA construct that induces the production of the desired double-stranded RNAs. The double-stranded RNA is then processed via established RNAi pathways, which ultimately target the desired mRNAs for selective cleavage and degradation. RNAi has been applied successfully to lepidopterans, both to inhibit expression of an endogenous gene (Rajagopal *et al.*, 2002) and to inhibit expression of a baculovirus gene during infection (Means *et al.*, 2003). In both cases, target gene transcripts were nearly undetectable after inoculation with the double-stranded RNA trigger.

C. Nucleotide Sugar Transporters

The addition of monosaccharides to *N*-glycan antennae occurs in the Golgi compartments. The nucleotide-sugar donor substrates utilized by glycosyltransferases are synthesized in the cytoplasm, except for CMP-Neu5Ac, which is produced in the nucleus, and each must be imported into the Golgi by specific nucleotide-sugar transporters (Gerardy-Schahn *et al.*, 2001). A gene encoding a putative CMP-sialic acid/UDP-galactose transporter was identified in the *D. melanogaster* genome, but the gene product actually transported UDP-galactose, not CMP-sialic acid (Aumiller and Jarvis, 2002; Segawa *et al.*, 2002). These studies did not reveal whether this fly transporter is expressed in tissue- or developmental stage-specific fashion, nor did they examine its expression levels.

The ability of Sf9 cells infected with the recombinant baculoviruses, AcSWT-1 and AcSWT-2c, and the transformed cell lines, SfSWT-1 and SfSWT-3, to produce sialylated *N*-glycoproteins indicates that these cells must have nucleotide-sugar transporters capable of importing CMP-Neu5Ac into the Golgi apparatus. However, the nature of these transporters remains unknown. Meanwhile, engineering insect cells to express an exogenous CMP-Neu5Ac transporter could increase the level of CMP-Neu5Ac import into the Golgi and possibly improve the efficiency with which *N*-glycans are sialylated during baculovirus infection.

D. Bisected, Tri- and Tetraantennary N-Glycans

In addition to producing glycoproteins with complex, terminally sialylated *N*-glycans, glycoprotein functionality can be extended by supplying additional *N*-glycan-processing activities. For example, the antibody-dependent cellular cytotoxicity of an antineuroblastoma IgG1 monoclonal antibody being developed to treat some forms of cancer correlates with the presence of a bisecting GlcNAc in its *N*-glycans (Lifely *et al.*, 1995). The enzyme GlcNAcT-III catalyzes the transfer of this bisecting GlcNAc to the core mannose residue of biantennary *N*-glycans (Fig. 1). Doxycyclin induction of CHO cells transformed with the GlcNAcT-III gene under the control of a tetracycline-repressible promoter increased the proportion of *N*-glycans with bisecting GlcNAc and concomitantly increased the antibody-dependent cellular toxicity of the IgG1 produced by this transformed cell line (Umana *et al.*, 1999).

The enzymes GlcNAcT-IV and GlcNAcT-V transfer additional *N*-acetylglucosamine residues to the mannose residues on the α -1,3

and α -1,6 branches, leading to the formation of tri- and tetraantennary *N*-glycans (Fig. 1). The glycoprotein hormone erythropoietin (EPO) had more activity *in vivo* if it had a tetraantennary rather than a biantennary *N*-glycan (Takeuchi *et al.*, 1989) and it also had a longer *in vivo* half-life (Misaizu *et al.*, 1995). The degree of sialylation of the tetraantennary form of EPO correlated positively with its *in vivo* bioactivity, presumably due to reduced clearance rates (Yuen *et al.*, 2003). Hence, engineering insect cells to produce *N*-glycans with more than two sialylated antennae could similarly improve the *in vivo* bioactivity of baculovirus vector-produced therapeutic glycoproteins. CHO cells engineered to express higher levels of GlcNAcT-IV and GlcNAcT-V produced glycoproteins with larger proportions of tri- and tetraantennary *N*-glycans (Fukuta *et al.*, 2000), indicating that branching can be increased by introducing genes encoding the appropriate glycosyltransferases.

E. Protein Specificity of N-Glycan Processing by Engineered Insect Cell Lines?

Lepidopteran insect cell lines engineered to express mammalian *N*-glycan-processing activities have been shown to sialylate a variety of different recombinant glycoproteins, as detailed earlier. However, one study has challenged the breadth of this capability. Legardinier *et al.* (2005) used the commercial version of SfSWT-1 cells (MIMICTM, Invitrogen) as a host for baculovirus-mediated expression of equine luteinizing hormone/chorionic gonadotropin (eLH/CG) and were unable to detect sialylation of this product using lectin-based assays. This result could indicate that these cells cannot universally sialylate any recombinant *N*-glycoprotein that one might want to produce using a baculovirus expression vector. Further work is clearly needed to examine this possibility and assess the breadth of the recombinant glycoprotein sialylation capabilities of SfSWT-1 and other transgenic lepidopteran insect cell lines. Another possibility raised by the results of Legardinier and coworkers is that SfSWT-1/MIMICTM cells might be genetically unstable under some conditions. With respect to this latter possibility, it is noteworthy that the eLH/CG preparations produced by Legardinier and coworkers were galactosylated, as this finding indicated that their cells had retained at least one of the five transgenes that were originally introduced. To our knowledge, the stability of the transgenes in SfSWT-1/MIMICTM cells has not been rigorously monitored. However, a previous study showed that another transformed Sf9 cell line retained its transgene for at least 55 serial passages in culture (Jarvis *et al.*, 1990). Similarly, we have observed GST-SfManI

sialylation using SfSWT-1 cells that have been routinely subcultured three times a week and subjected to well over 100 serial passages in the laboratory (D. L. Jarvis, unpublished data). Thus, while it remains a distinct possibility, it seems relatively unlikely to us that the results of Legardinier *et al.* reflect genetic instability of the MIMICTM cells used in their study.

IV. CONCLUDING REMARKS

Baculovirus expression vectors are a popular and safe means of producing large quantities of recombinant protein. However, the use of baculovirus vectors for the production of therapeutic glycoproteins is limited by the nature of insect cell glycosylation. Several studies have now shown that insects have the potential to produce complex, sialylated *N*-glycan. However, the *N*-glycans produced by the insect cell lines that serve as hosts for baculovirus vectors frequently consist of simple paucimannose structures. To overcome the deficiencies of insect glycosylation, baculovirus expression vectors and/or their insect-cell hosts have been supplemented with genes encoding enzymes required for complex, sialylated *N*-glycan biosynthesis. These approaches have been used successfully to produce glycoproteins with mammalianized *N*-glycans in the baculovirus–insect cell system. These results, along with efforts to address other problems with insect glycosylation, such as the presence of allergenic core α -1,3-fucose, will facilitate the adaptation of baculovirus expression vectors for the production of glycoproteins for biomedical purposes.

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