We have characterized the glycans at individual sites on the hemagglutinin of three influenza A variants to obtain information on the role of cell-specific glycosylation in determining the receptor binding properties of this virus. The variants differ in whether they have a glycosylation site at residue 129 on the tip of the hemagglutinin and whether amino acid 184 (near to the receptor binding site) is His or Asn. We found that all sites on each variant are glycosylated in Madin-Darby bovine kidney cells, that the glycosylation is site-specific, and that the glycans at the same site in each variant are highly similar. One site that is buried in the hemagglutinin trimer contains only oligomannose glycans. The remaining sites carry complex glycans of increasing size as the distance of the site from the viral membrane decreases. Most of these complex glycans are terminated with α-galactose residues, a consequence in bovine cells of the removal of terminal sialic acids by the viral neuraminidase. Although the glycans at residue 129 are among the smallest on the molecule, they are large enough to reach the receptor binding pocket on their own and adjacent monomers. The results suggest that the reduction in receptor binding observed with Madin-Darby bovine kidney cell-grown virus is due to the combined effect of large complex glycans at the tip of the hemagglutinin and a His to Asn substitution close to the receptor binding pocket.

The influenza virus hemagglutinin (HA)1 is the virion surface glycoprotein that attaches the virus to its receptors on host cells and fuses the viral envelope with the membranes of endocytic vesicles to initiate the infectious process. It is also the virion component that stimulates the formation of protective antibodies. It is not surprising, therefore, that the nature and extent of glycosylation of the HA have been implicated in altering its receptor binding properties and in the emergence of viral variants with enhanced cytopathogenicity (1–3) and virulence (4) and in masking its antigenic sites (5).

The amino acid sequence of the HA and hence the location of its N-glycosylation sites is determined by the viral genome. This RNA genome is replicated by a polymerase that lacks editing functions, so that mutations in all of the viral genes occur at a high frequency. One of the consequences of this high mutation frequency is that virus populations contain mutants that differ from the majority in the number and position of the N-linked glycans on the HA. The structures of these oligosaccharides appear to be determined by their position on the HA (6, 7) and by the array of biosynthetic and trimming enzymes provided by the host cell in which the virus is grown. Thus, the plasticity of the viral genome and the host-specific glycosylation machinery can, together, create virus populations that are more heterogeneous in structure and function than could be developed by either process alone. This diversity is considered to be responsible for survival of these viruses in a variety of biological niches and for their ability to overcome the inhibitory effects of neutralizing antibodies and antiviral agents.

The HA appears to have regions that must be glycosylated, others that must be free of oligosaccharides, and still others in which glycosylation may be either advantageous or detrimental to the survival of the virus. Glycosylation sites at certain positions on the HA of influenza A viruses isolated from various animals and humans are highly conserved and therefore appear to be essential for the formation and/or maintenance of functional HA (8). Conversely, the generation of glycosylation sites in certain regions of the HA reduces its transport to the cell surface, and its stability and/or function (9). It is in the regions in which glycosylation is neither prohibited nor required for the formation of functional HA that oligosaccharide diversity may have a major selective effect, depending on the specific environment in which the virus is expected to grow.

We have been using laboratory-isolated variants of an H1 strain to study this type of selection and to evaluate the combined effects of genetic mutation and cell-specific glycan processing on the properties of the HA. The properties of these variants are as follows. The three, designated Fo, Cf, and Co, grow equally well in chick embryo fibroblasts (CEF's) (3, 10). The Fo variant produces low virus yields on Madin-Darby bovine kidney (MDBK) cells. It has a glycosylation site at
residue 129 and an Asn at residue 184. This variant is lost from MDBK cell-grown virus populations, due to its low affinity for MDBK-cell receptors (1) and is replaced by Cf, which grows to high titer in MDBK cells. Cf has lost the glycosylation site at 129 by an Asn to Asp substitution but is identical to Fo at residue 184 (2). The remaining variant, Co, also grows well in MDBK cells despite having a glycosylation site at residue 129. This variant has His at residue 184 instead of Asn (11, 12), as do most of the HAs sequenced to date. Thus, the low receptor binding of the Fo variant appeared to be due to either the combined effects of MDBK cell glycosylation and an amino acid substitution at 184 or to the glycans on Fo being different from those on Co.

We therefore undertook the characterization of the glycans at individual sites on the HA1 subunits of these three variants grown in MDBK cells. The glycosylation of the HA2 subunit was not examined. Our aim was to gain information about what, in addition to the spectrum of glycosylating enzymes present in the cell, determines the structure of the glycans on the HA and to explain how these glycans affect the binding activities of these viruses.

In this report we relate the glycan structures that we have found to their location in the three-dimensional structure of this glycoprotein (13). Last, our work prompts new insights into the role of the viral neuraminidase in determining the structure of glycans put onto the HA by cells of different species.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—MDBK cells were grown as described previously (1). The three plaque-type variants of AWSN33 (H1N1) used in this study have been previously described (1, 3, 10), and the predicted amino acid sequences of their HA1 subunits have been published (2, 12). Virus stocks were maintained in CEPs in which the sequence of the HA at the critical sites was known to remain unchanged.

**HA Purification**—Virus was diluted to a concentration of 5 × 10^6 HA units/ml in saline/Tri/EDTA, pH 7.2, and incubated for 20 min at 37 °C with 0.02 mg/ml N\textsubscript{o}-tosyl-L-lysine chloromethyl ketone hydrochloride (Sigma) to remove the viral neuraminidase. Trypsinized virus was pelleted through a 30% sucrose cushion and toned-trypsin (Sigma) to remove the viral neuraminidase.

**HA Glycosylation**—The virus was repelleted and washed twice in STE containing 2 mM chloride and phenylmethylsulfonyl fluoride. The suspensions were made 50 mM in HCl, pH 8.3, containing dithiothreitol, detergent, and protease exoglycosidases used were 

**Isolation and Radiolabeling of Carbohydrates**—Carbohydrates were released from glycopeptides by hydrazinolysis and radiolabeled with Na\textsubscript{2}H\textsubscript{18}O (Amersham International plc, Little Chalfont, UK) as described previously (19).

**Mass Spectrometry of Glycopeptides**—Individual series and clusters of ions in the mass spectrum were assigned to glycopeptides containing a glucosamine residue using the software package MASCOT (Matrix Science, London). Glycopeptides were analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry as described previously (19).

**Bio-Gel P-4 Gel Filtration Chromatography of Neutral Oligosaccharides**—Neutral and charged carbohydrates were separated by high voltage electrophoresis, and the isolated neutral carbohydrates were separated by reversed-phase high-pressure liquid chromatography (HPLC)."
Site-specific Glycosylation of Influenza A Virus HA

Isolation and Identification of Individual Glycosylation Sites

Individual glycopeptides corresponding to each glycosylation site of the HA1 subunit were isolated from tryptic digests of Co-, Cf-, and Fo-derived HA by gel filtration chromatography (Fig. 1). Glycopeptides were designated GP20/21, GP65, GP129, and GP271 according to the fractions of the eluate (Fig. 1). Glycopeptides were desalted and subsequently reversed-phase HPLC of glucosamine-containing pools (Fig. 1) were further separated by reversed-phase HPLC (a, b, c, and d, respectively). Numbered peaks are those that were subsequently identified as glycopeptides, the numbers indicating the glycosylation sites contained in these glycopeptides.

RESULTS

Isolation and Identification of Individual Glycosylation Sites

Individual glycopeptides corresponding to each glycosylation site of the HA1 subunit were isolated from tryptic digests of Co-, Cf-, and Fo-derived HA by gel filtration chromatography and subsequent reversed-phase HPLC of glucosamine-containing fractions of the eluate (Fig. 1). Glycopeptides were designated GP20/21, GP65, GP129, and GP271 according to the glycosylation site that each contained (Table I).

Previous observations had suggested that Asn° in the sequence Asn-Pro-Ser served as a glycosylation site in two of the variants (Cf and Fo (2)). However, a glycopeptide that corresponded to such a glycosylation site was not present in the digests. We subsequently identified a tryptic peptide containing Asn° (data not shown), indicating that this sequence is not glycosylated.

It was apparent from the amino acid sequence of GP20/21 that either Asn 20 or 21 could serve as an N-linked carbohydrate attachment site (see Table I). For all three variants, N-terminal sequencing of this glycopeptide gave a positive identification of Asn at position 20 but a blank in the cycle of Edman degradation for residue 21, indicating that residue 21 carried the carbohydrate moiety. A similar observation was made by Deshpande et al. (4) for a related sequence in the HA from an avian H5 strain of influenza.

Structural Characterization of N-Glycans

Isolation of Neutral Glycans

The charged and neutral radiolabeled N-glycans from each glycopeptide were separated by high voltage electrophoresis. In all cases, only a very small proportion of the glycans were charged. Site 21 carried the largest proportion of charged glycans (<5%), and this charge was due mainly to sulfation (determined by neuraminidase treatment and chemical desulfation; data not shown).

Bio-Gel P-4 gel filtration chromatography of the neutral glycans showed that the elution profiles of the individual glycosylation sites differed markedly, indicating site-specific glycosylation (Fig. 2). The simplest elution profiles were from glycosylation site 65 (Fig. 2b), which showed little heterogeneity. The elution volume of the major species (K in Fig. 2b) was characteristic of the oligomannose structure Man, GlcNAc2 (23).

Table II presents a summary of the glycans found on each of the three variants. The three HAs exhibit only small variation in the distribution of the structures present at each of the sites, indicating that the glycans put onto sites 21, 65, and 271 were not significantly affected by the presence or absence of a glycan at site 129.

Oligomannose Oligosaccharides

The structural predictions made from the Bio-Gel P-4 elution profiles of the glycans from site 65 were confirmed by sequential exoglycosidase treatment. Species K and L were reduced to single 9-gu species on treatment with A. saitoi α-1,2-mannosidase, indicating the removal of four and three terminal α-1,2-linked Man residues, respectively. This component was further reduced to a 5.5-gu structure on treatment with jack bean α-mannosidase, indicative of the removal of the four remaining α-linked Man residues, leaving only the Man residue β-linked to the chitobiose core. This behavior was exactly that expected of authentic Man, GlcNAc2 and Man, GlcNAc2.

Complex-type Oligosaccharides

Mass Spectrometric Analysis—Initial attempts to sequence the neutral glycans at site 129 of the Co and Fo variants by sequential exoglycosidase treatment (devised on the basis of the hydrodynamic volumes obtained from Bio-Gel P-4 gel filtration chromatography) were unsuccessful. When the glycopeptides were reexamined by mass spectrometry, it became apparent that these glycosylation sites contained uncommon complex-type N-glycans. The mass profiles for the two variants (Fig. 3) were highly similar, suggesting that they carried highly similar structures at this glycosylation site. Two major (4167 Da (B) and 4327 Da (C)) and two minor (4005 Da (A) and 4489 Da (D)) mass species were found that, assuming complete trypsinization of the HA and no mass variation due to peptide, represent an oligosaccharide series differing by 162 Da (1 hexose unit). These results suggested that the structures at this site terminated in Gal-Gal sequences. Although these data
gave no indication of the anomericity of the linkage between these Gal residues, they were likely to be \( \alpha \)-linked, since previous studies have identified \( \alpha \)-1,3-galactosyltransferase activity in bovine cells (24, 25).

Serial Lectin Affinity Chromatography—The sequential use of immobilized lectins with defined carbohydrate-binding specificities affords a great deal of structural information with minimal loss of material and is thus a useful alternative to sequential exoglycosidase treatment for the structural characterization of complex mixtures of oligosaccharides (reviewed in Ref. 26). Neutral glycans from sites 21, 129, and 271 were first fractionated on immobilized \( A. \) aurantia lectin, which binds structures with a fucose residue \( \alpha \)-1,6-linked to the chitobiose core (“core-fucosylated”) (27). The major fractions from \( A. \) aurantia lectin affinity chromatography were subsequently subfractionated on immobilized \( D. \) stramonium lectin. \( D. \) stramonium lectin binds strongly to tri- and tetraantennary oligosaccharides containing the pentasaccharide unit Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{6} \)(Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{2} \)Man and/or lactosamine units (Gal\( \beta \)\( _{4} \)GlcNAc) extending one or more of the outer arms and less strongly to oligosaccharides containing Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{4} \)-(Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{2} \)Man (28). Strongly bound material is eluted with GlcNAc oligomers, and weakly bound material is eluted as a retarded fraction without the addition of GlcNAc oligomers. 

\( D. \) stramonium lectin binding properties are abolished by outer arm fucosylation. Finally, the major glycan fractions from \( D. \) stramonium lectin affinity chromatography were fractionated on immobilized \( G. \) simplicifolia lectin I. This lectin binds strongly to glycans containing two or more terminal \( \alpha \)-linked Gal or GalNAc residues and weakly to those containing only a single terminal \( \alpha \)-Gal/GalNAc.

The serial lectin affinity profiles of glycans from glycosylation sites 21, 129, and 271 (Fig. 2, Table I, and Table II) were similar for the three HAs at a given glycosylation site, and the majority of fractionated on immobilized \( A. \) aurantia lectin, which binds structures with a fucose residue \( \alpha \)-1,6-linked to the chitobiose core (“core-fucosylated”) (27). The major fractions from \( A. \) aurantia lectin affinity chromatography were subsequently subfractionated on immobilized \( D. \) stramonium lectin. \( D. \) stramonium lectin binds strongly to tri- and tetraantennary oligosaccharides containing the pentasaccharide unit Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{6} \)(Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{2} \)Man and/or lactosamine units (Gal\( \beta \)\( _{4} \)GlcNAc) extending one or more of the outer arms and less strongly to oligosaccharides containing Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{4} \)-(Gal\( \beta \)\( _{4} \)GlcNAc\( \beta \)\( _{2} \)Man (28). Strongly bound material is eluted with GlcNAc oligomers, and weakly bound material is eluted as a retarded fraction without the addition of GlcNAc oligomers. 

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The serial lectin affinity profiles of glycans from glycosylation sites 21, 129, and 271 (Fig. 2, Table I, and Table II) were similar for the three HAs at a given glycosylation site, and the majority of

### Table I

| Glycopeptide | Composition  | Predicted sequence | N-terminal sequence | Site |
|--------------|--------------|--------------------|---------------------|------|
| GP2021       | D\(_{5}\)EcS\(_{2}\)G\(_{2}\)HT\(_{2}\)YVI\(_{2}\)L\(_{2}\)K | DTICIGYHANNSTDVTDTILEK | DTICIGYHAN-STDT | 21   |
| GP65         | D\(_{5}\)EcS\(_{2}\)KTP\(_{2}\)IL\(_{2}\) | CNITGWLLGNPECDSSLFPAR | C-ITGWLLGNPECDS | 65   |
| GP129        | D\(_{5}\)EcS\(_{2}\)OH\(_{2}\)RT\(_{2}\)PV\(_{2}\)F | ESSHPHTFPNGTVTCSSH | ESSHP-HTFPNGTVS | 129  |
| GP271        | D\(_{5}\)EcS\(_{2}\)G\(_{2}\)HT\(_{2}\)A\(_{2}\)MI\(_{2}\)F\(_{2}\)K | GFESGHTSSASMHECNTK | GFESGHTS-ASMHE | 271  |

### Table II

| Site | Species | Size | Proportion |
|------|---------|------|------------|
| 21   | V       | 2    | 5          | 4           |
|      | A       | ->26 | 3            | 10          |
|      | B       | ->26 | 10           | 15          | 18  |
|      | C       | 25.9 | 23           | 27          | 32  |
|      | D       | 22.8 | 32           | 22          | 28  |
|      | E       | 19.7 | 18           | 13          | 9   |
|      | F       | 16.8 | 6            | 5           | 0   |
|      | G       | 16.1 | 6            | 5           | 0   |
| 65   | K       | 12.5 | 100          | 78          | 100 |
|      | L       | 11.7 | 0            | 22          | 0   |
| 129  | E       | 19.7 | 13           | 9           |
|      | F       | 16.8 | 22           | 19          |
|      | G       | 16.1 | 34           | 34          |
|      | H       | 15.2 | 12           | 14          |
|      | I       | 14.6 | 9            | 15          |
|      | J       | 13.8 | 9            | 9           |
| 271  | B       | ->26 | 1            | 0           | 0   |
|      | C       | 25.9 | 5            | 4           | 4   |
|      | D       | 22.8 | 16           | 14          | 14  |
|      | E       | 19.7 | 45           | 36          | 46  |
|      | F       | 16.8 | 17           | 24          | 25  |
|      | G       | 16.1 | 16           | 22          | 11  |

**FIG. 2.** Bio-Gel P-4 gel filtration chromatography of neutral oligosaccharides from individual glycosylation sites. a, b, c, and d depict sites 21, 65, 129, and 271, respectively. The numbers and ticks at the top of a represent an internal calibration with a partial dextran hydrolysate, allowing elution positions to be expressed in gu. The gu positions indicated in the top panel apply to all four chromatograms. a and d, the Co variant; b, the Cf variant; c, the Fo variant.
the glycans at all three sites were core-fucosylated as judged by their binding to *A. aurantia* lectin. Most of the core-fucosylated glycans at site 129 from Co and Fo failed to bind to *D. stramonium* lectin, suggesting limited branching (biantennary structures). This, together with the mass spectrometry data and the hydrodynamic volumes, suggested the presence of bisecting GlcNAc residues. The behavior of these *A+D−* glycans on *G. simplicifolia* lectin I indicated that they terminated in 0–2 *α*-Gal residues.

As with the glycans from site 129, the majority of the core-fucosylated oligosaccharides at site 271 were unbound by *D. stramonium* lectin, while most of the remaining glycans bound weakly to this lectin. The majority of the *A+/D−* structures were bound strongly by *G. simplicifolia* lectin I, indicating that they had two or more terminal *α*-Gal residues. The hydrodynamic volumes of the major species on Bio-Gel P-4 gel filtration (Fig. 2d) indicated multiantennary structures and to abolish the *D. stramonium* lectin binding properties associated with these types of structures, the glycans would have to carry outer arm fucosylation. Alternatively, these species could be bisected biantennary structures with 0–2 lactosamine units (Galβ4GlcNAc) extending one or both outer arms.

In contrast to sites 129 and 271, most of the core-fucosylated glycans from site 21 bound strongly to *D. stramonium* lectin, and most or all of this *A+/D−* material bound strongly to *G. simplicifolia* lectin I. These results, in combination with the hydrodynamic volumes of the glycans on Bio-Gel P-4 gel filtration chromatography (Fig. 2a), suggested that the major oligosaccharides at site 21 were highly branched, core-fucosylated structures, with terminal *α*-Gal residues on at least two outer arms and 0–2 lactosamine units extending one or more outer arms.

**Glycosidase Analysis**—In the final characterization of the complex glycans, exo- and endoglycosidase treatments were used to confirm and extend the structural predictions made from the experiments described above. A combination of sequential exoglycosidase treatment and MALDI-TOF mass
indicating the loss of one (Fig. 5c) resulted in a mass shift of 146 Da, consistent with the removal of a single fucose residue. The mass of this last species (F) was 934 Da, consistent with the core pentasaccharide Man6GlcNAc6.

Further evidence for these structures was provided by the masses of the in-source decay fragment ions (29, 30) present in the spectra shown in Fig. 5, b and c. The presence of the ion at m/z 1321.5 in the spectrum shown in Fig. 5c, caused by the loss of GlcNAcFuc in a B-type cleavage (31), and the absence of an ion corresponding to the loss of unsubstituted GlcNAc confirmed the presence of a core fucose at the reducing terminus. The in-source fragmentation pattern of the spectrum in Fig. 5c closely matched that of a reference sample of a bisected bi-antennary glycan containing a core fucose and lacking terminal Gal residues.

In order to determine whether the additional lactosamine units on the site 21 oligosaccharides extended from a single outer arm or were distributed over several arms, the A+/D+ fraction was treated with E. freundii endo-β-galactosidase. This enzyme specifically hydrolyzes the β-1,4-linkage in Galβ4GlcNAc only when it is present within an unbranched polylactosamine sequence, with no outer arm fucosylation (32, 33). Bio-Gel P-4 gel filtration chromatography of the glycan pools before and after endo-β-galactosidase treatment (Fig. 6, a and b, respectively) provided evidence for the distribution of single lactosamine units over several outer arms. The digestion products comprised several poorly resolved species (region Y in Fig. 6b), ranging in size from ~23 to ~19 gu, as would be expected from endo-β-galactosidase treatment of the structures shown in Fig. 6c. The structures at this site did not contain any outer arm fucosylation, since the digestion products were identical when the A+/D+ fraction was pretreated with almond α-fucosidase (data not shown).

The A+/D− fraction from site 271 was treated with a mixture of coffee bean α-galactosidase, jack bean β-galactosidase, and jack bean β-hexosaminidase (3 units/ml). The Bio-Gel P-4 profiles of the A+/D− glycan pools after this treatment (Fig. 7b) showed a major component at ~13 gu and a minor component at ~8.5 gu (species P and Q, respectively, in Fig. 7b). This result would be consistent with the structures depicted in Fig. 7c if the β-hexosaminidase had successfully removed the outer GlcNAc residues, but had failed to remove all of the innermost GlcNAc residues and the bisecting GlcNAc. The activity of the β-hexosaminidase toward an authentic bisected biantennary oligosaccharide substrate corresponding to structure P (13 gu) was only ~30%, showing that this was indeed the case. Digestion products identical to those shown in Fig. 7b were obtained when the A+/D− fraction was treated first with almond α-fucosidase and then with the enzyme mixture described above (data not shown), indicating that these structures were not substituted on the outer arms with fucose residues.

### DISCUSSION

The carbohydrate analysis described here shows conserved glycosylation at individual sites on the HA1 subunits from the three virus variants studied. The major glycans observed at sites 21, 65, and 271 from Co, Cv and Fo as well as those at site 129 of Co and Fo are shown in Fig. 8. The most striking feature of this figure is the site specificity that it illustrates. The highly processed polylactosamine complex-type structures observed at site 21 indicate that MDBK cells possess the full range of enzyme activities necessary for extensive processing of N-linked glycans, yet none of the oligosaccharides at the other sites are processed to this extent.

The most extreme example of restricted processing is site 65, which contains almost exclusively Manα3GlcNAcβ2, a structure
which has undergone very little processing from the glucosylated precursor common to all N-linked carbohydrates. This illustrates that, while the extent to which N-linked carbohydrates on influenza virus glycoproteins can be processed depends upon the array of glycosidases and glycosyltransferases available in the host, the nature of the protein backbone will determine the extent to which this potential is realized. In the case of site 65, the limited oligosaccharide processing is most likely due to the inaccessibility of the relevant enzymes to this site; since this site is almost buried within the monomer, between the head and stem regions of the HA1 subunit (see Fig. 8), it is probably no longer accessible after folding and trimerization of the HA polypeptides. Similar conclusions have been drawn by Keil et al. (6), who found oligomannose structures on a glycosylation site at the interface between HA monomers.

Although some secretory proteins have been found to contain

**Fig. 6.** Treatment of site 21 A+/D+ glycan pools with endo-β-galactosidase. A+/D+ neutral glycan pools from site 21 were analyzed by Bio-Gel P-4 gel filtration chromatography before (a) and after (b) endo-β-galactosidase treatment. Annotation for the chromatograms is as described in the Fig. 2 legend. c shows predicted structures for species B–E in a. Dotted lines indicate cleavage positions for endo-β-galactosidase, and monosaccharides are depicted as described in the legend to Fig. 5.

**Fig. 7.** Treatment of site 271 A+/D+ glycan pools with defined exoglycosidase mixture. A+/D+ neutral glycan pools were treated with a mixture of coffee bean α-galactosidase, jack bean β-galactosidase, and jack bean β-hexosaminidase and analyzed by Bio-Gel P-4 gel filtration chromatography before (a) and after (b) enzyme treatment. Predicted structures for species D–G are shown in c. Dotted lines indicate cleavage positions for the enzyme mixture, giving rise to species P (13 gu) and Q (8.5 gu). Monosaccharides are depicted as described in the legend to Fig. 5. b is offset to align the internal calibration with a.
oligosaccharides with one or two N-acetyllactosamine repeats, these structures are almost exclusive to membrane-bound glycoproteins in mammalian systems (34–36), and it has been suggested that the proximity of a glycosylation site to the membrane is important for the addition of polylactosaminoglycans (37). This hypothesis is supported by the observation that the Trypanosoma brucei type II variant surface glycoproteins have polylactosaminoglycans only at the site closest to the membrane (17). The HA is a membrane-bound glycoprotein, and of all the glycosylation sites on the HA1 subunits, site 21 is the most membrane-proximal (see Fig. 8). In addition, site 21 contained most of the charged oligosaccharides found on the HA. This charge is due to sulfation, which is most commonly found on GlcNAc residues in N-acetyllactosamine repeats. Thus, the carbohydrate structures at site 21 are not surprising when one considers the position of this glycosylation site in the three-dimensional structure of the HA.

The oligosaccharides at site 271 were not processed to the same extent as those at site 21. These two sites are apparently equally exposed on the HA surface and should be equally accessible to the glycosyl transferases involved in N-linked oligosaccharide processing. Site 129, which is the most membrane-distal glycosylation site on the HA1 subunit, contains glycans that are processed to a relatively high degree but less so than those at site 271. Thus, there appears to be an inverse correlation between the extent of oligosaccharide processing and the distance of the site from the membrane.

The level of α-galactosylation observed in this study is unusually high, although the presence of these moieties could be anticipated, since the host in which the viruses were grown is a bovine cell line and this species is known to possess α-1,3-galactosyltransferase activity (24, 25). Substrate specificity studies on α-1,3-galactosyltransferase purified from calf thymus demonstrated that the oligosaccharide acceptor for this enzyme is Galβ4GlcNAc-R; the activity of this enzyme is mutually exclusive with α-2,6-sialyltransferase and is blocked by the presence of α-1,3-fucosylation on the GlcNAc residue (38). Thus, terminal α-1,3-galactosyl residues can be explained by the activity of the viral neuraminidase, which ensures that terminal sialic acid residues are removed from HA, leaving an abundance of oligosaccharide acceptors for the action of α-1,3-galactosyltransferase. In addition, the lack of outer arm fucosylation would further promote α-galactosylation. The presence of terminal α-Gal residues on the majority of the glycans in this study explains the previous observation that HA N-glycans obtained from virus grown in MDBK cells are

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**Fig. 8.** The HA monomer depicting the major glycan structures observed at each of the N-linked glycosylation sites of the HA1 subunit for the three virus variants. The diagram shows the α-carbon backbone of the HA polypeptides. The RBP is indicated with a large arrow, and the location of residue 184 with a small arrow and a closed circle. The positions of glycosylation sites 21, 65, 129, and 271 are indicated by open circles. Monosaccharides are depicted as described in the legend to Fig. 5.
poor acceptors for sialyltransferase (39).

As indicated above, one of the aims of this study was to determine the mechanism by which the combination of an amino acid substitution at residue 184 (His to Asn) and a glycan at residue 129 on the HA reduces the receptor binding activity of the Fo virus. Since site 129 of Co- and Fo-HA contain highly similar arrays of N-glycans, it is apparent that the presence of Asn rather than His at position 184 does not influence the nature of the oligosaccharides at site 129. Furthermore, it is clear that the presence of an N-glycan at site 129 does not alter the glycosylation at the other sites. This points to the conclusion that the replacement of a highly conserved His at position 184 with an Asn residue affects the HA RBP such that the presence of a large, MDBK cell-synthesized glycan at site 129 can sterically hinder the interaction of this RBP with its sialic acid-containing receptor. The carbohydrates attached to the HA1 subunit of these variants by CEFs are known to be significantly smaller than those synthesized in MDBK cells (40), and the CEF-synthesized glycans on fowl plague virus are also smaller than the structures reported here (7). CEF-synthesized oligosaccharides may be too small to interfere with receptor binding in the way that the larger MDBK cell-synthesized oligosaccharides do. This would explain why all three virus variants are able to grow to equally high titer in CEFs and why Fo virus grown in CEFs is capable of binding MDBK more strongly than Fo virus grown in MDBK cells (1).

The hypothesis that large oligosaccharides near the RBP of influenza A virus HA can interfere with receptor binding activity, whereas smaller oligosaccharides may have no influence, is supported by the work of Kemble et al. (41). These authors proposed that a large oligosaccharide 7 Å from the RBP interfered with receptor binding activity by “filling or masking the receptor pocket on a neighboring monomer,” (41) whereas a smaller oligosaccharide did not. Analysis of minimum energy models of the Fo- and Co-HA, based on the available crystal structure of an H3 HA complexed with sialylactose (42), showed that glycosylation site 129 is about 11 Å from the RBP (43). Furthermore, we were able to show that an MDBK cell-synthesized oligosaccharide at this site was able to reach both the RBP of the monomer to which it was attached and that of the adjacent monomer.

The high degree of conservation of His184 in nature and its proximity to the RBP residue His183 suggest that a His to Asn substitution at this site may be detrimental to the receptor binding activity of the HA. We have looked at the RBPs of the minimum energy models of the Co- and Fo-HA and have found that the side chain of residue 184 points downwards and away from the RBP. This observation argues against this amino acid substitution resulting in conformational alterations to the RBP that could account for reduced receptor binding activity. It may, however, be that electrostatic effects arise from interactions between His183 and His184 in the HA. This type of interaction has been demonstrated for RNase A, where proton exchange between two adjacent residues in the active site (His142 and His119) ensures that one of these residues is in the correct protonatable state to interact with the substrate and is therefore crucial to the substrate binding activity of this molecule (reviewed in Ref. 44). Although His183 does not interact directly with sialic acid, it forms part of a hydrogen bond network that defines the RBP surface topology (45). If there is proton exchange between His183 and His184 in the HA analogous to that in the RNase A active site, it is possible that replacing this His with Asn might disrupt the RBP architecture. This could perturb the RBP such that a glycan at site 129 now becomes detrimental to receptor binding activity.

This is the first report that provides a detailed description of site-specific glycosylation of the HA by mammalian cells. Our results clearly reveal the dual role of the amino acid sequence and the glycosylating enzymes in determining the final structure of the biologically active trimer. For example, one can predict that the amino acid sequence of the HA is likely to be a major determinant of the glycan structures found at site 65 and other sites buried in or between monomers in the HA trimer. However, given the smaller size of the HA when synthesized by other cells (CEF and Madin-Darby canine kidney cells), the structures of the glycans at the other sites will undoubtedly depend, to varying degrees, on the processing enzymes of the host cell. Since the Fo variant used in this study is less cytotoxic than either C variant (1), we postulated that it might contain somewhat larger complex-type oligosaccharides or glycans with different structures from those on the C viruses. However, we have shown here that this is not the case. Differences in the degree to which the C and F variants reduce host protein synthesis (46) also had no detectable effect on the ability of the glycosylating enzymes to process the HA N-glycans.

In contrast, a rather large effect on the final HA structure appears to result from the action of the viral neuraminidase. The effects of this viral enzyme activity go beyond the removal of sialic acid residues from the HA, since its activity provides substrates for outer arm substitutions by host glycosyltransferases. The data presented here might suggest that the action of the neuraminidase may enhance the difference between the glycans put onto the HA by cells of different species. Whether this has biological consequences in terms of epitope masking, trimer stability, or fusion activity remains to be investigated.

Last, many of the conclusions to be drawn from this study are similar to those made earlier by Keil et al. (6, 7), who examined the glycans at individual sites on an avian H7 influenza virus HA grown in CEFs. However, more work along the lines reported here is needed before information can be generalized to other influenza A strains. For example, H1 strains newly isolated from the human population contain eight or nine N-linked glycosylation sites, many of which are conserved during antigenic drift, whereas those isolated from avian species have only four sites (47). It is not known whether all of the N-glycan attachment sites on these human isolates are occupied, nor is it known whether the conserved sites common to human and avian strains would carry the same glycans if grown in the same host. Answers to questions such as these should contribute to our understanding of the epidemiology of influenza, to improved methods for the characterization of new isolates, and to the improvement of vaccines.

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REFERENCES
1. Crecelius, D. M., Deom, C. M., and Schulze, I. T. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 36–40
2. Deom, C. M., Caton, A. J., and Schulze, I. T. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 3771–3775
3. Aytaç, S., and Schulze, I. T. (1991) J. Virol. 65, 3022–3028
4. Deshpande, K. L., Fried, V. A., Ando, M., and Webster, R. G. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 36–45
5. Skelley, J. J., Stevens, D. J., Daniels, R. S., Douglas, A. R., Knossow, M., Wilson, I. A., and Wiley, D. C. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 1779–1783
6. Keil, W., Niemann, H., Schwarz, R. T., and Klenk, H.-D. (1984) Virology 133, 77–91
7. Keil, W., Geyer, R., Dah browski, J., Dah brown, U., Niemann, H., Stirm, S., and Klenk, H.-D. (1988) Virology 171, 371–3770
8. Gallagbar, P. J., Henneberry, J. M., Sambrook, J. F., and Gething, M.-J. (1992) J. Virol. 66, 7136–7145
9. Gallagbar, P., Henneberry, J., Wilson, I., Sambrook, J., and Gething, M. J. (1988) J. Cell Biol. 107, 2559–2575
10. Noronha-Blob, L., and Schulze, I. T. (1976) Virology 69, 314–322
11. Hiti, A. L., Davis, A. R., and Nayak, D. P. (1981) Virology 111, 113–124
12. Schulze, I. T., Steward, R., Rajakumar, A., and Aytaç, S. (1988) Virus Res. 2,
Site-specific Glycosylation of Influenza A Virus HA

Harvey, D. J., Naven, T. J. P., Kuster, B., Bateman, R. H., Green, M. R., and Critchley, G. (1995) Rapid Commun. Mass Spectrom. 9, 1556–1561

Demon, B., and Costello, C. E. (1988) Glycoconjugate J. 5, 397–409

Fukuda, M. N., Watanabe, K., and Hakimori, S.-I. (1978) J. Biol. Chem. 253, 6814–6819

Scudder, P., Lawson, A. M., Weidemann, E. F., Carruthers, R. A., Childs, R. A., and Feizi, T. (1987) Eur. J. Biochem. 168, 585–593

Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., and Kobata, A. (1981) J. Biol. Chem. 256, 8476–8484

Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987) J. Biol. Chem. 262, 12059–12076

Fukuda, M. N., Dell, A., Oates, J. E., and Fukuda, M. (1985) J. Biol. Chem. 260, 6623–6631

Fukuda, M., Guan, J.-L., and Rose, J. K. (1988) J. Biol. Chem. 263, 5314–5318

Blanken, W. M., and Van den Eijnden, D. H. (1985) J. Biol. Chem. 260, 12927–12934

Lakshmi, M. V., and Schulze, I. T. (1978) Virology 88, 314–324

Deem, C. M., and Schulze, I. T. (1985) J. Biol. Chem. 260, 14771–14774

Kemble, G. W., Henis, Y. I., and White, J. M. (1993) J. Cell Biol. 122, 1253–1265

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988) Nature 333, 426–431

Mir-Shekari, S. Y. (1995) Glycosylation of the Influenza A/WSN/33 Virus Hemagglutinin. Ph.D. thesis, University of Oxford

Blackburn, P., and Moore, S. (1982) in The Enzymes (Boyer, P. D., ed.) Vol. 15, pp. 317–433, Academic Press, Inc., New York

Keim, S., Paulson, J. C., Rose, U., Brossmer, R., Schmidt, W., Bandgar, B. P., Schreiner, E., Hartmann, M., and Zibral, E. (1992) Eur. J. Biochem. 205, 147–153

Crecelius, D. M. (1983) Host Cell Determined Physical and Replicative Properties of Two Variants of Influenza Virus. Ph.D. thesis, St. Louis University

Inkster, M. D., Hinshaw, V. S., and Schulze, I. T. (1993) J. Virol. 67, 7436–7443