Host-dependent Variation of Asparagine-linked Oligosaccharides at Individual Glycosylation Sites of Sindbis Virus Glycoproteins*

Peggy Hsieh, Marsha Rich Rosner§, and Phillips W. Robbins

From the Department of Biology and Center for Cancer Research and the §Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

We examined the Asn-linked oligosaccharides at individual glycosylation sites of the two envelope glycoproteins of Sindbis virus, E1 and E2. The analysis was done by separating tryptic glycopeptides by reverse phase high performance liquid chromatography and analyzing the oligosaccharides from isolated glycopeptides by gel filtration chromatography. Both E1 and E2 have two glycosylation sites each in virus grown in chick embryo fibroblasts, baby hamster kidney cells, and Chinese hamster ovary cells. Our results indicate that previously observed host-dependent variation in the oligosaccharides of Sindbis virus glycoproteins are probably attributable to differences in oligosaccharide composition at individual sites of E1 and E2 and not to differences in the frequency of glycosylation of these sites.

One of the two glycosylation sites in E1 has exclusively complex type oligosaccharides regardless of the host cell type. However, the second glycosylation site in E1 has high mannose oligosaccharides in virus grown in chick embryo fibroblasts, complex oligosaccharides in virus from baby hamster kidney cells, and both complex and high mannose oligosaccharides in virus from Chinese hamster ovary cells. E2 has one glycosylation site which has primarily high mannose oligosaccharides and one site which has primarily complex type oligosaccharides. Certain sites which have predominantly complex type glycans also have a small number of two specific classes of oligosaccharides which are cleaved by endo-β-N-acetylglucosaminidase H and may be high mannose type. Our findings are discussed with regard to regulation of oligosaccharide processing in animal cells.

Recent studies from a number of laboratories have yielded considerable information about the biosynthesis of asparagine-linked oligosaccharides in animal cells (reviewed in Ref. 1). All Asn-linked oligosaccharides share a common "core" region, ManαGlcNAcβ. However, high mannose type oligosaccharides contain additional α-linked mannose residues while complex type oligosaccharides contain other external sugars, GlcNAc, Gal, sialic acid, and fucose (reviewed in Ref. 2). Both complex and high mannose type oligosaccharides are synthesized via a common precursor oligosaccharide, GlcαManα-ManαGlcNAcβ, which is transferred in the endoplasmic reticulum from a lipid carrier to newly synthesized polypeptides (3, 4). Oligosaccharide processing begins in the endoplasmic reticulum with the removal of the three terminal glucose residues (5) and possibly a single mannose residue (6). The majority of oligosaccharides are further processed by removal of up to four α-linked mannose residues (7, 8) following transport of glycoproteins from the endoplasmic reticulum to the Golgi apparatus. Further modifications in the Golgi apparatus, including the addition of a GlcNAc residue to ManαGlcNAcβ, the removal of two additional mannose residues, and the addition of external sugars, result in the formation of complex type oligosaccharides (reviewed in Refs. 1 and 2).

While the general pathway of oligosaccharide processing is relatively well understood, considerably less is known about its regulation. One approach to the problem is to compare Asn-linked oligosaccharides from glycoproteins of the same virus grown in different host cells. Host-dependent variation in oligosaccharide composition has been observed for G protein of vesicular stomatitis virus (9), hemagglutinin of influenza virus (10, 11), and E1 and E2 glycoproteins of Sindbis virus (12-15).

Sindbis virus is a particularly useful system for studying glycosylation in animal cells. The virus utilizes host glycosylation machinery (16) and contains both high mannose and complex type oligosaccharides (14). The mRNA sequences of E1 and E2 have been determined and indicate that there are two potential glycosylation sites in E1 and two in E2 (17). We have examined the Asn-linked oligosaccharides of Sindbis virus grown in CEF, BHK, and Chinese CHO cells. Reverse phase HPLC was used to separate tryptic glycopeptides each containing a single glycosylation site from E1 or E2. Gel filtration chromatography was then used to analyze the oligosaccharides from each site. Our findings reveal that host-dependent variation of Sindbis virus oligosaccharides is due to differences in oligosaccharide composition at individual sites and not to differences in the relative frequencies of glycosylation. In addition, our results suggest that particular steps in the oligosaccharide processing pathway may be rate-limiting and are consistent with the hypothesis that accessibility of oligosaccharides may regulate the extent of processing which occurs in vivo (see accompanying paper (31)).

**EXPERIMENTAL PROCEDURES**

Cell Preparation and Viral Infection—Primary chick embryo fibroblasts were grown as monolayers in glass roller bottles at 37 °C in Eagle's minimal essential medium (Gibco) supplemented with 2% fetal bovine serum.

†To whom correspondence should be addressed at Center for Cancer Research E17-235, Massachusetts Institute of Technology, Cambridge, MA 02139.

*This work was supported by Grants CA14142 and CA14051 from the National Cancer Institute, Department of Health, Education, and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed at Center for Cancer Research E17-235, Massachusetts Institute of Technology, Cambridge, MA 02139.
Oligosaccharide Variation of Sindbis Virus Glycoproteins

tryptophase phosphate broth, 1% calf serum, and 1% heat-inactivated chicken serum (Gibco). BHK-21 cells, grown in monolayers in glass roller bottles at 37°C in Eagle's minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco). CHO-K1 cells were obtained from Dr. R. C. Burke and grown as monolayers in plastic roller bottles at 37°C in a minimal essential medium (KC Biological) containing 5% fetal calf serum. BHK-21 cells were infected on secondary CEF cells. The oligosaccharides of virus grown in primary CEF cells, analyzed by the methods described below, were identical with those of the original virus stock (data not shown). Thus, the selection process on CHO cells did not alter oligosaccharase processing in the CHO-derived viral stock.

Confluent monolayers were infected with 0.1 plaque-forming unit/cell in 10 ml of medium containing 5% fetal calf serum/roller bottle. Virus was allowed to adsorb to cells for 1 h at 37°C. Then 9 ml of medium containing 5% fetal calf serum and 1 ml of 0.2% [3H]mannose (20-30 Ci/mmol, New England Nuclear) was added. The cells were incubated at 37°C.

The medium was collected and filtered through a 0.45-μm Millex filter (Millipore). All subsequent steps were performed at 4°C. The filtrate was layered onto a step gradient of sucrose in 10 ml of 50% w/v sucrose in HEN buffer and 10 ml of 10% w/v sucrose in HEN buffer. The gradient consisted of 10 ml of 25% w/v sucrose in HEN buffer and 10 ml of 10% w/v sucrose in HEN buffer. Gradients were centrifuged at 120,000 × g for 75 min in an SW 27 rotor. Virus recovered from the interface between the 10% and 50% sucrose layers was precipitated by 10-fold dilution in cold water, pelleted by centrifugation at 120,000 × g for 30 min, and resuspended in a small volume of HEN buffer. Aliquots of radiolabeled virus were stored at −70°C.

Separation of E1 and E2 Glycoproteins—E1 and E2 from [3H]mannose-labeled virions were separated according to the method of Burke and Keegstra (13). Intact virions which had been precipitated in cold water and pelleted were resuspended in 0.5% Triton X-100 in 0.01 M potassium phosphate, pH 6.0. The suspension was centrifuged at 145,000 × g for 60 min at 4°C. The supernatant containing E1 was removed and stored at −70°C. The pellet was extracted once more with 0.5% Triton in 0.01 M potassium phosphate and centrifuged. The final pellet containing E2 was resuspended in 0.5% Triton, 0.2 M Tris-Cl, pH 8.0, and stored at −70°C. Sodium dodecyl sulfate-polycarbamid gel electrophoresis revealed that preparations of E1 contained a single labeled polypeptide of M r = 50,000 while preparations of E2 were composed primarily of a radioabeled polypeptide of M r = 45,000 and a small amount (less than 10%) of contaminating E1 glycoprotein.

Sodium dodecyl sulfate-polycarbamid gel Electrophoresis—Sodium dodecyl sulfate-gel electrophoresis was carried out according to the method of Laemmli (18). To resolve E1 and E2, 7.5% gels were used. Trypsin Digestion and Reverse Phase HPLC—[3H]Mannose-labeled Sindbis virions were denatured in the presence of 1-3 μg of bovine serum albumin and 100 μg of unlabeled Sindbis virions per ml. The suspension was adjusted to 0.1 M Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 20 mM dithiothreitol, and 1% β-mercaptoethanol (Bio-Rad) and heated at 100°C for 5 min under a nitrogen atmosphere. The mixtures were incubated at 37°C for 1-2 h and then precipitated overnight at −20°C by the addition of 2 volumes of acetone. Samples were pelleted, dried in vacuo and resuspended in 180 μl of 50 mM ammonium carbonate, pH 8.0. They were digested with two aliquots of 30 μg each of trypsin (Worthington, type THTPCK) unless otherwise noted. After addition of each aliquot of enzyme, the samples were incubated at 37°C for 2 h. Prior to HPLC, lyophilized trypic peptides were dissolved in 100 μl of 0.1 M sodium phosphate, pH 2.2, and centrifuged to remove particulate matter.

Reverse phase HPLC was carried out as described previously (19). A C-18 Ultrasphere-ODS (4.6 × 25 cm) reverse phase HPLC column (Beckman) was used. Separation of glycopeptides was carried out at room temperature at a flow rate of 1.0 ml/min. 100% solvent A (0.1 M sodium phosphate, pH 2.2) was run for 10 min followed by a linear gradient of 0 to 50% solvent B (acetonitrile (Baker)) in solvent A. Fractions of 0.5 ml were collected. Radioactivity in the fractions was measured by liquid scintillation counting. Recoveries of glycopeptides were typically 70-75%.

To examine oligosaccharides at a single glycosylation site, fractions from a single HPLC peak were pooled and adjusted to neutral pH with 5.0 M NaOH. Samples were heated at 37°C under air for 15-20 min and then lyophilized with 7 μg of bovine serum albumin added as carrier. The lyophilized samples were reconstituted in 100 μl of water and desalted on columns (2 × 0.5 cm) of Sephadex G-10 prior to pronase and endo H digestion.

Oligosaccharides were incubated with pronase (Calbiochem) as previously described (20). Endo H was prepared according to the method of Tarentino et al. (21), and the final Sephadex G-75 fraction (30 μg of protein/ml) was used for incubation as described previously (20). Digestion with jack bean α-mannosidase (Sigma, Type III) was as described elsewhere (22). Digestion with endo D purchased from Seikagaku Fine Biochemicals (Tokyo) was as described previously (15).

RESULTS

Reverse Phase HPLC Separation of Tryptic Glycopeptides—We used reverse phase HPLC to resolve tryptic glycopeptides, each of which contain a single glycosylation site from E1 or E2. The sequences of mRNA coding regions for E1 and E2 glycopeptides (17) identified a total of four potential glycosylation sites. However, when tryptic peptides of E1 and E2 glycopeptides from virions grown in CEF cells and labeled in vivo with [3H]mannose were analyzed by HPLC, five different peaks were resolved (Fig. 1, A and B). These same five peaks, numbered I through V in order of elution, were present when [3H]mannose-labeled tryptic peptides from whole Sindbis virus virions were resolved by HPLC (Fig. 1C). HPLC peaks I, II, and IV originate from E2 while peaks III and V originate from E1. The appearance of a small amount of peak III material in Fig. 1B results from contaminating E1 glycoprotein in preparations of E2.

Tryptsin time course experiments carried out with virus grown in CEF cells strongly suggested that the five HPLC peaks represented only four glycosylation sites in Sindbis virus, two in E1 and two in E2. [3H]Mannose-labeled glycopeptides of CEF-derived virus were treated for increasing lengths of time with increasing amounts of trypsin and analyzed by reverse phase HPLC (Table I). Under all conditions of trypsin digestion tested, only five glycopeptides were resolved, each of which eluted at a characteristic concentration of acetonitrile. With the exception of the 2-h digest, HPLC peaks II, III, and V showed no change in relative size with increased trypsin digestion. In contrast, peak I increased in size and peak IV decreased in size as the concentration of trypsin and length of incubation increased. However, the sum of peaks I and IV remained constant. To show that the glycopeptides of peaks I and IV were related, HPLC fractions corresponding to peak IV were pooled and treated with an additional 60 μg of trypsin for 4 h. When this material was rechromatographed by HPLC, greater than 90% of the [3H] counts/min migrated as a single peak at a position corresponding to that of peak I (data not shown). This demonstrated that
tured and treated with a total of 60 pg of trypsin for tryptic glycopeptides from ["H]mannose-labeled virions grown in CEF-grown virus; BHK cells; E, tryptic glycopeptides from ["H]mannose-labeled virions grown in CHO cells. Oligosaccharides obtained after pronase and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF cells were also separated by reverse phase HPLC (Fig. 1, A and B). Comparison of these results with those obtained from CEF-derived virus (Fig. 1C) revealed that the same five HPLC peaks were resolved regardless of the viral host. This result would be expected if the same glycosylation sites in E1 and E2 were recognized by oligosaccharide transferases of CEF, BHK, and CHO cells since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

Oligosaccharide Composition at Individual Glycosylation Sites—To examine host-dependent variation of oligosaccharides, the composition of oligosaccharides at each of the four glycosylation sites was analyzed. Tryptic peptides from E1 and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF, BHK, or CHO cells were separated by reverse phase HPLC, and fractions corresponding to a single HPLC peak were pooled and subjected to pronase digestion. The resulting glycopeptides were incubated with endo H, an enzyme which cleaves between the proximal GlcNAc residues of high mannose or complex type included sizing on Bio-Gel P-4 columns, since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

Oligosaccharide Composition at Individual Glycosylation Sites—To examine host-dependent variation of oligosaccharides, the composition of oligosaccharides at each of the four glycosylation sites was analyzed. Tryptic peptides from E1 and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF, BHK, or CHO cells were separated by reverse phase HPLC, and fractions corresponding to a single HPLC peak were pooled and subjected to pronase digestion. The resulting glycopeptides were incubated with endo H, an enzyme which cleaves between the proximal GlcNAc residues of high mannose or complex type included sizing on Bio-Gel P-4 columns, since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

Oligosaccharide Composition at Individual Glycosylation Sites—To examine host-dependent variation of oligosaccharides, the composition of oligosaccharides at each of the four glycosylation sites was analyzed. Tryptic peptides from E1 and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF, BHK, or CHO cells were separated by reverse phase HPLC, and fractions corresponding to a single HPLC peak were pooled and subjected to pronase digestion. The resulting glycopeptides were incubated with endo H, an enzyme which cleaves between the proximal GlcNAc residues of high mannose or complex type included sizing on Bio-Gel P-4 columns, since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

Oligosaccharide Composition at Individual Glycosylation Sites—To examine host-dependent variation of oligosaccharides, the composition of oligosaccharides at each of the four glycosylation sites was analyzed. Tryptic peptides from E1 and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF, BHK, or CHO cells were separated by reverse phase HPLC, and fractions corresponding to a single HPLC peak were pooled and subjected to pronase digestion. The resulting glycopeptides were incubated with endo H, an enzyme which cleaves between the proximal GlcNAc residues of high mannose or complex type included sizing on Bio-Gel P-4 columns, since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

Oligosaccharide Composition at Individual Glycosylation Sites—To examine host-dependent variation of oligosaccharides, the composition of oligosaccharides at each of the four glycosylation sites was analyzed. Tryptic peptides from E1 and E2 glycoproteins of ["H]mannose-labeled virus grown in CEF, BHK, or CHO cells were separated by reverse phase HPLC, and fractions corresponding to a single HPLC peak were pooled and subjected to pronase digestion. The resulting glycopeptides were incubated with endo H, an enzyme which cleaves between the proximal GlcNAc residues of high mannose or complex type included sizing on Bio-Gel P-4 columns, since our earlier studies established that HPLC separation is based largely on amino acid composition (19). Of special interest was the observation that relative peak sizes differed in virus from different hosts. In particular, the relative size of peak III was larger in CEF- and CHO-derived virus than in BHK-derived virus (Fig. 1, C-E). Since the number of mannose residues per oligosaccharide chain varies, the observed differences in peak sizes could reflect either (a) less frequent glycosylation of sites in some host cells as opposed to others or (b) host-dependent variation of carbohydrate composition at individual sites.

**Table I**

| Time course of trypsin digestion of Sindbis virus glycopeptides |
|---------------------|---------------------|
| ["H]mannose-labeled Sindbis virus glycopeptides were denatured and digested with trypsin as described under "Experimental Procedures." One to four 30-μg aliquots of trypsin, 1 mg/ml, were added to four identical samples of virus. Following addition of each aliquot of fresh enzyme, the samples were incubated at 37 °C for 2 h. Samples were then boiled for 5 min and analyzed by reverse phase HPLC as described under "Experimental Procedures." The total amount of trypsin added to each sample was as follows: after 2 h, 30 μg; after 4 h, 60 μg; after 6 h, 90 μg; after 8 h, 120 μg. HPLC recoveries for all four samples were between 71-75%. Peaks I and II were separated by one fraction containing less than 10% of the total counts/min in I and II. Peaks IV and V were separated by one fraction which constituted at most 25% of the total radioactivity in IV and V. Such fractions were divided in half and assigned equally to peaks IV and V. | **HPLC peak** | 2 h | 4 h | 6 h | 8 h |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| I                   | 32                  | 37                  | 40                  | 42                  |
| IV                  | 16                  | 11                  | 4                   | 5                   |
| I+IV                | 48                  | 48                  | 44                  | 47                  |
| II                  | 10                  | 14                  | 17                  | 16                  |
| III                 | 27                  | 20                  | 20                  | 19                  |
| V                   | 15                  | 18                  | 19                  | 19                  |
The identification of these rare glycopeptides was carried out by endo D digestion of putative high mannose oligosaccharides from CHO-derived virus as described previously by Hunt (15). The results of α-mannosidase and endo H digestion demonstrate that the overwhelming majority of oligosaccharides which co-migrate with high mannose standards on gel filtration are authentic high mannose oligosaccharides.

The majority of oligosaccharides at one of the glycosylation sites in E2, HPLC peaks I+IV, are high mannose oligosaccharides in virus from all three hosts (Fig. 2). While this site has only high mannose oligosaccharides in virus from CEF hosts, Fig. 2A, the peak I+IV preparations from BHK- and CHO-grown virus also contained some complex type oligosaccharides (Fig. 2, B and C). However, when material from peak I of BHK-derived virus was purified by a second HPLC step prior to pronase digestion, the relative amount of complex type oligosaccharides decreased to one-third its original value. This result suggests that some of the complex oligosaccharides at this site in BHK- and CHO-derived virus are contaminants from neighboring HPLC peaks II or V which have exclusively complex type glycans. The high mannose oligosaccharides from HPLC peaks I or V exhibited host-dependent variation. Thus, in CEF- and CHO-derived virus, larger oligosaccharides containing 7, 8, or 9 mannose residues constituted the majority of oligosaccharides, while in BHK-derived virus smaller Man₆GlcNAc₂ and Man₇GlcNAc₂ oligosaccharides predominated.

The second glycosylation site in E2 (HPLC peak II), had primarily complex type oligosaccharides in virus from all three hosts (Fig. 3). However, some host-dependent variation was observed. The complex glycopeptides from CHO-derived virus were smaller than those from CEF- or BHK-derived virus. These unusual glycopeptides were resistant to mild acid hydrolysis and probably lack sialic acid (data not shown). This observation differs from recent results of Hunt (15) and may be characteristic of the particular CHO cell line employed here. A small number of noncomplex type oligosaccharide

![Figure 2](image_url)

**FIG. 2.** Gel filtration of oligosaccharides from glycopeptides of HPLC peaks I+IV. (°H)Mannose-labeled tryptic peptides of E2 glycoprotein from virus grown in CEF or BHK cells or intact virions grown in CHO cells were separated by reverse phase HPLC as described in Fig. 1. Fractions corresponding to peaks I and IV were pooled separately. Glycopeptides were digested with pronase and endo H and analyzed by gel filtration on Bio-Gel P-4 as described under "Experimental Procedures." Oligosaccharide profiles shown are representative of profiles from either peak I or \( W \) for CEF- and CHO-grown virus (see text for discussion). For BHK-grown virus, conditions of trypsinization were sufficient to yield peak I exclusively. The exclusion volume was at fraction 63; the inclusion volume was at fraction 173. M, Man; N, GlcNAc. Arrows indicate elution positions of high mannose oligosaccharide standards. S₁, S₂, and S₃ complex glycopeptides eluted at positions 72, 78, and 87, respectively. A, oligosaccharides from peaks I and IV of E2 from CEF-grown virus; B, oligosaccharides from peaks I and IV of E2 from BHK-grown virus; C, oligosaccharides from peaks I and IV of CHO-grown virus.

![Figure 3](image_url)

**FIG. 3.** Gel filtration of oligosaccharides from glycopeptides of HPLC peak II. (°H)Mannose-labeled tryptic peptides from E2 or intact virions were separated by HPLC and the glycopeptides from peak II analyzed by gel filtration on Bio-Gel P-4 as described in Fig. 2. A, glycopeptides from peak II of E2 from CEF-grown virus; B, glycopeptides from peak II of E2 from BHK-grown virus; C, glycopeptides from peak II of CHO-grown virus. G, Glc; M, Man; N, GlcNAc.

The second glycosylation site in E2 (HPLC peak II), had primarily complex type oligosaccharides in virus from all three hosts (Fig. 3). However, some host-dependent variation was observed. The complex glycopeptides from CHO-derived virus were smaller than those from CEF- or BHK-derived virus. These unusual glycopeptides were resistant to mild acid hydrolysis and probably lack sialic acid (data not shown). This observation differs from recent results of Hunt (15) and may be characteristic of the particular CHO cell line employed here. A small number of noncomplex type oligosaccharide
species were found at this site in virus from CEF hosts (Fig. 3A). After treatment with endo H, these structures migrated at positions corresponding to Glc1Man9GlcNAc1, Man6GlcNAc1, and Man9GlcNAc1. Particularly noticeable was the scarcity at this site of other typical high mannose oligosaccharides containing 6, 7, or 8 mannose residues. The high mannose species at HPLC peak II in CEF-derived virus were not artifacts due to contaminating high mannose glycopeptides from neighboring HPLC peak I since rechromatography of peak II material prior to pronase digestion did not alter the oligosaccharide composition (data not shown).

In order to characterize more fully the oligosaccharides present at complex glycosylation sites, the oligosaccharides from peak II of CEF-grown virus which migrated after endo H treatment as Man9GlcNAc, Man6GlcNAc, and Glc1Man9GlcNAc were pooled separately after gel filtration chromatography and treated with jack bean α-mannosidase. The products of the digests were analyzed by gel filtration chromatography (data not shown). The only products of the Man9GlcNAc digestion were a disaccharide which migrated as Man6GlcNAc, and free mannose, with the ratio of label in disaccharide to that in free mannose being 1:1.1. This result confirms the presence of Man9GlcNAc2 oligosaccharides at a "complex" glycosylation site in virus grown in CEF cells. In the case of material migrating as Glc1Man9GlcNAc or Man6GlcNAc, only 1 to 4 mannose residues were removed by α-mannosidase, and only a trace (<2% of radioactivity) of Man9GlcNAc disaccharide was generated. Similar results have been obtained when authentic Glc1Man9GlcNAc was digested with α-mannosidase (20). On the basis of these preliminary findings it is possible that the glycosylation site carries a small number (less than 10% of total oligosaccharides) of high mannose oligosaccharides containing a terminal glucose residue, most probably Glc1Man9GlcNAc and Glc1Man6GlcNAc, which resulted from aborted processing. Another possibility which is consistent with our data is that these oligosaccharides are hybrid structures (25) which are cleaved by endo H, but contain outer sugars such as GlcNAc and Gal. These two possibilities are currently under investigation.

The most striking example of host-dependent variation in oligosaccharide type occurred at the glycosylation site in E1 represented by HPLC peak III (Fig. 4). In CEF-grown virus, the site had exclusively high mannose oligosaccharides, but in BHK-grown virus, the vast majority (>90%) of oligosaccharide chains were complex type. The only high mannose oligosaccharides present at this site in BHK-grown virus migrated as Man9GlcNAc and Man6GlcNAc after treatment of glycopeptides with endo H. In CHO-derived virus, a situation intermediate between that of virus grown in CEF or BHK cells existed. Approximately 50% of the oligosaccharide chains were complex type, and among the remaining high mannose oligosaccharides, small Man9GlcNAc structures predominated.

The second glycosylation site in E1, represented by HPLC peak V, had exclusively complex type oligosaccharides in virus from all three cellular hosts (Fig. 5). Virus grown in BHK cells...
was enriched for sialic acid-containing complex oligosaccharides compared to CEF-derived virus, while the complex oligosaccharides of CHO-derived virus appeared to lack sialic acid entirely.

The relative frequency of glycosylation was determined for each of the four glycosylation sites of Sindbis virus. These estimates were obtained by calculating the amount of radio-labeled mannose present in each HPLC peak and normalizing for the oligosaccharide composition at each site. Three of the four sites, HPLC peaks I+IV, II, and V, are glycosylated at approximately the same frequency in all three cellular hosts. The remaining site in E1, represented by HPLC peak III, is glycosylated about 80% as often as the other three sites in CEF and CHO cells and about 50% as often in BHK cells. Therefore, host-dependent variations in the glycosylation frequencies of different sites are insufficient to account for the oligosaccharide variation observed in virus from different hosts.

**DISCUSSION**

Reverse phase HPLC and gel filtration chromatography were used to characterize the oligosaccharide compositions of the four glycosylation sites of E1 and E2 glycoproteins of Sindbis virus grown in CEF, BHK, and CHO cells. E2 has one high mannose site (HPLC peaks I+IV) and one complex site (peak II), regardless of the host. Similarly, E1 has one complex site (peak V) in virus from all three hosts. However, the second glycosylation site in E1 (peak III) has high mannose oligosaccharides in CEF-grown virus, complex type oligosaccharides in BHK-grown virus and both complex and high mannose type oligosaccharides in CHO-grown virus. This finding is consistent with the results of Burke and Keegstra (13) who examined total oligosaccharides from E1 glycoprotein of Sindbis virus grown in CEF and BHK cells.

Three of the four glycosylation sites are glycosylated at roughly equal frequencies in virus from CEF, BHK, and CHO hosts. The site in E1 which exhibits variation in oligosaccharide type (HPLC peak III) is glycosylated approximately 80% as often as the other sites in CEF and CHO cells and half as often in BHK cells. Thus, host-dependent variation in oligosaccharide type is primarily due to differences in oligosaccharide composition at individual glycosylation sites and not to differences in frequencies of glycosylation. Our estimate of the relative glycosylation frequency for CEF-derived virus is similar to that made previously by Setton and Keegstra (27).

The glycosylation sites of E2 resolved by HPLC can be assigned to specific asparagine residues based on previous work from other laboratories. The mRNA sequence of E2 determined by Rice and Strauss (17) identifies two potential glycosylation sites at Asn-196 and Asn-318. Keegstra et al. (12) have determined the amino acid composition of the high mannose and complex pronase glycopolypeptides of E2 from BHK-grown virus. Thus, we can unambiguously assign HPLC peaks I and IV to the site at Asn-318 which has high mannose oligosaccharides and peak II to the site at Asn-196 which has complex oligosaccharides. HPLC resolves two glycosylation sites in E1, which are indicated by nucleotide sequencing to reside at Asn-138 and Asn-245 (17). On the basis of expected sizes for tryptic peptides, it is likely that the site with host-dependent oligosaccharide type (HPLC peak III) is located at Asn-245 while the site which always has complex type oligosaccharides (peak V) is located at Asn-139. Unambiguous identification of the E1 sites will require amino acid sequencing.

The results presented here and in the accompanying paper (31) suggest that oligosaccharide processing is modulated by (a) the presence of at least one rate-limiting step in processing and (b) physical accessibility of oligosaccharides at individual glycosylation sites. Examination of oligosaccharides at a glycosylation site in E2 where 90% of the oligosaccharides are complex type in CEF-derived virus reveals that Man₇GlcNAc₂ is the only high mannose species present in significant quantities. The striking absence at this site of other typical high mannose oligosaccharides suggests that α-mannosidases IA and IB of the Golgi apparatus (7, 8) act rapidly to remove the four α,1,2-linked mannose residues from Man₇GlcNAc₂ intermediates. The accumulation of Man₇GlcNAc₂ suggests that the next processing step, the addition of a GlcNAc residue by N-acetylgalactosaminyltransferase I (28, 29) occurs more slowly at some complex sites than at others. Direct proof that N-acetylgalactosaminyltransferase I is rate-limiting for formation of mature complex type oligosaccharides is required.

The processing enzymes of the Golgi apparatus must distinguish between the glycosylation sites which will have predominantly high mannose oligosaccharides in the mature glycoprotein from those which will have complex type oligosaccharides. While we have not addressed the question of oligosaccharide accessibility here (see accompanying paper (31)), the oligosaccharide composition of the four glycosylation sites of E1 and E2 are consistent with a steric model for the control of oligosaccharide processing. The model predicts that, for the majority of glycosylation sites, oligosaccharides are either accessible to processing enzymes and therefore destined to acquire a complex type structure or are relatively inaccessible and left as high mannose structures. Two observations presented here support this concept. First, as has been noted for other viral glycoproteins (9, 30), a given glycosylation site usually carries almost exclusively either complex or high mannose type oligosaccharides regardless of the host. Second, comparison of oligosaccharides at the two sites in CEF-grown Sindbis virus which have predominantly high mannose glycans suggests differential susceptibility to the Golgi α,1,2-mannosidases. At the site which always has high mannose oligosaccharides regardless of the viral host (HPLC peaks I+IV), the majority of glycans are large, less extensively processed structures (Fig. 2A). At a site which has high mannose oligosaccharides in CEF-grown virus but has complex type oligosaccharides in BHK-grown virus (peak III), smaller Man₇GlcNAc₂ structures predominate in CEF-grown virus (Fig. 4A). Similarly, at the same two glycosylation sites in CHO-grown virus, one finds large oligosaccharides at the E2 site which always has high mannose oligosaccharides (Fig. 2C) and smaller structures at the E1 site which exhibits host dependent variation in oligosaccharide type (Fig. 4C).

The findings presented here together with work from other laboratories (12-15) demonstrate that different cell lines have varying capacities to process oligosaccharides of Sindbis virus glycoproteins. This observation is best illustrated by the E1 glycosylation site (HPLC peak III) which has primarily complex oligosaccharides in BHK-grown virus, but carries high mannose chains in CEF-grown virus (Fig. 5). Possible explanations for differences in processing among cell types include (a) intrinsic differences in cellular processing enzymes, (b) differences in the duration of intracellular transit of glycoproteins, and (c) differences in the physical accessibility of oligosaccharides during processing. Studies to evaluate these possibilities are in progress.

**Acknowledgments**—We thank S. C. Hubbard and M. D. Snider for helpful discussions and careful reading of the manuscript and D. Young for typing.
REFERENCES

1. Hubbard, S. C., and Ivatt, R. J. (1981) Annu. Rev. Biochem. 50, 555-583
2. Kornfeld, R., and Kornfeld, S. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed.) pp. 1-34, Plenum Press, New York
3. Robbins, P. W., Hubbard, S. C., Turco, S. J., and Wirth, D. F. (1977) Cell 12, 893-900
4. Tabas, I., Schlesinger, S., and Kornfeld, S. (1978) J. Biol. Chem. 253, 716-722
5. Grinna, L. S., and Robbins, P. W. (1979) J. Biol. Chem. 254, 8814-8818
6. Godelaine, D., Spiro, M. J., and Spiro, R. G. (1981) J. Biol. Chem. 256, 10161-10168
7. Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W., and Touster, O. (1982) J. Biol. Chem. 257, 11655-11663
8. Tabas, I., and Kornfeld, S. (1979) J. Biol. Chem. 254, 11655-11663
9. Etchison, J. R., and Holland, J. J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4011-4014
10. Klenk, H.-D., Schwarz, R. T., Schmidt, M. F. G., and Wollert, W. (1978) in Topics in Infectious Diseases (Laver, W. G., Bachmanner, H., and Weil, R., eds) Vol. 3, pp. 83-99, Springer-Verlag, Vienna
11. Nakamura, K., and Compans, R. W. (1979) Virology 95, 8-23
12. Keegstra, K., Sefton, B., and Burke, D. J. (1975) J. Virol. 16, 613-620
13. Burke, D. J., and Keegstra, K. (1976) J. Virol. 20, 676-686
14. Burke, D. J., and Keegstra, K. (1979) J. Virol. 29, 546-554
15. Hunt, L. A. (1981) Virology 133, 534-543
16. Schlesinger, S., Gottlieb, C., Fell, P., Gelb, N., and Kornfeld, S. (1976) J. Virol. 17, 239-246
17. Rice, C. M., and Strauss, J. H. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2062-2066
18. Laemmli, U. K. (1979) Nature (London) 227, 680-685
19. Rosner, M. R., and Robbins, P. W. (1982) J. Cell. Biochem. 18, 37-86
20. Hubbard, S. C., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4568-4576
21. Tarentino, A. L., Trindle, R. B., and Maley, F. (1978) Methods Enzymol. 54, 574-580
22. Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4554-4559
23. Spiro, R. G. (1966) Methods Enzymol. 8, 3-26
24. Tai, T., Yamashita, K., and Kobata, A. (1977) Biochem. Biophys. Res. Commun. 78, 434-441
25. Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1978) J. Biol. Chem. 253, 4508-4511
26. Yamashita, K., Tachibana, Y., and Kobata, A. (1978) J. Biol. Chem. 253, 3862-3869
27. Sefton, B., and Keegstra, K. (1974) J. Virol. 14, 522-530
28. Narasimhan, S., Stanley, P., and Schachter, H. (1977) J. Biol. Chem. 252, 3926-3933
29. Tabas, I., and Kornfeld, S. (1978) J. Biol. Chem. 253, 7779-7786
30. Rosner, M. R., Grinna, L. S., and Robbins, P. W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 67-71
31. Hsieh, P., Rosner, M. R., and Robbins, P. W. (1983) J. Biol. Chem. 258, 2555-2561
Host-dependent variation of asparagine-linked oligosaccharides at individual glycosylation sites of Sindbis virus glycoproteins.

P Hsieh, M R Rosner and P W Robbins

J. Biol. Chem. 1983, 258:2548-2554.

Access the most updated version of this article at http://www.jbc.org/content/258/4/2548

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/4/2548.full.html#ref-list-1