Asparagine-Linked Oligosaccharide Processing in Lepidopteran Insect Cells. Temporal Dependence of the Nature of the Oligosaccharides Assembled on Asparagine-289 of Recombinant Human Plasminogen Produced in Baculovirus Vector Infected *Spodoptera frugiperda* (IPLB-SF-21AE) Cells†

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Received January 22, 1991; Revised Manuscript Received March 14, 1991

ABSTRACT: Previous studies from this laboratory have established that lepidopteran insect cells possess the glycosylation machinery needed to assemble N-linked complex-type oligosaccharides on Asn^{289} of recombinant human plasminogen (r-HPg). In the present paper, we show that the nature of the N^{289}-linked glycosylation of [R^{561}E]r-HPg expressed in *Spodoptera frugiperda* (IPLB-SF-21AE) cells is dependent upon the length of time of infection of the cells with the recombinant baculovirus/HPg-cDNA construct. At the earliest postinfection (p.i.) time period studied, i.e., 0–20 h, virtually all (96%) of the oligosaccharides released with glycopeptidase F from N^{289} of the expressed r-HPg were of the high-mannose type and comprised nearly the full range of such structures, containing 3–9 mannose units. At a time window of 60–96 h, p.i., essentially all of the oligosaccharides (92% of the total) assembled on N^{289} of rHPg were of the biantennary, triantennary, and tetraantennary complex classes, with varying extents of outer arm completion. At an intermediate time period window, of 20–60 h, p.i., a mixture of complex-type oligosaccharides, totaling approximately 77% of the glycans, with various levels of branching and outer arm completion, and high-mannose type of oligosaccharides, totaling approximately 23% of the glycans, was assembled on N^{289} of the r-HPg produced. These studies demonstrate that lepidopteran insect cells contain the glycosyltransferase genes required for assembly of N-linked complex oligosaccharide and that these transferases are utilized under proper conditions. The time dependency of the assembly of complex-type oligosaccharides on r-HPg indicates that an activation of the appropriate glycosyl transferases and/or transferase genes can take place. Thus, one consequence of the infective process with the recombinant baculovirus/HPg-cDNA construct is to alter the normal glycosylation characteristics of insect cells and to allow complex-type oligosaccharide processing to occur.

Human plasmin (HPm)[1] is a serine proteolytic enzyme that functions in the dissolution of fibrin blood clots. This enzyme is present in plasma as the zymogen, HPg, and its conversion to HPm occurs consequent to limited proteolytic cleavage of a single peptide bond, $R^{561}V$, in the HPg molecule. The cDNA (Malinowski et al., 1984; Forsgren et al., 1987) and gene (Petersen et al., 1990) for HPg have been cloned and sequenced, and this protein has been expressed in a fully functional form in several lepidopteran insect cell lines (Davidson et al., 1990a; Hink et al., 1990). The completely processed zymogen consists of 791 amino acids (Wiman, 1973, 1977; Wiman & Wallen, 1975; Sottrup-Jensen et al., 1978; Malinowski et al., 1984; Forsgren et al., 1987; Petersen et al., 1990), which are organized into a series of regulatory and catalytically active domains (Sottrup-Jensen et al., 1978).

HPg exists in human plasma as two glycoforms, separable by affinity chromatography on Sepharose–lysine (Brockway & Castellino, 1972). One form of the protein is N-glycosylated at amino acid N^{289} and O-glycosylated at amino acid T^{346}, and the other is glycosylated only at T^{346} (Hayes & Castellino, 1979a–c), despite the presence in this latter form of the required tripeptide consensus sequence for N-linked glycosylation at N^{289} (Powell & Castellino, 1983). While it was previously believed that insect cell derived proteins would only contain high-mannose type oligosaccharide [for a review, see Luckow and Summers (1988)], we found that a major fraction of r-HPg expressed in *Spodoptera frugiperda* (IPLB-SF-21AE) cells contained the same N-linked bisialyl–biantennary complex-type oligosaccharide as was found as the sole N^{289}-linked complex-type oligosaccharide of human plasma HPg (Davidson et al., 1990a). This observation has been verified and extended with r-HPg produced in another insect cell line, IZD-MBO503, from *Mamestra brassicae*, wherein it was also discovered that fucosylation of N-linked complex-type oligosaccharide could also occur in insect cells.[2] During the course of our studies on r-HPg expressed in insect cells, we observed a strong dependence of the times of infection of such cells with the recombinant baculovirus/HPg-cDNA construct on the nature of oligosaccharides assembled on N^{289} of r-HPg. This highly important phenomenon indicates that insect cell glycosylation machinery can be influenced, perhaps specifically, by the infective process. We decided to more fully characterize

†Supported by Grant HL-13423 from the National Institutes of Health, Grant 89/833 from the American Heart Association, and the Kleiderer/Pezold Family endowed professorship (to F.J.C.).
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[1] Abbreviations: HPm, any form or variant of human plasmin; HPg, any form or variant of human plasminogen; r, recombinant; wt, wild type; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; [R^{561}E]r-HPg, a recombinant form of human plasminogen containing a substitution of E for R at amino acid sequence position 561 (this renders the plasminogen resistant to activation to plasmin and is the form of r-HPg used in this investigation); SF, *Spodoptera frugiperda*; SNA, *Sambucus nigra* agglutinin; NDV, Newcastle disease virus; GF, glycopeptidase F; WGA, wheat germ (*Triticum vulgaris*) agglutinin; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; p.i., postinfection; ELISA, enzyme-linked immunoadsorbent assays; HPAEC, high-pH anion-exchange chromatography; EACA, ε-aminocaproic acid.

[2] D. J. Davidson, and F. J. Castellino, manuscript submitted for publication.

0006-2960/91/0430-6167$02.50/0

the types of temporal alterations in glycosylation that occur in insect-expressed r-HPg, due to the practical consequence of providing the opportunity to control the glycosylation of recombinant proteins, and the more basic consequence of investigating this type of control of glycosylation at the level of glycosyltransferase availability. The current paper provides a summary of the important features of this study.

Materials and Methods

Proteins. Human plasminogen was purified from fresh human plasma by affinity chromatography on Sepharose–lysine (Brockway & Castellino, 1972). The first affinity chromatography resolved glycoform was employed herein.

Glycopeptidase F (*Flavobacterium meningosepticum*), biotinylated SNA, and rabbit anti-human HPg were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Goat anti-human plasminogen and peroxidase-labeled WGA were products of the Sigma Chemical Co. (St. Louis, MO). The avidin–alkaline phosphatase complex employed for visualization of biotinylated lectin blots was obtained from Bio-Rad (Richmond, CA).

The clam exoglycosidase mixture employed for total enzymatic digestions of asialyl oligosaccharides was obtained from Dr. Subhash Basu, of this department. It was employed for this purpose under conditions previously detailed (Davidson et al., 1990a).

Exoglycosidases were obtained from the following sources: NAc(β1,2)-D-glucosaminidase (*Diplococcus pneumoniae*), (β1,4)-D-galactosidase (*D. pneumoniae*), and (α2,3/6/8)-D-neuraminidase (*Arthrobacter ureafaciens*), from Boehringer Mannheim Biochemicals; (α1,2/3/6)-D-mannosidase (*Canavalia ensiformis*, jack bean), (α1,6>2/3/4)-L-fucosidase (bovine epididymis), (β1,4)-D-mannosidase (*Turbo cornufus*), and NAc(β1,2/3/4/6)-D-glucosaminidase (*C. ensiformis*, jack bean), from the Sigma Chemical Co.; and (α1,2)-D-mannosidase (*Aspergillus phoenicis*), from Oxford GlycoSystems (Rosedale, NY). The reaction conditions required for these enzymes were as suggested by the manufacturers and by a previous study (Parekh et al., 1989). NDV (α,2,3)-D-neuraminidase was prepared and utilized in our laboratory as previously described (Davidson & Castellino, 1991).

Cells. IPLB-SF-21AE cells were obtained from Dr. Eliot Rosen (Notre Dame, IN).

[$R^{561}E$]r-HPg Expression in Insect Cells. S. frugiperda (IPLB-SF-21AE) cells were maintained as monolayers in serum-free Excell 400 medium and infected with recombinant baculoviruses at multiplicites of 4 plaque-forming units/cell. The infection was allowed to proceed for a time of 20 h, after which the culture medium was collected and replaced with fresh medium. This cell-conditioned medium was collected at 60 h and again replaced with fresh medium, which was collected after 96 h. This procedure allowed examination of glycosylation events in r-HPg that occurred during infection time windows of 0–20, 20–60, and 60–96 h. After the infection times indicated above, the r-HPg was purified from the cell-conditioned media by batch purification on Sepharose–lysine affinity chromatography columns (Deutsch & Mertz, 1970).

DNA Methodology. The method for construction of the baculovirus expression plasmid (pAV6), for insertion of the cDNA for [R^{561}E]r-HPg into pAV6, and for obtaining the recombinant baculovirus for subsequent infection of the insect cells has been described (Whitefleet-Smith et al., 1989). The procedure for altering the cDNA for wt-r-HPg to that which would translate into the activation-resistant variant, [R^{561}E]r-HPg, and for screening the resulting transformants, has been published previously (Davidson et al., 1990b).

Oligosaccharide Methodology. All methodologies employed for oligosaccharide determinations, including deglycosylation of SF-21AE-expressed by HPg with GF, oligosaccharide mapping by HPAEC, isolation and complete enzymatic hydrolysis of oligosaccharides, monosaccharide analyses, sequential exoglycosidase digestions, and lectin visualization of Western blots of r-HPg, have been described previously (Davidson et al., 1990a; Davidson & Castellino, 1991). The oligosaccharide standards used in this investigation were purchased from the Dionex Corp. (Sunnyvale, CA) or Oxford GlycoSystems (Rosedale, NY).

Quantitation of the amount of monosaccharides released from a glycan after exoglycosidase treatment was accomplished by first measuring the amount of glycan hydrolyzed by the area of the HPAEC peak and comparing this to a standard dose–response curve for that particular oligosaccharide, obtained under the same conditions.

ELISA. The r-HPg contents of the various culture media were determined by an ELISA method, the details of which have been described (Whitefleet-Smith, et al., 1989). Here, we employed a polyclonal goat anti-HPg as the first antibody, which was adsorbed to the wells of microtiter plates. This was followed by addition of the cell-conditioned media containing the r-HPg. A second antibody, rabbit anti-HPg, was added to label the HPg bound to the first antibody. Quantitation of the second antibody present was accomplished by addition of an anti-rabbit IgG/alkaline phosphatase complex, followed by the indicator substrate, *p*-nitrophenyl phosphate. Dose–response curves were constructed with various concentrations of purified plasma HPg.

Results

The general strategy employed in this study was to express r-HPg in infected IPLB-SF-21AE cells for various periods of time and to determine the nature of the oligosaccharides assembled on N^{289} of this protein. In an investigation such as this, wherein considerable heterogeneity of the oligosaccharides attached to this position is observed (vide infra), it is important to isolate the protein in high yield in order to ascertain that the population of oligosaccharides ultimately identified is reflective of that present in the entire pool of r-HPg molecules secreted by the cells. This is readily accomplished with r-HPg, by employing affinity chromatography with Sepharose–lysine, where >90% of the protein present in the cell-conditioned media is routinely obtained in a purified form.

After isolation of the r-HPg, the N^{289}-linked oligosaccharides were released by GF treatment, and a glycan fingerprint was obtained by HPAEC. It has been amply demonstrated that this latter methodology can efficiently separate oligosaccharides that differ in size, composition, and specific sugar linkages (Hardy et al., 1988; Townsend et al., 1988; Spellman et al., 1989; Basa & Spellman, 1990; Davidson et al., 1990a; Davidson & Castellino, 1991). Each fraction obtained was then characterized as to its monosaccharide composition and then subjected to sequential degradation with exoglycosidases, several of which were linkage-specific. From all of the above information, we were, in most cases, able to assign unambiguous structures to each oligosaccharide component.

In the first stage of this investigation, IPLB-SF-21AE cells were infected with the pAV6/HPg-cDNA construct for a time period of 20 h, after which time the medium was collected. The cell-conditioned medium was analyzed by ELISA in order to determine the concentration of r-HPg, and the value obtained was 0.75 μg/mL (0.0375 μg/(mL·h). The r-HPg-containing culture medium was passed over a column of Se-

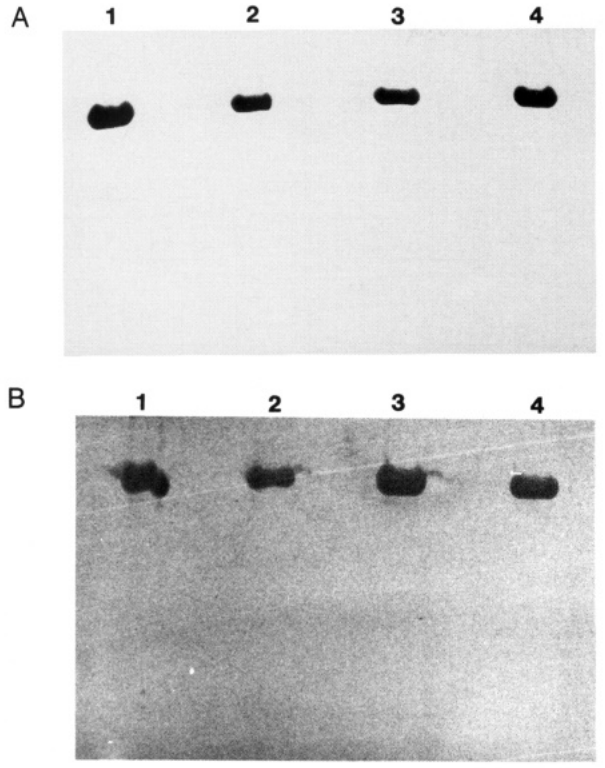

FIGURE 1: Electrophoretic analysis of the recombinant plasminogens employed in this study. (A) Nonreduced $DodSO_4$/PAGE of the purified r-HPg preparations employed. Lane 1, human plasma HPg, affinity chromatography glycoform 1; lane 2, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 0–20 h, p.i.; lane 3, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 20–60 h, p.i.; lane 4, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 60–96 h, p.i. Approximately 5 μg of each protein was applied in each lane. (B) Nonreduced Western immunoblots of the purified r-HPg preparations employed. The proteins from $DodSO_4$/PAGE separations were transferred to an Immobilon P sheet, and ultimate staining depended upon their reactivities with anti-HPg. Lane 1, human plasma HPg, affinity chromatography glycoform 1; lane 2, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 0–20 h, p.i.; lane 3, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 20–60 h, p.i.; lane 4, IPLB-SF-21AE-expressed [R^{561}E]r-HPg, 60–96 h, p.i. Approximately 2 μg of each protein was present in each lane.

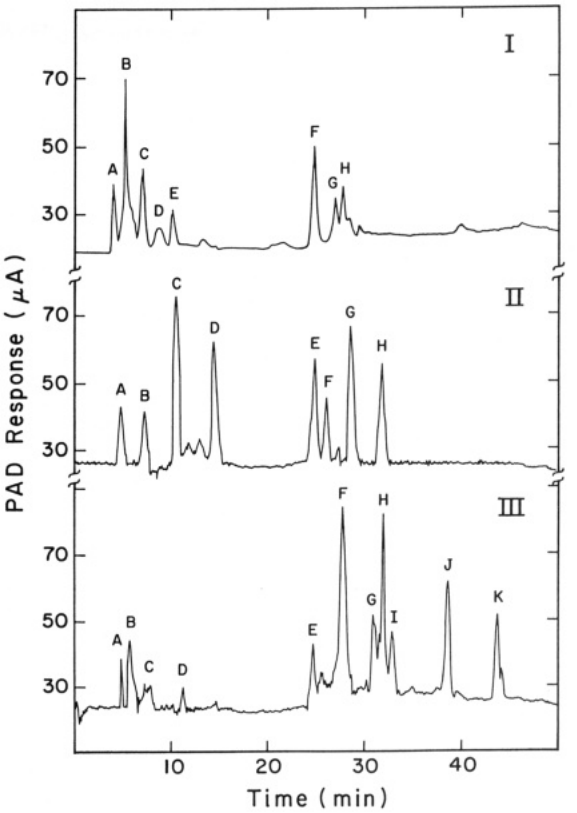

FIGURE 2: High-pH anion-exchange chromatographic (HPAEC) mapping of the oligosaccharides released from IPLB-SF-21AE-expressed [R^{561}E]r-HPg after treatment with gycopeptidase F. (I) 0–20 h, postinfection. (ii) 20–60 h, postinfection. (III) 60–96 h, postinfection. Gradient 1, described in Davidson et al. (1990a), was employed to effect the resolution. The ordinate is expressed as the arbitrary scale response of the pulsed amperometric detector (PAD).

pharose–lysine, weakly bound proteins were then washed from the column with a high-salt buffer (0.3 M sodium phosphate, pH 7.4), and the r-HPg was eluted with a buffer of 0.1 M sodium phosphate/0.3 M EACA, pH 7.4. The r-HPg was obtained in a 95% yield in a high state of purity, as shown by the $DodSO_4$/PAGE gel and Western immunoblot in Figure 1, panels A and B, respectively. This r-HPg sample was treated with GF, until the time at which WGA-dependent staining of Western blots of the r-HPg (Davidson et al., 1990a; Davidson & Castellino, 1991) indicated the absence of the GlcNAc(β1,4)GlcNAc reducing terminal structure. The oligosaccharide pool was then subjected to fingerprint analysis on HPAEC, under the conditions previously described (Davidson et al., 1990a; Davidson & Castellino, 1991), and the elution profile obtained is illustrated in Figure 2-I. From this, and the retention times of a variety of standard oligosaccharides, structures (referred to in Table I) were tentatively assigned to each of the eight components present in Figure 2-I that were present in an overall relative yield of >1% (based upon HPAEC dose–response curves of the tentative structures with the corresponding standard). The complete list of structures obtained for all components of this study is given in Figure 3.

The peak tubes from the chromatogram of Figure 2-I of each of the eight indicated fractions were first treated with

Table I: Monosaccharide Compositions of the Oligosaccharides Released from SF-21AE-Expressed Recombinant Human Plasminogen at 0–20 h Postinfection

fraction (%)[a]	structure[b]	Sia[c]	Gal[d]	GlcNAc[d]	Man[d]
I-A (21)	**1**			2.00	3.08
I-B (1)	**2**			2.00	3.86
I-C (6)	**4**			2.00	5.09
I-D (5)	**5**			2.00	6.13
I-E (11)	**6**			2.00	7.00
I-F (52)	**7**			2.00	8.96
I-G (2)	**10**	1.00	2.00	3.88	3.14
I-H (2)	**11**	2.00	2.06	3.92	3.02

[a] The fraction numbers (relative percent of total oligosaccharide) correspond to those in Figure 2-I. [b] The structure numbers correspond to those of Figure 3. [c] Cleaved from the oligosaccharide with neuraminidase (*A. ureafaciens*). [d] Complete digestion of the desialyl oligosaccharide with a clam liver glycosidase mixture (Davidson et al., 1990a). The value was set at 2.00 for fractions 1.6 and the Man content obtained from the ratio of Man/GlcNAc.

A. ureafaciens neuraminidase, and the amount of Sia liberated was determined by HPAEC. The remaining sample was then subjected to complete enzymatic hydrolysis by a clam exoglycosidase mixture and the monosaccharide composition determined by HPAEC (Davidson et al., 1990a; Davidson & Castellino, 1991). The data obtained are provided in Table I and represent the maximum amounts of each monosaccharide liberated. It is clear that the monosaccharide compositions are in agreement with the proposed structures of Figure 3 and that fractions A–F of Figure 2-I, which represent approximately 96% at the total N-linked oligosaccharide of the 0–20 h, p.i., r-HPg sample, are of the high-mannose class. These structures were verified by sequential exo-

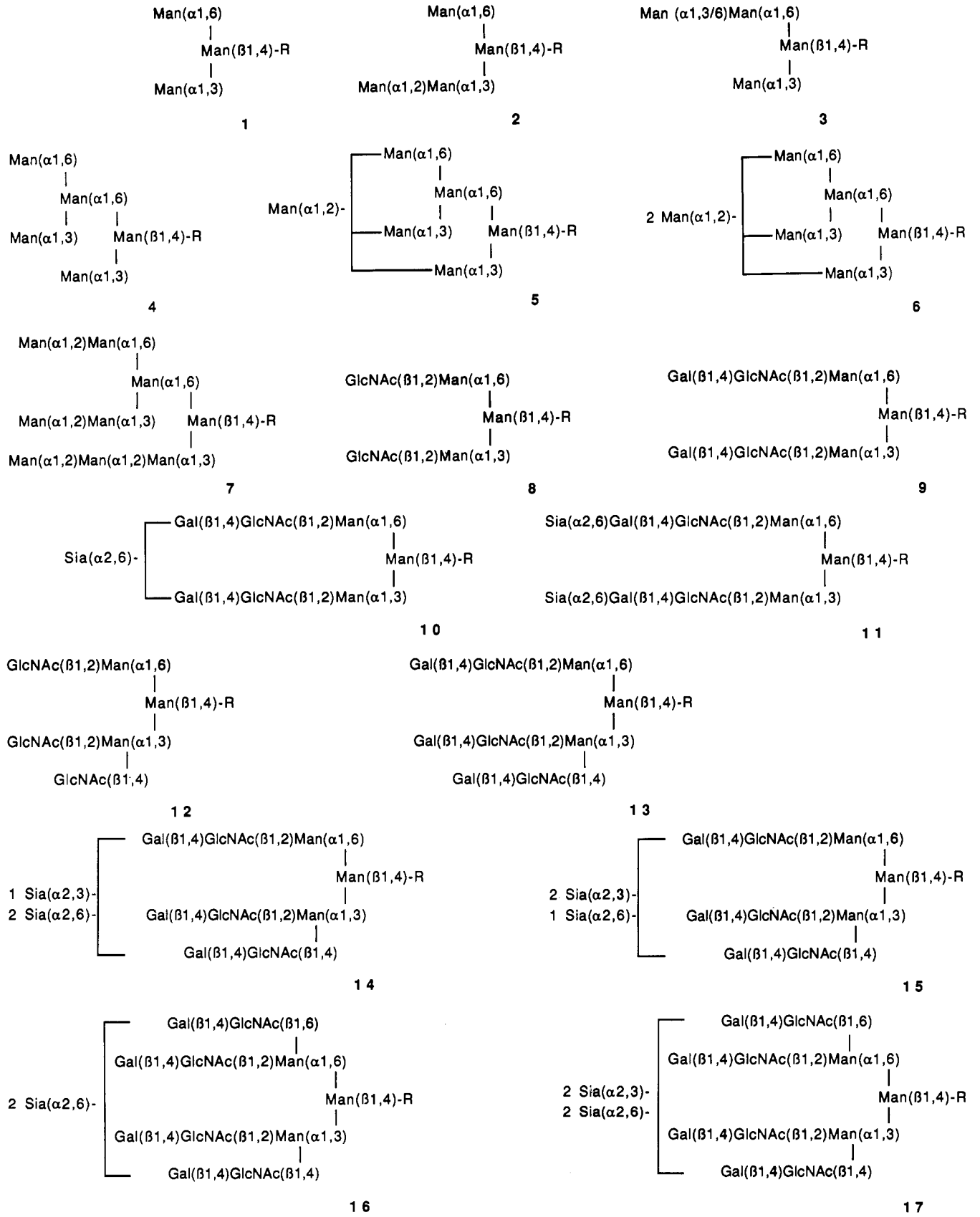

FIGURE 3: Structures of the oligosaccharides released from IPLB-SF-21AE cell expressed [$R^{561}E$]r-HPg at all postinfection times of the insect cells with the recombinant baculovirus (pAV6)/HPg-cDNA construct. R = GlcNAc(β1,4)GlcNAc.

glycosidase analysis, and the results are listed in Table II. The details of our methods of structural analysis follow.

Fraction IA (Figure 2-I and Table I) was unreactive with all of the exoglycosidases used in Table II, except jack bean α-mannosidase, which liberated approximately 2 mol of α-Man/mol of glycan. This sample was not reactive with *D. pneumoniae* (α1,2)-mannosidase, demonstrating that none of this α-Man was present in (α1,2) linkage. The sample that remained after digestion with jack bean α-mannosidase was reactive only with *T. cornufus* (β)-mannosidase, which led to

Table II: Sequential Exoglycosidase Digestion of Oligosaccharides Released from SF-21AE-Expressed Recombinant Human Plasminogen at 0–20 h Postinfection[a]

fraction[b]	Sia (α2,3):R[c]	Gal (β1,4)[d]	GlcNAc (β1,2):R[e]	α-Man (α1,2):R[f]	β-Man[g]	GlcNAc[h]
I-A				0:2.00	1.00	1.96
I-B				1.00:1.94	0.99	2.06
I-C				0:4.02	0.96	2.00
I-D				0.98:3.96	1.10	1.98
I-E				1.88:4.00	0.96	2.00
I-G				3.92:4.06	0.94	1.88
I-H	0:1.08	2.02	1.89:0	0:2.00	0.96	1.92
I-I	0:2.00	1.82	1.76:0	0:1.77	0.80	1.68

[a] For pools containing complex carbohydrate, the sequence of enzymes added was as in footnotes *c–h*. For high-mannose pools, the sequence of addition was as in footnotes *f–h*. [b] The fraction numbers correspond to those in Figure 2-I. [c] (α2,3)-D-Neuraminidase (NDV) followed by (α2,3/6/8)-D-neuraminidase (*A. ureafaciens*). This provides the number of (α2,3)-linked Sia residues:the number (*R*) of α-Sia residues in linkages other than (α2,3). [d] (β-1,4)-Galactosidase (*D. pneumoniae*). [e] NAc(β1,2)-Glucosaminidase (*D. pnemoniae*) followed by NAc(β1,2/3/4/6)-D-glucosaminidase (jack bean). This provides the number of (β1,2)-linked GlcNAc residues:the number (*R*) of β-GlcNAc residues in linkages other than (β1,2). [f] (α1,2)-Mannosidase (*A. phoenicis*) followed by (α1,2/3/6)-D-mannosidase (jack bean). This provides the number of (α1,2)-linked Man residues:the number (*R*) of α-Man residues in linkages other than (α1,2). [g] (β1,4)-Mannosidase (snail). [h] NAc(β1,2/3/4/6)-D-Glucosaminidase (jack bean).

maximal release of approximately 1 mol of β-Man/mol of glycan. Upon treatment with jack bean NAc(β)-glucosaminidase, the remaining oligosaccharide liberated approximately 2 mol of β-linked GlcNAc/mol of glycan. The proposed standard structure was treated in parallel with fraction I-A, and the oligosaccharides from each remaining sample after each step of exoglycosidase treatment were also subjected to HPAEC analysis to verify their identities. Fraction I-A oligosaccharide exhibited chromatographic and enzymatic behavior exactly the same as the proposed standard (structure **1** of Figure 3) and was finally assigned as such.

Fractions I-B–I-F (Figure 2-I and Table I) were treated enzymatically in the same manner as fraction I-A, above, and the results are listed in Table II. Fractions I-D–I-F contained a portion of the α-Man in (α1,2) linkage, as would be expected if these components arose from mannosidase I processing of the $(Man)_9(GlcNAc)_2$ structure originally transferred to the protein. Fraction I-C did not contain Man in (α1,2) linkage, also as expected if it arose from processing of the parent $(Man)_9(GlcNAc)_2$ oligosaccharide from the classical pathway. Fraction I-B, however, contained one of its four Man residues in (α1,2) linkage, demonstrating that it could not have originated from the classical pathway and likely arose from the alternate processing pathway (Yamashita et al., 1983). Each of these components was assigned one the structures contained in Figure 3, as listed in Table I.

The HPAEC elution positions and monosaccharide compositions of fractions I-G and I-H (Figure 2-I and Table I) demonstrated that they were of the complex type. In these cases, *A. ureafaciens* neuraminidase treatment resulted in release of approximately 1 and 2 mol of Sia/mol of glycan, respectively. None of this Sia was (α2,3)-linked, since the original samples were unreactive with NDV neuraminidase. The Sia in each glycan was assigned to an (α2,6) linkage because of the lack of reactivity of these oligosaccharides with NDV neuraminidase, and due to the comigration on HPAEC of this oligosaccharide with standard structures containing (α2,6)-linked Sia. Following this treatment, the remaining samples were successively reactive with *D. pneumoniae* (β1,4)-galactosidase, wherein approximately 2 mol of (β1,4)-linked Gal was released per mole of oligosaccharide, and *D. pneumoniae* NAc(β1,2)-glucosaminidase, leading to release of 2 mol of (β1,2)-linked GlcNAc/mol of glycan. The remaining samples were treated as above for the high-mannose structures, and the data in Table II were obtained. On the basis of these HPAEC analyses, monosaccharide compositional determinations, and sequential exoglycosidase digestions in parallel with proposed standards, fractions I-G and I-H were assigned as monosialylated (structure **10**, Figure 3) and bisialylated (structure **11**, Figure 3) biantennary oligosaccharides, respectively.

Table III: Monosaccharide Compositions of the Oligosaccharides Released from SF-21AE-Expressed Recombinant Human Plasminogen at 20–60 h Postinfection

fraction (%)[a]	structure[b]	Sia[c]	Gal[d]	GlcNAc[d]	Man[d]
II-A (6)	**1**			2.00	3.00
II-B (5)	**4**			2.00	5.04
II-C (23)	**9**		2.00	4.20	3.08
II-D (16)	**13**		3.00	5.10	3.06
II-E (12)	**7**			2.00	9.04
II-F (5)	**10**	1.00	2.07	3.89	2.95
II-G (22)	**11**	2.00	2.08	4.00	3.02
II-H (11)	**14**	3.12	3.00	4.95	3.03

[a] The fraction numbers (relative percent of total oligosaccharide) correspond to those in Figure 2-II. [b] The structure numbers correspond to those of Figure 3. [c] Cleaved from the oligosaccharide with neuraminidase (*A. ureafaciens*). [d] Complete digestion of the desialyl oligosaccharide with a clam liver lycosidase mixture (Davidson et al., 1990a).

The above provides examples of the methods that we employed to obtain structures of oligosaccharides assembled on r-HPg from IPLB-SF-21AE cells. These same analyses were employed for all other r-HPg samples investigated herein, and the data for each will be summarized but not elaborated upon, except in cases where unusual features were present.

The next r-HPg sample examined resulted from its expression in SF-21AE cells in the same manner as above, except that the infection times of the cells with the pAV6/HPg-cDNA construct was a window between 20 and 60 h (media removed from the infected cells at 20 h and replaced with fresh media, which was collected at 60 h). The expression level of this r-Hpg sample over this time period was 1.46 μg/mL of culture medium [0.0365 μg/(mL·h)]. $DodSO_4$/PAGE and Western immunoblot analyses of this purified r-HPg are shown in Figure 1, panels A and B, respectively, which demonstrate its high state of purity. The HPAEC map of the oligosaccharides released from this r-HPg sample is shown in Figure 2-II, and differs considerably from the 0–20-h sample (Figure 2-I), in that higher relative amounts of complex-type oligosaccharide are observed from retention time analysis of this chromatograph, as compared with standard glycans. The monosaccharide compositions of each of the eight selected components that were present in >1% relative yield of this r-HPg are provided in Table III, and sequential exoglycosidase analysis of each component is provided in Table IV. For this

Table IV: Sequential Exogycosidase Digestion of Oligosaccharides Released from SF-21AE-Expressed Recombinant Human Plaminogen at 20–60 h Postinfection[a]

fraction[b]	Sia (α2,3):R[c]	Gal (β1,4)[d]	GlcNAc (β1,2):R[e]	α-Man (α1,2):R[f]	β-Man[g]	GlcNAc[h]
II-A				0:2.04	0.96	1.98
II-B				0:4.00	0.94	1.98
II-C		2.00	1.88:0	0:2.03	1.02	1.96
II-D		3.06	1.89:1.08	0:1.92	0.96	2.04
II-E				4.12:3.98	1.00	1.91
II-F	0:1.00	1.99	1.87:0	0:1.89	0.90	1.91
II-G	0:2.07	2.00	2.06:0	0:2.03	1.03	2.00
II-H	0.94:2.00	1.94	1.87:0.93	0:1.87	0.90	1.95

[a] For pools containing complex carbohydrate, the sequence of enzymes addedwas in footnotes *c–h*. For high-mannose pools, the sequence of addition was as in footnotes *f–h*. [b] The fractions numbers correspond to those in Figure 2-II. [c] (α2,3)-D-Neuraminidase (NDV) followed by (α2,3/6/8)-D-neuraminidase (*A. ureafaciens*). This provides the number of (α2,3)-linked Sia residues:the number (R) of α-Sia residues in linkages other than (α2,3). [d] (β-1,4)-Galactosidase (*D. pneumoniae*). [e] Nac(β1,2)-Glucosaminidase (*D. pneumoniae*) followed by NAc (β1,2/3/4/6)-D-glucosamindase (jack bean). This provides the number of (β1,2)-linked GlcNAc residues:the number (R) of β-GlcNAc residues in linkages other than (β1,2). [f] (α1,2)-Mannosidase (*A. phoenicis*) followed by (α1,2/3/6)-D-mannosidase (jack bean). This provides the number of (α1,2)-linked Man residues:the number (R) of α-Man residues in linkages other than (α1,2). [g] (β1,4)-Mannosidase (snail). [h] NAc(β1,2/3/4/6)-D-Glucosaminidase (jack bean).

sample, the total relative level of complex-type oligosaccharides present was approximately 77%. In addition to the biantennary complex-type glycans seen previously [Figure 2-I and Davidson et al. (1990a)], an amount totaling approximately 27% of triantennary oligosaccharide (asialyl and trisialyl) is present. In each case, the oligosaccharides were confidently assigned to one of the structures of Figure 3. The only unusual feature of the structures resides in the sequential release of (β1,2)-linked GlcNAc in fractions II-D and II-H. In theory, 2 mol of residues/mol of glycan should be released at this stage of the sequential digestion from each oligosaccharide, and this was observed here. This demonstrates that the GlcNAc from the third arm of the oligosaccharide was indeed attached to the core Man in a (β1,4) linkage, and not in a (β1,6) linkage. If this latter GlcNAc(β1,6)Man linkage was present, the residue of (β1,2)-linked GlcNAc attached to this same Man would not be released by the *D. pneumoniae* NAc(β1,2)-glucosaminidase (Yamashita et al., 1981). Since the full complement of (β1,2)-linked GlcNAc was indeed observed, the GlcNAc present in the third arms in fractions II-D and II-H must be attached in (β1,4) linkages to the core Man. To our knowledge, the only GlcNAc(β1,4)Man linkage found in triantennary complex oligosaccharides of this type is

GlcNAc(β1,4)**Man(α1,3)**Man(β1,4)GlcNAc(β1,4)-GlcNAc

and not the alternative:

GlcNAc(β1,4)**Man(α1,6)**Man(β1,4)GlcNAc(β1,4)-GlcNAc

However, this latter structure has been found in triantennary *GlcNAc-bisecting* glycans (Yamashita et al., 1983). In addition, as further proof of the proposed linkage, both fractions II-D and II-H comigrated on alkaline HPAEC with standards which contained the GlcNAc(β1,4)Man(α1,3) linkage. With the precedents already established for linkage-specific retention times with this methodology (Hardy et al., 1988; Townsend et al., 1988; Spellman et al., 1989; Basa & Spellman, 1990; Davidson & Castellino, 1990; Davidson et al., 1990a), we would expect that a triantennary oligosaccharide with a GlcNAc(β1,4)Man(α1,6) linkage would not possess the same retention time as the same oligosaccharide containing a GlcNAc(β1,4)Man(α1,3) linkage. Thus, we conclude that, for fractions II-D and II-H of Figure 2-II and Table III, the (β1,4)-linked GlcNAc of the third arm of the glycan is attached to the Man(α1,3) branch of the core structure, as indicated in structures **13** and **14**, respectively, of Figure 3.

Table V: Monosaccharide Compositions of Oligosaccharides Released from SF-21AE-Expressed Recombinant Human Plasminogen at 60–96 h Postinfection

fraction (%)[a]	structure[b]	Sia[c]	Gal[d]	GlcNAc[d]	Man[d]
III-A (1)	**1**			2.00	2.98
III-B (2)	**3**			2.00	4.02
III-C (2)	**4**			2.00	4.96
III-D (3)	**9**		2.00	3.96	2.98
III-E (3)	**7**			2.00	9.02
III-F (32)	**11**	2.00	2.06	4.10	2.92
III-G (8)	**14**	3.09	3.00	4.98	3.03
III-H (16)	**16**	2.00	3.98	6.02	2.94
III-I (6)	**6**	2.00	4.08	5.94	2.89
III-J (16)	**15**	3.00	2.98	4.87	3.06
III-K (11)	**17**	4.04	4.00	5.91	3.00

[a] The fraction numbers (relative percent of total oliosaccharide) corresponds to those in Figure 2-III. [b] The structure numbers correspond to those of Figure 3. [c] Cleaved from the oligosaccharide with neuraminidase (*A. ureafaciens*). [d] Complete digestion of the desialyl oligosaccharide with a clam liver glycosidase mixture (Davidson et al., 1990a).

The final r-HPg analyzed resulted from expression between a p.i. time window of 60 and 96. The expression level of this sample was 0.63 μg/mL [0.0175 μg/(mL·h)]. This was a slightly lower level of expression than for r-HPg produced at the earlier p.i. times and may be the result of loss of cell viability, although morphologic evidence for a significant amount of cell death was not apparent. The $DodSO_4$/PAGE and Western immunoblot analyses (Figures 1, panels A and B, respectively) of the resulting r-HPg demonstrated that it was obtained in a highly purified state. The HPAEC fingerprint of the oligosaccharides released from this r-HPg after GF treatment is illustrated in Figure 2-III. From this, it is clear that the majority of glycans present are of the complex type, a finding that is verified by the monosaccharide compositions of Table V and the sequential exoglycosidase analysis of each component, summarized in Table VI. Here, the total relative amount of complex-type oligosaccharide is approximately 92%. Structural assignments for each of the 11 components of Figure 2-III and Table V were relatively uncomplicated, with the only uncertainty surrounding exact assignments of the bisialyl–tetraantennary oligosaccharides represented by fractions III-H and III-I. These samples exhibited identical behavior in the sequential exoglycosidase analyses of each and the desialylated samples comigrated with the proper standard, asialyl–tetraantennary oligosaccharide. It is concluded that fractions III-H and III-I differ only in the

Table VI: Sequential Exoglycosidase Digestion of Oligosaccharides Released from SF-21A-Expressed Recombinant Human Plaminogen between 60 and 96 h Postinfection[a]

fraction[b]	Sia (α2,3):R[c]	Gal (β1,4)[d]	GlcNAc (β1,2):R[e]	α-Man (α1,2):R[f]	β-Man[g]	GlcNAc[h]
III-A				0:1.96	1.02	1.94
III-B				0:2.94	1.04	2.03
III-C				0:3.88	0.93	1.95
III-D		2.00	1.96:0	0:1.88	1.02	1.87
III-E				3.86:4.06	1.00	1.94
III-F	0:2.01	1.93	2.06:0	0:2.12	0.98	1.94
III-G	1.08:2.01	2.96	1.91:1.08	0:2.06	1.02	2.00
III-H	0:2.06	3.97	1.00:2.90	0:1.94	1.00	1.95
III-I	0:2.10	4.01	0.99:3.02	0:1.93	0.92	1.98
III-J	2.00:0.94	2.86	2.05:0.94	0:1.93	0.97	1.91
III-K	1.90:2.08	4.00	0.94:3.09	0:1.90	0.90	2.03

[a] For pools containing complex carbohydrate, the sequence of enzymes added was as in footnotes *c–h*. For high-mannose pools, the sequence of addition was as in footnotes *f–h*. [b] The fraction numbers correspond to those in Figure 2-III. [c] (α2,3)-D-Neuraminidase (NDV) followed by (α2,3/6/8)-D-neuraminidase (*A. ureafaciens*). This provides the number of (α2,3)-linked Sia residues:the number (R) of α-Sia residues in linkages other than (α2,3). [d] (β-1,4)-Galactosidase (*D. pneumoniae*). [e] NAc(β1,2)-Glucosaminidase (*D. pneumoniase*) followed by NAc(β1,2/3/4/6)-D-glucosaminidase (jack bean). This provides the number of (β1,2)-linked GlcNAc residues:the number (R) of β-GlcNAc residues in linkages other than (β1,2). [f] (α1,2)-Mannosidase (*A. phoenicis*) followed by (α1,2/3/6)-D-mannosidase (jack bean). This provides the number of (α1,2)-linked Man residues:the number (R) of α-Man residues in linkages other than (α1,2). [g] (β1,4)-Mannosidase (snail). [h] NAc(β1,2/3/4/6)-D-Glucosaminidase (jack bean).

arms of the glycan to which Sia is attached, and both are assigned to structure **16** of Figure 3. Appropriately, the comments above, regarding sequential release of (β1,2)-linked GlcNAc by *D. pneumoniae* NAc(β1,2)-glucosaminidase, also apply to the structural assignments to glycans **14** and **15** (Figure 3) of fractions III-G and III-J from Figure 2-III and Table V. In this same regard, this enzyme also catalyzed sequential release of only one of the two (β1,2)-linked GlcNAc residues from fractions III-H, III-I, and III-K. This is consistent with the presence of the GlcNAc(β1,6)Man(α1,6) linkage in these samples, as shown in their assigned structures (**16**, **16**, and **17**, respectively, of Figure 3) and discussed in the preceding section.

Discussion

It has been commonly accepted that insect cells are not capable of assembling N-linked complex oligosaccharide on endogenous proteins (Butters et al., 1981; Ryan et al., 1985), on several recombinant proteins (Kuroda et al., 1986; Wojchowski et al., 1987; Luckow & Summers, 1988; Jarvis & Summers, 1989), and on proteins contained on aglycosyl viruses employed to infect such cells (Hsieh & Robbins, 1984). However, after infection of IPLB-SF-21AE cells with a recombinant baculovirus/HPg-cDNA construct, we observed that a significant fraction (approximately 40%) of the r-HPg contained N^{289}-linked sialylated complex-type oligosaccharide, suggesting that genes for appropriate trimming glucosidases and mannosidases, as well as genes for NAc-glucosaminyltransferases(s), galactosyltransferase(s) and sialyltransferase(s), were present in these insect cells and were capable of activation under appropriate, and as yet, not fully defined, conditions (Davidson et al., 1990a). This discovery was extended to r-HPg produced in *M. brassicae* IZD-MBO503 cells,[2] wherein >60% of the N^{289}-linked glycans of the expressed r-HPg were of the complex–bisialyl–biantennary class. A fraction of this material contained Fuc, thereby suggesting that a fucosyltransferase gene was also present and capable of being activated and utilized. In this same report, more qualitative studies demonstrated that r-HPg contained in *S. frugiperda* SF-9 cells and *Manduca sexta* CM-1 cells also contained Sia. Thus, we believe that the trimming and processing enzymes needed for N-linked complex-type oligosaccharide formation are able to be activated and utilized in, at the least, lepidopteran insect cells.

During the course of our investigations, we observed that the exact oligosaccharide structures assembled on r-HPg exhibited a dependence on the time of infection of the insect cells with the recombinant baculovirus/HPg-cDNA construct. This important effect has been more clearly defined and elaborated upon in this paper.

At the earliest postinfection times (0–20 h) the majority (96%) of the oligosaccharides assembled on Asn^{289} of r-HPg were of the high-mannose class and represented virtually the full range of these structures, from $(Man)_3(GlcNAc)_2$ to $(Man)_9(GlcNAc)_2$, with the exception of $(Man)_8GlcNAc_2$, which was not observed in a significant amount. Most of these structures appear to result from mannosidase-catalyzed degradation of the parent $(Man)_9(GlcNAc)_2$ that is assembled on the protein from the classical pathway. However, since the $(Man)_4(GlcNAc)_2$ glycan contains an (α1,2)-linked Man residue, this oligosaccharide likely originates from the alternate glycosylation pathway (Yamashita et al., 1983), thus showing its functional presence in lepidopteran insect cells. Only a small amount (4%, total) of complex-type biantennary oligosaccharide [containing 1 and 2 mol of (α2,6)-linked Sia/mol of oligosaccharide] is present on r-HPg at this postinfective time period. Thus, these results are in reasonable accord with previous literature [reviewed in Luckow and Summers (1988)], which suggested that only high-mannose oligosaccharide is assembled on proteins derived from insect cells.

This view must be altered, however, when the glycosylation of r-HPg obtained at later postinfective times is considered. In the r-HPg expressed between 20 and 60 h, p.i., approximately 77% of the oligosaccharides assembled on N^{289} are of the complex type, the dominant forms being asialyl–, monosialyl–, and bisialyl–biantennary carbohydrate. Considerable levels of asialyl– and trisialyl–triantennary oligosaccharides are also present, demonstrating that insect cells are capable of a high degree of oligosaccharide processing. This is in agreement with previous studies from this laboratory, which showed that N^{289} of r-HPg, expressed for 40 h in these same IPLB-SF-21AE cells (Davidson et al., 1990a) and for 48 h in *M. brassicae* IZD-MBO503 cells,[2] contained a large relative amount of biantennary complex-type oligosaccharide, the structure of which was identical with that assembled on N^{289} of human plasma HPg (Hayes & Castellino, 1979a–c).

Even more dramatic results are found when the oligosaccharide structures assembled on N^{289} of r-HPg, expressed

between 60 and 96 h, are examined. Here, 92% of the total oligosaccharide was of the complex type, with biantennary, triantennary, and tetraantennary glycans present, containing various amounts of (α2,3)- and (α2,6)-linked outer arm Sia residues. Thus, it is clear that insect cells contain most of the glycosyltransferase genes found in mammalian cells and that these enzymes are not normally functionally present in insect cells. Whether utilization of these enzymes depends upon control exerted at the gene level or at the protein level is not known, but it is clear that these enzymes can become functional under certain conditions. Such conditions were realized in this investigation by the infection of the insect cells with a baculovirus/HPg-cDNA construct and were greatly dependent upon the time of exposure of these cells to the recombinant virus. This time-dependent process could be suggestive of a relatively slow activation at the gene or protein level but certainly demonstrates that mechanisms are available for alteration of the nature of the oligosaccharides present on recombinant proteins in insect cells. Also, the slower rate of translation at this later time window could also be a contributing factor to the type of glycosylation observed. Further, cell culture environmental conditions could additionally influence the nature of the glycosylation of proteins, as has been found in previous work [reviewed in Goochee and Monica (1990)]. Thus, it may be possible to control the type of glycosylation assembled on recombinant proteins expressed in insect cells by conditions that can be easily manipulated.

The significant aspects of this study lie in the observations that complex processing of glycans can occur in lepidopteran insect cells, resulting in assembly of glycans equivalent to those produced in mammalian cells. We have yet to find hybrid-type oligosaccharides or GlcNAc-bisecting glycans in insect-derived r-HPg, but such reactions may be discovered in future investigations under different culture conditions, and/or with other recombinant proteins and other insect cell lines. Appropriately, fucose attachment can occur, as we have found in studies with glycosylation of r-HPg in *M. brassicae* IZD-MBO503 cells.[2] We also demonstrate that the complex-type processing glycosyltransferases are not immediately available and that mechanisms exist which result in their activation. Thus, future work on this type of control of glycosylation should allow understanding of manners for manipulating the nature of glycosylation of proteins in cultured cells. Such knowledge would allow approaches toward cellular attachment of physiologically relevant oligosaccharides on recombinant proteins and thus avoid possible problems associated with their functional, immunological, and clearance differences with natural proteins, possibly related to generation of a nonphysiological group of protein glycoforms.

Registry No. r-HPg, 9001-91-6; Asn, 70-47-3; glycosyltransferase, 9033-07-2.

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