INFLUENZA VIRUS RECEPTORS

AND GENOMIC PACKAGING

by

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Degree Conferred:.....

To my husband, James, who has supported me through every moment of this work, I would never have been able to do it without you. And to Mom, Dad and Paul for their unconditional love and understanding always.

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Abstract

The influenza A viral genome is composed of eight negative-sense RNA chromosomes (called vRNAs), all of which must be packaged to produce an infectious virion. It is not clear whether individual vRNAs are packaged specifically or at random, however, and the total vRNA capacity of the virion is unknown. We have created modified forms of the viral nucleoprotein (NP), neuraminidase (NA), and nonstructural (NS) vRNAs that encode green or yellow fluorescent proteins, and have studied the efficiency with which these are packaged using a plasmid-based influenza assembly system. We found that, under conditions where virions are limiting, pairs of alternatively-tagged vRNAs compete for packaging, but do so in a nonspecific manner. Moreover, 3-5% of transduction-competent viruses were found to incorporate two alternative reporters, regardless of whether those reporters represented the same or different vRNAs – a finding compatible with random, but not with specific packaging. Probabilistic estimates suggest that, in order to achieve this level of dual-transduction by chance alone, each influenza A virion must package an average of 9-11 vRNAs.

Influenza A and C viruses each utilize specific forms of sialic acid on cell surfaces as receptors to bind and infect their host cells. The extent to which various specific types of sialoglycoproteins function as influenza receptors, however, is largely unknown. Sialomucins are a distinct class of surface proteins that contain regions that are densely substituted with O-linked sialylated oligosaccharides, which could provide an ideal substrate for

polyvalent interactions between influenza virus and the cell. Here we demonstrate that pretreatment with an enzyme, which selectively degrades sialomucins as a class from cell surfaces, profoundly inhibits the attachment of influenza A or C viruses as measured by hemagglutination. Surprisingly, however, neither enzymatically depleting surface sialomucins from CHO or MDCK cells nor the overexpression of a specific surface sialomucin (CD34) in CHO cells had any discernible effect on influenza infectivity, as measured by the levels or kinetics of viral RNA expression. Despite their predominant role in influenza attachment to erythrocytes, we find no evidence that sialomucins function as receptors for influenza A or C viruses on mammalian cells.

Abstract Approved: 

Thesis Advisor

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Chapter 1

Influenza Virus Structure and Biology

The viral disease now known as influenza or the "flu" has circulated the globe for thousands of years. It is spread from person to person via an aerosol route and can result in a range of clinical responses from asymptomatic infection to viral-induced pneumonia that can progress to a fatal outcome. The most typical symptoms occur after an incubation period from 24 hours to 5 days, whereupon the patient presents with rapid onset of headache, chills, and a dry cough, followed soon after by a high fever (up to 41°C), myalgias, malaise and anorexia. Nasal obstruction, sneezing and rhinorrhea can also occur. Fever typically tends to decline after 2 to 3 days at which time respiratory symptoms can intensify and lead to a more productive cough. These symptoms, as well as malaise, may linger for 1 to 2 additional weeks. Long-term damage due to the disease is rare, even in patients with chronic lung disease. However, complications can arise during acute infection, so that young children, elderly and those with other health problems can be especially at risk for pneumonia, bronchitis, sinus and ear infections and even death (39).

Influenza viruses are classified into types A, B, and C based on antigenic differences among their matrix (M) and nucleoprotein (NP) antigens. All three strains are known to infect humans, though influenza A and C viruses have also been shown to infect other mammalian species naturally. Influenza B virus tends to cause the same symptoms as influenza A (described above), though the

frequency of hospitalization resulting from influenza B infection is about four-fold lower than for infection by influenza A. Influenza C virus tends to cause a less severe form of upper respiratory illness and is rarely associated with lower respiratory disease (39).

Although only a small percentage of influenza cases are fatal, the sheer prevalence of this disease makes it the leading viral killer of humanity. In addition to its annual mortality, certain strains of influenza have caused widespread pandemics of disease and death in its yearly circle of the globe. One such viral epidemic, only later attributed to influenza, was recorded by Hippocrates as early at 412 BC. More recent epidemics have originated in the China, presumably due to close contact between humans and livestock known to harbor influenza (39). However, no documented epidemic to date has been as virulent as the virus circulating between 1918-1919, which is thought to have killed between 20 and 40 million people worldwide and altered the course of World War I. Although influenza virus was first isolated in 1933 and has been studied intensively since, scientists still do not have a clear understanding of the viral attributes which make one strain of influenza more virulent than another (52).

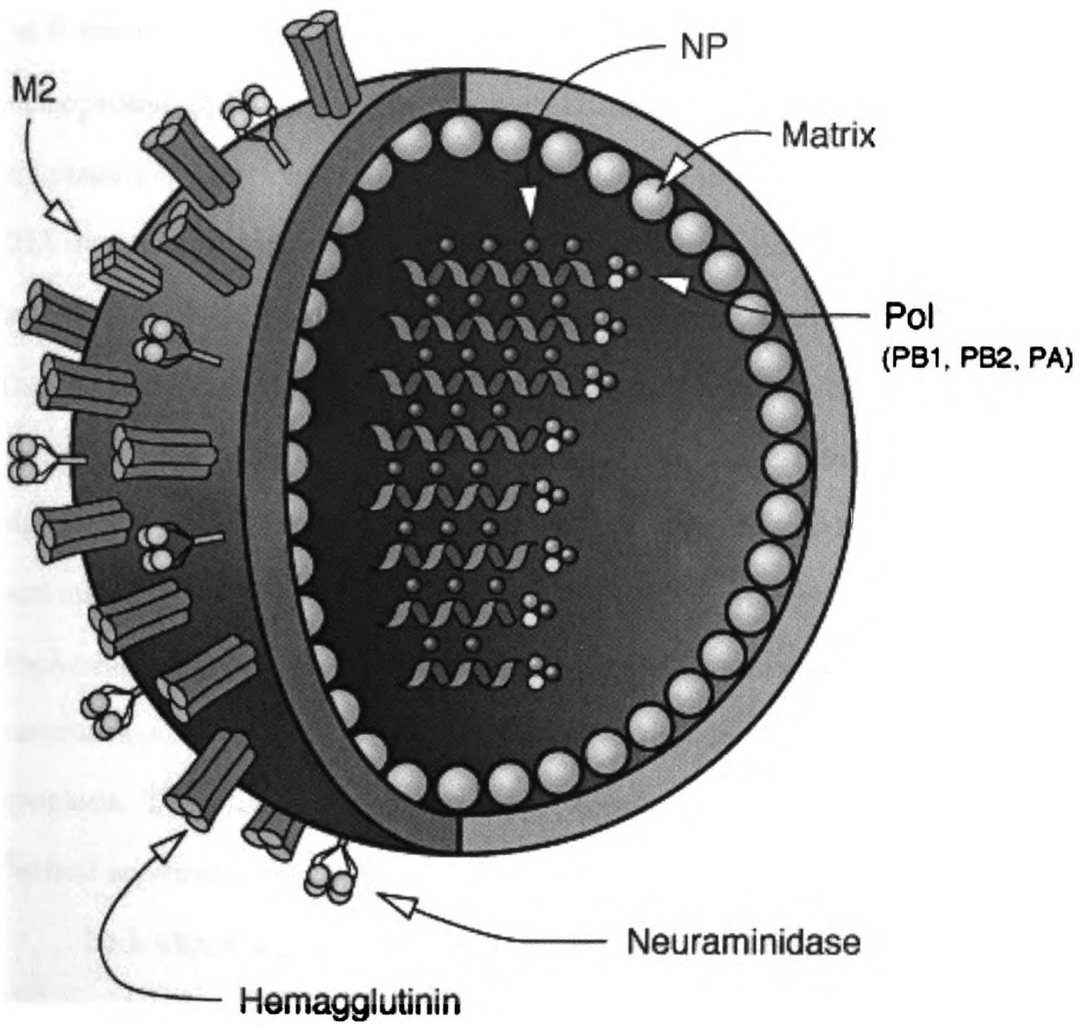
Influenza virus is a member of the Orthomyxoviridae (derived from the Greek *orthos*, "standard, correct" and *myxo*, "mucus") family characterized so named for their ability to bind to mucus. Influenza A and B each have a genome composed of 8 negative-sense viral RNAs (vRNAs), that together encode 11 proteins, whereas influenza C virus has 7 segments encoding 9 known

proteins (51, 65). The negative-sense RNA genomes are templates for transcription of positive-sense mRNA and of cRNA, a full-length anti-genomic (+) copy of each vRNA segment, which is used as a template for new vRNA synthesis. Influenza A and B are morphologically indistinguishable, whereas influenza C may be distinguished by its single surface glycoprotein spike ordered into a hexagonal structure.

Influenza viruses are enveloped viruses; the lipid bilayer is derived from the host cell during the budding process following infection (19). Influenza surface glycoprotein molecules are incorporated into the bilayer during assembly and radiate outward as spike projections. Influenza A and B viruses (Figure 1) have both Hemagglutinin (HA), assembled into trimers, and Neuraminidase (NA), assembled into homotetramers, as surface proteins. Influenza C has only a single, multi-purpose surface glycoprotein, Hemagglutinin Esterase Fusion protein (HEF), which assembles into hexagonal arrays (19, 27, 40). HA and HEF molecules must be cleaved by a trypsin-like protease into two subunits in order to become competent for infection (57). Influenza M2 protein is also expressed as a virion-surface molecule, though very few copies are present, and functions as an ion channel to facilitate uncoating during the early stages of viral infection (129).

Underlying the lipid bilayer is a protein coat derived primarily from Matrix protein (M1), the most abundant structural component of the influenza virus, which interacts with both the lipid bilayer and the viral core (35, 131, 138, 139). Housed inside the protein coat is a ribonucleoprotein core (RNP), which is

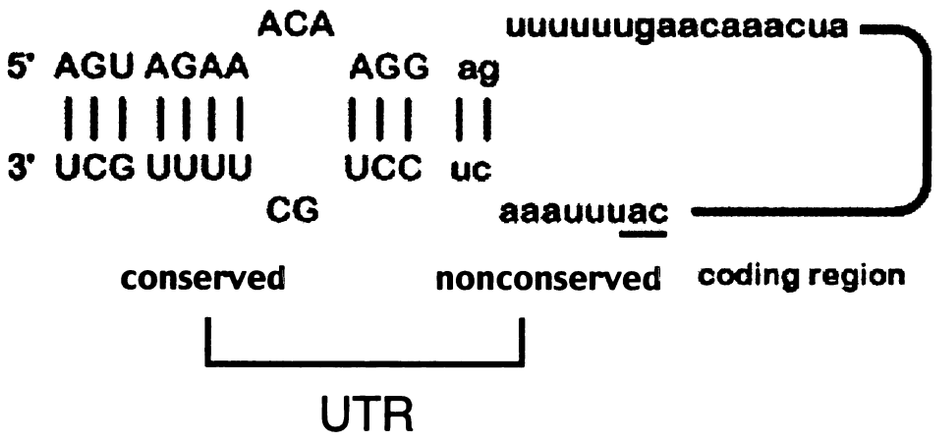
Figure 1. Influenza A virus. Schematic representation of virion structure, proteins, and genomic RNA segments.


M2
NP
Matrix
Pol
(PB1, PB2, PA)
Neuraminidase
Hemagglutinin

visible upon thin sectioning of the virus and appears to assemble into long helical filaments (3, 80). These can be separated according to size, into either 8 or 7 segments for influenza A and B or C, respectively (103). These RNPs are composed of the vRNA segments, encapsidated with three viral proteins (PB1, PB2 and PA), which together make up viral the polymerase complex, bound to 3′ and 5′ termini (31, 37). The remaining lengths of the vRNAs are associated with nucleoprotein (NP), an RNA-binding structural protein (5). Also incorporated into virions is NS2 protein, or NEP, which has recently been characterized as an RNA nuclear export protein, and is thought to be important for export of unspliced viral RNA molecules from the host cell nucleus to the cytoplasm (88). The one of the two other known influenza proteins is NS1, a protein that is not thought to exist within the virion. NS1 is an early-phase protein synthesized at high abundance and is important for inhibiting nuclear export of capped, spliced host mRNAs (61). The other known nonstructural viral protein, PB1-F2, has been discovered recently. It localizes to the mitochondria, though expression in variable depending upon the host species, and appears to induce cellular apoptosis. The authors theorize that this protein may be important to evasion of the host antiviral response by killing immune cells responding to infection (17).

Each vRNA segment consists of a protein-coding region flanked by 3′ and 5′ untranslated regions (UTR) (Figure 2). The distal 12 and 13 nucleotides of the 3' and 5' UTRs, respectively, are highly conserved among all eight gene segments and among influenza A strains (61). Influenza B chromosomes likewise show a conservation of sequence at the distal 3′ and 5′ ends; though these differ slightly

Figure 2. Influenza A viral RNA. Schematic representation of viral RNA sequences and structure. Untranslated region (UTR) encompasses both conserved sequences, common to all viral RNA segments, and nonconserved regions, specific to each segment, though conserved among strains.


ACA
uuuuuugaacaaacua
5' AGU AGAA
AGG ag
3' UCG UUUU
UCC uc
CG
aaauuuac
conserved
nonconserved
coding region
UTR

from the corresponding sequences of influenza A (81). These conserved regions have an inverted complementarity and are thought to anneal so that each chromosome forms a panhandle structure under certain conditions (5, 18, 28). The remainder of each UTR has a unique sequence for each chromosome end, but is also highly conserved among strains. The UTRs (including both the common and segment-specific sequences) at the ends of the viral chromosomes are thought to contain all of the necessary signals to direct RNA transcription, replication, and packaging. In fact, a heterologous gene can be replicated, transcribed, and packaged into an influenza virion when flanked by these UTR sequences alone (69, 79).

Influenza virus begins its infection cycle by using its HA molecule to bind to sialic acid residues on host cell surface glycoproteins. This is a low-affinity interaction thought to be enhanced by multi-valent binding to multiple HA trimers on the surface of the virus. Influenza is then endocytosed into clathrin-coated vesicles, which later fuse with endosomal compartments (75). The low-pH environment of the endosome has been shown to be important for productive infection, as raising the pH of the endosome chemically inhibits the uncoating process (75). The low pH activates the M2 ion channel and allows H+ ions to enter the virion where the acidic environment interrupts the interaction between M1 and the RNPs as well as M1-HA interactions (74). Once the virion interior is acidified, a conformational change, in a properly cleaved HA, occurs to release a fusion peptide that intercalates into the endosomal lipid bilayer (132). After complete viral-endosomal membrane fusion, the RNPs are released into the

cytoplasm and transported to the nucleus by the polymerase and nucleoprotein molecules, each of which carries a nuclear localization signal (reviewed in (70)) (74).

Once inside the nucleus, primary transcription of specific mRNAs occurs, namely those encoding NP, NS1 and the three P proteins of the polymerase complex (86). This transcription process requires initiation by host cell-derived RNA primers. It was determined that these primers were derived from host cell RNA polymerase II transcripts because viral transcription halts when Pol II inhibitors, such as actinomycin D and α-amanatin, are present during infection (12, 56, 95, 96). The short, capped RNA primers are generated by a viral cap-dependent endonuclease. Influenza proteins have been found to bind capped cellular mRNAs through PB2 protein, part of the polymerase complex, (9, 10, 13, 130) though the endonuclease cleavage itself appears to be mediated by PB1 (65, 66). It has also been shown that polymerase complex must bind the 3′ and 5′ ends of vRNA for activation of PB1 endonuclease activity (28, 29, 37). Using these stolen host mRNA primers, synthesis of viral mRNAs is carried out by PB1 until a stretch of uridine residues are encountered just prior to the 5′ end of the vRNA template (39, 104). Stuttering occurs at this location due to tight binding of the polymerase complex to the 5′ end of the vRNA, providing the physical barrier to full-length elongation and this results in polyadenylation of the viral mRNA (68, 104).

Replication of virion RNA occurs in two steps: First, synthesis of template RNA (cRNA), a full-length copy of the vRNAs; and second, transcription of

cRNAs back to vRNA. This requires a change from cap-primed mRNA transcription to unprimed initiation and a switch from termination at the poly-U sequence to anti-termination and full-length RNA synthesis (70). The switch from mRNA transcription to replication has been shown to be dependent upon protein synthesis, and specifically depends upon synthesis of new NP molecules that would not already be associated with vRNA (6, 116, 137). It is hypothesized that interaction with free NP might somehow change the structure of the trimeric polymerase complex such that it switches from being a transcriptase to a replicase, this putative conformational change may also function in some manner to inhibit anti-termination or stuttering at the poly-U stretch (6). Although NP has never been demonstrated to bind viral RNA sequence-specifically in vitro, it does appear to associate preferentially with full-length cRNA and vRNA in infected cells (55, 97). Because of this, it has been hypothesized that the nucleation site of NP on vRNA or cRNA molecules might be their respective 5′ and 3′ ends, which are lacking in the mRNAs that, perhaps as a result, do not associate with NP.

These steps of transcription and replication, however, are not mutually exclusive. Influenza gene expression can be divided temporally into early and late phases with transcription and replication occurring in both phases to some extent (38, 116, 120). Primary transcription, as described above, is the first event to occur after RNP import into the nucleus, followed closely behind by the transcription of template cRNA, which is transcribed off parental vRNA (60, 117, 118). Each of the 8, or 7, cRNAs are synthesized in approximately equimolar

ratios during the early hours after infection, after which synthesis declines sharply. Specific vRNAs, which encode proteins important early in infection (ie. NP and NS), are synthesized first while vRNAs which encode late proteins (ie. M1) are delayed. The selective replication of vRNA from cRNA seems to control which vRNA templates are amplified and can thus be to transcribed to produce the corresponding mRNAs for protein translation. At this beginning of the late phase of infection selective replication seems to be eliminated and maximal replication of cRNA to vRNA and transcription of mRNA occurs for all of the segments. Synthesis of mRNAs then drops off dramatically but synthesis of vRNA segments continues at maximal level, accompanied by maximal translation of all proteins, particularly the late phase structural proteins, M1 and HA (120) (reviewed in(70)) (117).

Although all transcription and replication occurs in the nucleus of the host cell, it is important for these viral RNAs and proteins to leave the nucleus at various times during infection for translation of mRNAs and for packaging of genomic RNAs into nascent virion particles. The virus has multiple ways of accomplishing this export process in a carefully orchestrated manner. NS1, an early viral protein, is one of the primary regulators of viral and cellular RNA export. Like the Rev-like proteins of lentiviruses, NS1 regulates the nuclear export of mRNA; however, NS1 also inhibits pre-mRNA splicing (4, 32, 101). Both proteins have RNA binding and effector domains; however, NS1 inhibits the export of spliced mRNA and also inhibits the process of splicing whereas Rev protein facilitates the export of unspliced viral pre-mRNAs (36, 71, 72).

Therefore, in the early phase of infection, prior to NS1 translation, all viral and cellular mRNAs are exported normally, so that the translation of NS1, NP and P proteins can occur. Once NS1 is synthesized it is transported back into the nucleus and thought to be important in inducing retention of cellular mRNAs in the nucleus, where they are available for cap stealing, but are sequestered away from the splicing and export machinery. Very few cellular mRNAs are available for translation after NS1 enters the nucleus and host-cell protein synthesis essentially shuts down. NS1 is also thought to function as an mRNA export inhibitor for viral mRNAs, thus providing a mechanism by which translation of late viral proteins, such as HA, NA and M1, might be delayed until late in infection. However, the activity of NS1 would have to be inhibited at late time points in infection in order for these viral proteins to be translated. It is thought that NS1, which has been shown to exist in both phosporylated and non-phosphorylated forms, is modified posttranslationally at late time points to inhibit its function as a splicing and export inhibitor (98, 99). This would be important for activating the export of mRNAs encoding late viral proteins and also the splicing of NS and M mRNAs.

It is also important for unspliced viral RNAs to be exported at late time points of infection in order to localize them in the cytoplasm for packaging into newly synthesized virions. This process relies on a viral protein to mediate transport, as most mammalian cells do not actively export unspliced RNAs. Both NS1 and M1 mRNAs undergo alternative splicing into smaller mRNAs which encode proteins called NEP or M2, respectively (reviewed in (70)). As discussed

above, NS1 is responsible for inhibiting the splicing of cellular and viral mRNAs; however, NS1 mRNA has also been shown to contain sequences that allow it to be spliced, even in the presence of NS1 protein (67). Therefore, NS1 mRNA can be spliced, but is not exported until the NS1 protein is inactivated later in infection. At this point, NEP is translated and then transported back to the nucleus, where it appears to play a major role in export of vRNA, but not cRNA, from the nucleus (88). This mechanism of this selective export is not known. NEP has been shown to interact with the nuclear pore complex and also can function as an effector domain when fused to the RNA-binding domain of HIV Rev, creating a chimeric protein that can induce cellular export of RNAs containing the Rev Response Element (88). There is also some indirect evidence that M1 may play a role in nuclear export, as raising the pH of the endosome early in infection (thus inhibiting disruption of M1-RNP interactions) appears to keep the RNPs localized to the cytoplasm. Also, injection of M1 antibodies to the cytosol during infection appears to inhibit the transport of RNPs to the cytosol late in infection (14, 15, 74, 133).

Once RNPs are localized to the cytosol it is important that they be packaged into virion particles at the plasma membrane. Influenza buds from the apical surface of polarized cells, where the integral membrane proteins, HA, NA, and M2 are localized (11, 46, 49, 109-111). Packaging all of the necessary chromosomes to produce an infectious particle presents a problem in segmented viruses such as influenza. In RNA viruses with segmented genomes, such as reovirus, rotavirus, and orthomyxovirus (influenza), it is not known whether an

individual virion packages a random collection of viral chromosomes or if the viruses have evolved ways of selectively packaging one of each (70).

In influenza virus, there is evidence to support both the specific and random mechanisms of genomic packaging. The random packaging model is supported by the observation that the ratio of infectious particles to noninfectious ones is as low as 10%. Thus the full-complement of vRNAs could be packaged in a random manner if extra chromosomes were packaged into the virion (24). Virions have been engineered by transfection to contain two NS genes (24)and reassortants have been generated with two copies each of PB1 and NA vRNA, thus giving credence to the idea that influenza A virions are capable of packaging more than the 8 chromosomes that are necessary to create an infectious virion (114).

However, there is evidence to the contrary suggesting that influenza packages its viral genome in a segment specific manner. By isolating RNA from a population of virions it has been shown that influenza packages an equimolar ratio of each of the 8 vRNAs and that the relative amount of each genome in an infected cell does not reflect the extent to which that RNA is packaged into a virion (7, 76). Influenza defective-interfering (DI) particles can out-compete each other for packaging, in a segment-specific manner (90). The strongest support for the specific packaging model, comes from studies that passaging NS2 mutant virus at high multiplicity of infection (MOI) generates defective-interfering particles, which produce large amounts of DI particles in a single round of infection. This population of DI particles are characterized by large deletion in

the PA polymerase gene, which occurs when the DI PA gene, present at a low level in the initial viral stock, is preferentially replicated over the wild type PA gene during cRNA synthesis. The defective PA gene is an internally deleted vRNA that contains the UTRs as well as approximately 100-200 nucleotides of coding sequence on both the 3′ and 5′ ends (23, 83-85, 91). However, this increase in replication does not by itself account for the rapid population change from wild type to DI particles during a single round of infection. Odagiri and Tahsiro have shown that although replication of the wild type PA gene is only 20% that of the DI PA gene, the actual competition for packaging seems to occur at the assembly step where the wild type PA gene is packaged at only 5% of the level of the DI PA gene whereas other vRNAs are packaged at normal levels (90). In order to further support their claim that the competition occurs specifically between the DI and wild type PA vRNA at the level of packaging, Odagiri et al. constructed CAT genes with flanking UTRs from either the PA or PB2 vRNAs and transfected this RNA into cells infected with the NS2 mutant virus to determine if packaging efficiency of the CAT gene would be determined by its flanking UTR sequence. In fact, it was shown that packaging was almost undetectable when PA UTRs flanked the CAT gene, whereas a PB2-CAT RNA fusion was packaged at a high efficiency. These results give credence to the idea that the viral RNAs compete for packaging in a segment specific manner (90). However, it is important to note that this apparently competitive packaging was observed in a mutant virus and with a mutated form of the PA vRNA. Thus it

remains unknown whether specific competition occurs between like segments in the context of a wild-type virus.

The specific packaging model relies upon the notion that there are viral sequences, which presumably differ among all of the segments, that would direct packaging in a segment specific manner. By contrast, random packaging relies on the idea that there are sequences common to all the segments, which are identical though virus-specific, which allow them to be packaged into virions. Based on evidence from Luytjes et al., it appears that influenza can package a heterologous gene which is flanked only with the viral UTR sequences, which are known to include both virus-specific and vRNA-specific sequences (see Figure 2) (69). The distal 12 and 13 nucleotides of the 3′ and 5′ ends, respectively, are conserved among all segments; however, we also know that the sequences internal to those are unique to each chromosome, and thus could differentiate vRNAs for packaging.

In fact, there is much evidence that UTRs do play an important role in both replication and packaging and may interact specifically with the coding region as well. It has been shown that replacing a UTR from one segment with the UTR of another segment inhibits replication and packaging 4 to 50 fold, depending on the sequences used (140). Chimeric viral gene segments have also been created in which the NA gene from influenza A virus is flanked on both the 3′ and 5′ ends by UTRs from the NA gene of influenza B; these inter-species chimeras are not packaged into influenza A virions. By contrast, if the 3′ and 5′ UTRs from the non-structural gene (NS) of influenza B are used, the gene is

replicated and packaged, but at a decreased level as compared to the wild type segment (81). This suggests that the non-conserved UTR sequences and the coding region of each segment interact specifically in some unknown way to provide signals for efficient replication and packaging of a particular gene segment.

There is a great deal of evidence that the nonconserved region of the UTR is important for both replication and packaging. However, identification of sequences important for packaging are confounded because genetic approaches to map the packaging signals also tend to destroy or impair the promoter, which is crucial for normal replication levels (30). In chapter 2, I will discuss more about the determinants of influenza A gene packaging and studies that I carried out to examine whether packaging occurs in a competitive or random manner.

Influenza begins its infectious cycle by binding to cell surface glycoproteins bearing either neuraminic acid (sialic acid, SA) or 9-O-acetyl-N-neuraminic acid for influenza A and C, respectively (44, 106). This binding is mediated by the Hemagglutinin (HA) molecule in the case of influenza A and B or by the Hemagglutinin Esterase Fusion protein (HEF) for influenza C virus. Both HA and HEF must be processed from precursor molecules into two functional subunits by a trypsin-like protease (62, 70, 92). This cleavage can occur either intracellularly, when the appropriate protease is present in the Golgi, or in the extracellular environment following virion release. The specificity of HA binding to specific surface glycoproteins has not been explored in depth; however, there is evidence that human influenza A virus prefers to

bind to SA residues linked by NeuAc ∝2,6Gal linkage, whereas avian influenza appears to prefer a NeuAc∝2,3Gal linkage. The specificity is determined by a single amino acid in the HA molecule at position 226 (108). This specificity, along with limited expression of these molecules in certain hosts, appears to be one determinant of host range for influenza viruses. In fact, a molecular basis for the observation that pigs are susceptible to infection by both human and avian viruses is revealed by staining pig trachea with lectins that specifically bind to ∝2,6 or ∝2,3 linkages; such studies have shown that both types of sialic acid linkages occur in pigs, whereas avian hosts and human hosts both each express only one or the other (48, 124). Because receptor specificity seems to be determined by only a single amino acid, viruses that are passaged in the context of only one linkage type evolve such that binding will occur in the new environment. This has been documented by passaging viruses harvested from throat washing of human patients in amniotic, allantoic egg compartments, and MDCK tissue culture cell lines. Resultant stocks of viruses acquire receptor specificity for the environment in which they have been passaged.

Although receptor specificity at the level of single sialic molecule has been investigated, the specificity in terms of the preferred glycoprotein backbone has been less studied. Reading et al. have recently shown that influenza A appears to utilize the mannose receptor on the surface of macrophages as its primary functional receptor. They have shown that it is possible to block infection when including mannose as a competitor for binding during infection and have also shown a titratable effect of increased mannose expression correlating with

increased infectivity. Thus, in both primary macrophages and macrophage cell lines, it seems that influenza shows a specific affinity for the mannose receptor as a functional receptor for infection (102). Interestingly, this implies that although many cell surface proteins could serve as receptors, simply by virtue of expressing sialic acid residues, there appear to be additional characteristics contributed by the glycoprotein backbone which may make a specific glycoprotein a preferred and functional receptor.

In Madin-Darby Canine Kidney (MDCK) cells, a laboratory cell line typically used for influenza infections, it has been shown that influenza C virus utilizes a specific glycoprotein to mediate the majority of surface binding. A novel cell surface glycoprotein, gp40, was characterized in this cell line for its ability to bind influenza C virus in a nitrocellulose binding assay (141). A sub-line of MDCK cells, designated MDCK II, which are not infectable with influenza C virus and do not express gp40, in turn do not bind virus in an in vitro assay. Stable transfection of gp40 into MDCK II cells rescues binding in the in vitro assay; therefore, it has been hypothesized that gp40 is necessary for influenza C binding and and possibly infection in this cell line (142). In order to characterize the gp40 molecule, cells were treated with a variety of proteases that target specific classes of glycoproteins and binding efficiency was once again measured. It was found that both neuraminidase and O-sialoglycoprotein endopeptidase (OSGE) treatment inhibit binding significantly (141). Neuraminidase targets sialic acid residues overall, whereas OSGE specifically targets O-linked mucin-type glycoproteins expressed on the cell surface; therefore, gp40 has been

designated a member of this class (1, 77). Although, gp40 has not been directly shown to be a functional receptor in MDCK I cells, characteristics of mucin-class glycoproteins might make them attractive as influenza virus receptors.

Mucin family glycoproteins are highly branched, O-linked cell surface glycoproteins which contain multiple serine and threonine residues, which are glycosylated and terminate in sialic acid residues (reviewed in(16)). This branched structure with multiple sialic acid residues localized on one molecule could theoretically provide an environment which would allow for a multi-valent interaction between multiple hemagglutinin molecules and multiple sialic acid residues on one glycoprotein. This preferential binding to clusters of sialic acid residues on sialoglycoconjugates and mucins is also thought to be important for infection by simian rotaviruses (134). Because sugar-protein interactions are known to be relatively weak, multiple interactions would facilitate binding between influenza virus and a cell surface receptor. In Chapter 3, I will discuss work done to investigate the possibility that mucin-family glycoproteins serve a role as primary functional receptors for both influenza A and C viruses in two different cell lines. In chapter 4, I will discuss the results and conclusions from the projects described in chapters 2 and 3.

Chapter 2

Evidence For Chromosome-Nonspecific Packaging Of The Influenza A Genome

The genome of influenza A virus consists of eight single-stranded, negative-sense RNA chromosomes (vRNAs) that together encode the eleven known viral proteins (17, 58). To be infectious, an influenza A viral particle must package at least one copy of each chromosome. *Cis*-acting signals necessary for packaging appear to reside within the 3′ and 5′ untranslated regions (UTRs) of each vRNA (69) -- regions that also control vRNA transcription and replication by the viral RNA-dependent RNA polymerase (93). In the influenza strain A/WSN/33, for example, these UTRs include 12 and 13 bases at the extreme 3′ and 5′ ends, respectively, that are common to all vRNAs, as well as the adjacent 7-45 bases at each end whose sequence is unique to a given chromosome and highly conserved in other strains. When attached in appropriate pairs to the ends of a heterologous RNA, the UTR sequences are sufficient to direct packaging into influenza virions, and also to support subsequent rounds of replication (69). At least some specific bases required for packaging occur within the region that mediates polymerase binding (34, 64, 127), which presumably implies that interaction with the viral polymerase is an essential requirement for packaging.

One fundamental question in influenza genetics is whether the virus discriminates among its chromosomes during the packaging process. While a

variety of mechanisms could be envisioned, the issue is often framed in terms of two simple, hypothetical models that are intended to represent the extreme alternatives. Under the first model, called random packaging, each virion selectively incorporates viral chromosomes (as opposed to other viral or cellular RNAs) but does not differentiate among them, so that the likelihood of acquiring the full complement of vRNAs is determined entirely by chance. The second model, by contrast, asserts that the virus targets each of the eight chromosomes individually and independently, a phenomenon called specific packaging. The evidence in support of either model is limited and inconclusive. Evidence for specific packaging, for example, is adduced mainly from the finding that the various vRNAs are equimolar within viral particles even though their concentrations in the infected cells may differ (120), and from several reports that defective-interfering vRNA mutants can competitively inhibit packaging of their normal counterparts but not of other vRNAs (23, 83-85, 91).

The recent development of efficient, plasmid-based systems for assembling infectious influenza A virions now provides an opportunity to dissect the packaging mechanism in detail (43, 87). As an initial step, we have developed a quantitative assay in which pairs of genetically-marked vRNAs compete for packaging into transduction-competent particles. Here we report that, in this reverse genetic system, the influenza chromosomes compete for packaging, but do so in a random, non-specific manner.

Materials and Methods

Cells. 293T cells were grown in DMEM H-21 supplemented with penicillin and streptomycin (pen-strep), glutamine, and 10% heat-inactivated fetal calf serum (FCS). Uninfected MDCK cells were grown in Eagle's MEM supplemented with pen-strep, glutamine, non-essential amino acids (NEAA; Gibco/BRL), and 10% FCS. After infection, MDCK cells were maintained in L-15 medium supplemented with pen-strep, glutamine, NEAA, 15 mM HEPES (pH 7.5), 0.75 g/L $NaHCO_3$, and 0.125% (w/v) bovine serum albumin (BSA).

Construction of GFP and YFP reporters. The 17-plasmid influenza assembly system was a generous gift from Dr. Y. Kawaoka (Univ. of Wisconsin), and was used essentially as described (87). We created five chromosome-expression reporter plasmids by first deleting all vRNA sequences from the wild-type neuraminidase (NA) vRNA vector through polymerase chain reaction (PCR) amplification using the primers 5′-CGGCGGCGGGGTACCGTCTCCTCCCCCCCACCTTCGGAGGTCGACCAGT-3′ and 5′-CGGCGGCGGGGTACCGTCTCCAATAACCCGGCGGCCCAAAATGCC-3′, digesting with KpnI, and recircularizing by ligation. The resulting vector is equivalent to the plasmid pHH21, described in Neumann et al. (42, 87). We cleaved this vector at its two BsmB1 sites and inserted coding sequences for the green (GFP) and yellow (YFP) fluorescent proteins of *Aequorea victoria* that had been PCR-amplified from the plasmids EGFP-N1 or EYFP-N1 (Clontech), respectively, using primers that added flanking influenza-A UTRs and a BsmB1

site at each end. The NA reporters encoding GFP and YFP were created with primers 5′-CGGCGGCGGCGTCTCGTATTAGTAG AAACAAGGAGTTTTTTGAACAAATTACTTGTACAGCTCGTCC-3′ and 5′-CGGCGGCGGCGTCTCGGGGAGCGAAAGCAGGAGTTTAAATGGTGAGCA A GGGCGAGGAGC-3′. Nucleoprotein (NP) vRNA reporters were made with primers 5′-CGGCGGCGGCGTCTCGTATTAGTAGAAACAAGGGTATTTTTCTTTACTTGT ACAGCTCGTCC-3′ and 5′-CGGCGGCGGCGTCTCGGGGAGCAAAAGCAGGG TAGATAATCACTCACAGAGTGACATCGAAATCATGGTGAGCAAGGGCGA GG AGC-3′. The nonstructural (NS) vRNA reporter was made with primers 5′-CGGCGG CGGCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTTATTATTACTTGTACA GCTCGTCC-3′ and 5′-CGGCGGCGGCGTCTCGGGGAGCAAAAGCAGGGTGACAAAGAC ATAATGGTGAGCAAGGGCGAGGAGC-3′. NA and NP "competitor" vectors, encoding red fluorescent protein (RFP) from *Discosoma sp*, were created in an identical manner using PCR primers that amplified the entire 680-nucleotide RFP coding sequence from pDsRed-N1 (Clontech).

Generation of infectious influenza particles. 293T cells were transfected in 35-mm dishes at approximately 50% confluency, using Trans IT LT-1 transfection reagent (Panvera, Madison, WI). Following the protocol of Neumann et al. (87),

every transfection included 1 μg each of the eight wild-type vRNA vectors; 1 μg each of expression vectors for PB1, PB2, NP, NA, HA, and NS2 proteins; 0.1 ug each of vectors for M2 and PA proteins, and 2 μg of the M1 protein vector. To this mixture, we added various combinations of vRNA reporters as indicated in the text, along with sufficient pHH21 to maintain total DNA input constant in a given experiment. Transfection reagent (2 μl per μg of DNA) was added to 100 μl of serum-free Opti-MEM, mixed, and incubated at room temperature for 15 min. DNA was then added, mixed by pipetting, and allowed to sit at room temperature for 15 min before adding to cells. Six hr later, transfection mixture was removed, and cells were washed once and then refed with Opti-MEM containing 0.3% BSA and 0.01% FCS. Supernatants were collected at 48 hr post-transfection, clarified by microcentrifugation for 5 min, and then used for infection of MDCK cells either immediately or after storage at –80°C.

Infection of MDCK cells. Confluent monolayers of MDCK cells in six-well plates were infected with 293T supernatants. MDCK cells were washed once with phosphate-buffered saline containing calcium and magnesium (PBS+CM), inoculated with 250 μl of 293T supernatant, and incubated for 1 hr at 37°C. Viral innoculum was removed, and cells were then washed once with PBS+CM and refed with L-15 medium.

Flow cytometry. Infected cells were harvested 10 hr post-infection by removing medium, washing once with calcium- and magnesium-free PBS, and digesting with trypsin until they detached from the plate. Cells were then washed once with PBS+CM, pelleted by centrifugation, and resuspended in 2 ml ice-cold

PBS+CM for analysis. Flow cytometry was performed using a FACSCalibur cytometer (Becton Dickson) with a 525-nm shortpass dichroic filter to separate the signals and paired 550/30-nm and 510/20-nm bandpass filters to collect them.

Quantitative analysis of vRNAs in tranfected 293T cells. Reporter-derived vRNAs were quantified using a ribonuclease-protection assay (RPA) that detected antisense or sense forms of both the GFP and YFP sequences, but not of the red fluorescent protein (RFP) or of any authentic influenza vRNA. Primers 5′-CCCCCCAAGCTTGAAGGCT ACGTCCAGGAGCGC-3′ and 5′-CCGCCGGAATTCTCCTCGATGTTGTGGCGG-3′ were used in a PCR reaction with pEYFP-N1 (Clonetech) as template to amplify residues 271-521, which are common to both the GFP and YFP sequences. The PCR product was digested with EcoR1 and Hind3 and cloned into pBluescript II KS+ (Stratagene). This RPA plasmid was linearized with EcoRI or HindIII, and transcribed in vitro with T3 or T7 RNA polymerase and $\alpha[^{32}P]$-UTP. Total cellular RNA was isolated from 293T cells 48 hr after transfection, using TRIZOL (Gibco/BRL) according to the manufacturer's instructions. The RPA III kit (Ambion) was used to perform RPA with 10 µg of input RNA and 80,000 cpm of probe per reaction.

Results and Discussion

Influenza A virus will package and replicate heterologous RNA molecules that carry viral UTR sequences at their 3′ and 5′ ends. Such artificial chromosomes can also be packaged by influenza virus-like particles assembled using plasmid-based transfection systems. For the present study, we utilized the assembly system developed by Kawaoka and colleagues (87), which is based on 17 plasmid expression vectors that together encode the 8 wild-type vRNAs and 9 viral proteins needed to assemble an influenza A virion. We confirmed that, when 293T cells were transfected with these plasmids in the recommended stoichiometries (see Materials and Methods), infectious influenza A particles were efficiently produced, accumulating over 48 hr to concentrations of roughly 10^6 plaque-forming units (pfu)/ml in the 293T supernatant. Unless otherwise noted, this standard mixture of 17 vectors was included in all our transfections to provide a fixed supply of virion components, and other plasmids were added as detailed below.

The vRNA-expression vectors are derivatives of pHH21 (42, 87), which uses a mammalian RNA polymerase I promoter and terminator to transcribe the appropriate viral coding and UTR sequences. We used this backbone to create five new reporter vectors (Table 1) each specifying a variant of one of the three vRNAs that normally encode the viral neuraminidase (NA), nucleoprotein (NP), or nonstructural (NS) proteins, respectively. These reporter vRNAs had the same UTRs as their wild-type counterparts but lacked any viral genes; instead, they contained the 719-base negative-sense coding sequences for either the green

TABLE 1. Reporter vRNAs used in this study [1]

Construct	
NP-Y or NP-G	3′ UCGUUUUCGUCCCAUCUAUUAGUGAGUGUCUCACUGUAGCUUUAG [antisense GFP or YFP] UCUUUUUAUGGGAACAAAGAUGA 5′
NA-Y or NA-G	3′ UCGCUUUCGUCCUCAAAUU [antisense GFP or YFP] AAACAAGUUUUUUGAGGAACAAAGAUGA 5′
NS-G	3′ UCGUUUUCGUCCCACUGUUUCUGUAU [antisense GFP or YFP] AUUUUUUGUGGGAACAAAGAUGA 5′

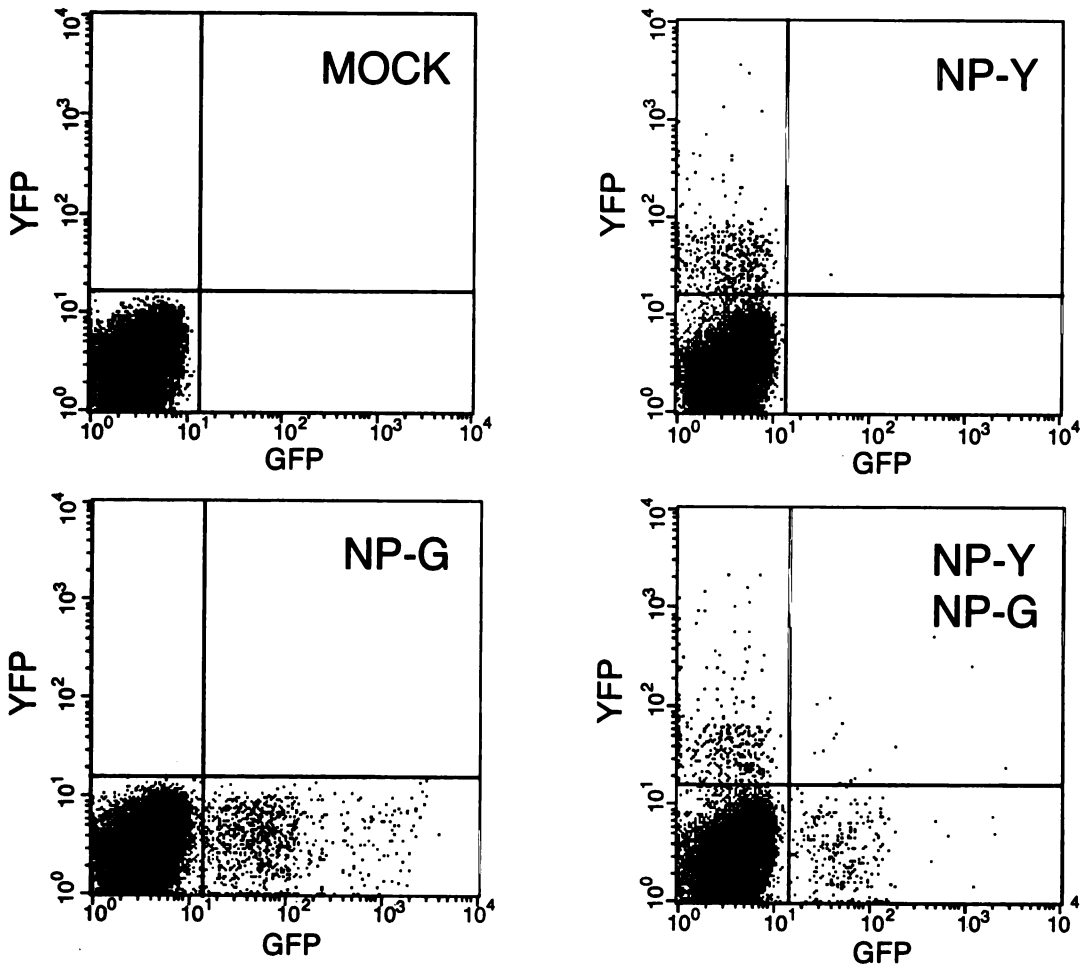
[1] Sequences shown are for the vRNAs expressed from each of the 5 reporter plasmids under control of the human PolI promoter and mouse PolI terminator elements. Each contains the 719-base coding sequence of GFP or YFP in antisense orientation, flanked by the 3′ and 5′ UTR sequences shown. The latter are derived from Influenza A/WSN/33. The conserved 3′ and 5′ bases common to all flu RNAs are underlined.

(GFP) or yellow (YFP) fluorescent proteins, which are identical at all but 7 bases. We thus obtained two alternatively-tagged NP reporters (NP-G and NP-Y), two NA reporters (NA-G and NA-Y), and a single NS reporter (NS-G). For purposes of this study, pairs of reporters that share the same UTRs will be termed "homologous," whereas those with dissimilar UTRs will be called "non-homologous" reporters.

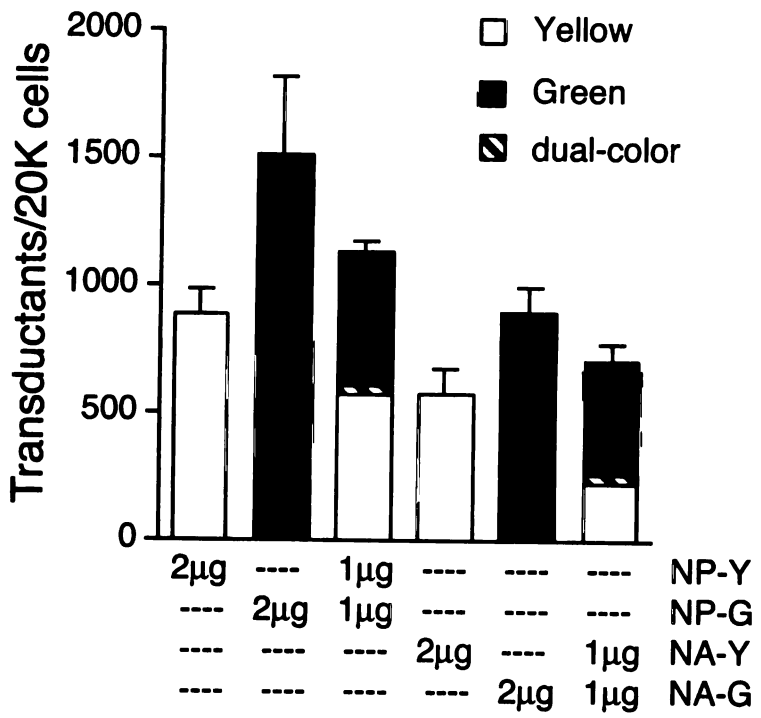
To verify that the reporters were biologically active, we transfected each into 293T cells along with the 17 wild-type vectors, collected supernatants 48 hr later, and used them to infect MDCK cells at a ratio of 0.1-1.0 pfu/cell. Ten hours later (to allow time for primary infection but not for secondary spread), the MDCK cells were harvested and screened individually for fluorescence using two-color flow cytometry. As illustrated in Figure 3A, roughly 3-8% of MDCK cells exposed to virions that had been assembled in the presence of any single reporter vRNA exhibited significant yellow or green fluorescence, implying that each of these cells had been infected by a virion carrying the reporter vRNA, which had then been transcribed by the viral polymerase to yield positive-sense mRNAs. The absolute number of transductants (i.e., fluorescent MDCK cells) obtained with a given reporter varied somewhat among experiments, presumably due to varying growth states of the cell populations, but it was highly reproducible within any single experiment (Figure 3B). Moreover, when viruses were assembled using two homologous GFP and YFP reporters (e.g., NP-Y and NP-G) in combination, we could readily discriminate two subpopulations of transductants that each expressed only one fluorescent marker or the other, as

Figure 3. A quantitative assay for influenza vRNA packaging. **(A)** Immunofluorescence flow cytometry of MDCK cells infected with plasmid-derived influenza virions. Cultured 293T cells were transfected with the 17-plasmid assembly system supplemented with 2 μg NP-Y, with 2 μg NP-G, with 1 μg each of NP-Y and NP-G, or with 2 μg of the control plasmid pHH21. Supernatants harvested 48 hr later were used to infect MDCK cells, which were themselves harvested and analyzed by flow cytometry 10 hr post-infection. Each point represents one of 20,000 viable MDCK cells from each population, analyzed for green (GFP) and yellow (YFP) fluorescence, each expressed on an exponential scale. The four quadrants of each plot represent (clockwise from lower left) nonfluorescent, yellow, dual-colored, and green fluorescent MDCK cells, respectively. Data from one representative experiment. **(B)** Quantitative transduction data for viruses prepared using the indicated amounts of various reporter plasmids. Each bar indicates the numbers of yellow (open), green (filled), and dual-colored (hatched) transductants obtained per 20,000 MDCK cells. Data are mean ± standard error of the mean (SEM) for triplicate transfections in a single experiment. Statistically significant differences between yields from homologous reporters were observed in some individual experiments, as here, but were not reproducible (see Table 2).

A)



B)

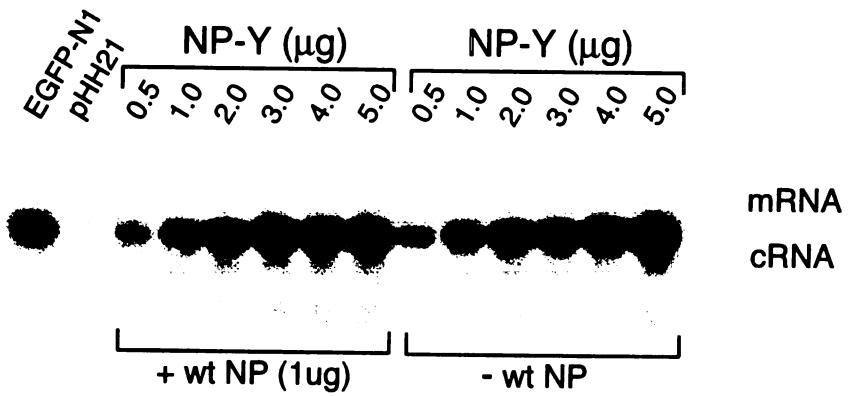

Transductants/20K cells
2000
1500
1000
500
0
Yellow
Green
dual-color
2µg ---- 1µg ---- ---- ---- NP-Y
---- 2µg 1µg ---- ---- ---- NP-G
---- ---- ---- 2µg ---- 1µg NA-Y
---- ---- ---- ---- 2µg 1µg NA-G

well as a small minority that expressed both (Figure 3A and 3B). These results confirmed that our reporter vRNAs could be packaged and transduced by influenza virions, and that the fluorescence they conferred could be used to precisely enumerate transduced cells during the first round of infection.

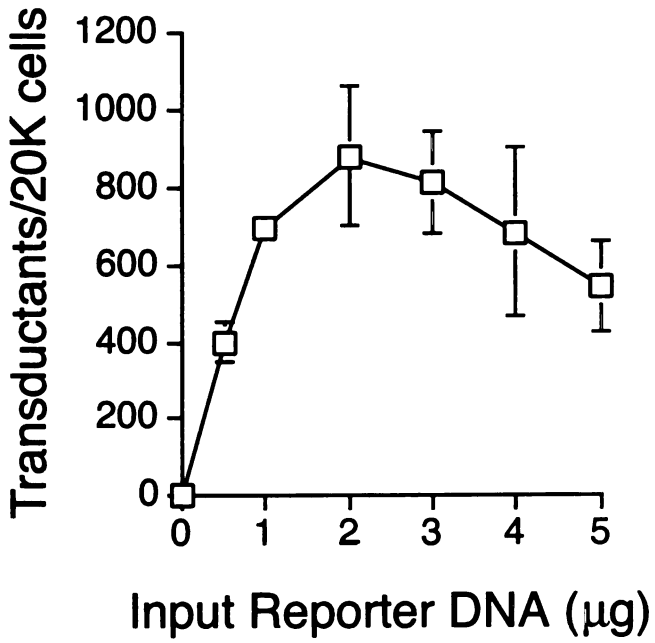
To determine whether this could be exploited as an assay for packaging, we first asked whether transduction frequency depended on the amount of reporter DNA introduced into the 293T producer cells, where all relevant packaging occurred. To that end, we transfected 293T cells with various amounts (0.5-5.0 μg) of NP-Y reporter along with the standard 17 plasmids, and harvested the cells and their supernatants 48 hr later. We then extracted RNA from these cells and probed it for reporter-derived sequences by using an RNase-protection assay (RPA). As shown in Figure 4A (left), positive-sense reporter RNAs (i.e., mRNAs and cRNAs) were readily detectable in amounts that increased in proportion to vector DNA input up to the highest level tested (Figure 4A). Similar results were also obtained with the NA-based reporters (data not shown). This indicated that transfection efficiency and reporter transcription in the 293T cells were not limiting within this range of vector input. When we measured the transducing activity of supernatants from these cells (Figure 4B), however, we found that the yield of fluorescent MDCK cells rose to a maximum with transfection of 1-2 μg of reporter DNA and then appeared to plateau, suggesting that the capacity of the virions to incorporate reporter vRNAs had reached saturation. The yield of transductants then declined somewhat at the highest plasmid dosages, perhaps indicating that highly overexpressed reporter vRNAs could competitively inhibit

Figure 4. Saturable packaging and transduction of reporter vRNA. **(A)** Dose-dependent expression of reporter transcripts. 293T cells were transfected with the indicated amounts of NP-Y reporter vector along with either all 17 standard plasmids (at left) or all except the wild-type NP vRNA vector (at right). Cellular RNA harvested 48 hr post-transfection was probed for positive-sense reporter transcripts (mRNA and cRNA) by RPA. Control cells were transfected either with a GFP-mRNA expression vector (EGFP-N1) or with pHH21. **(B)** Virions isolated from supernatants of the same cells (whose transfections included the wild-type NP vRNA vector) were assayed for MDCK-transducing activity at 10 hr post-infection, using flow-cytometry. **(C)** Total plaque-forming activities of the supernatants in panel B, assayed in MDCK monolayers.

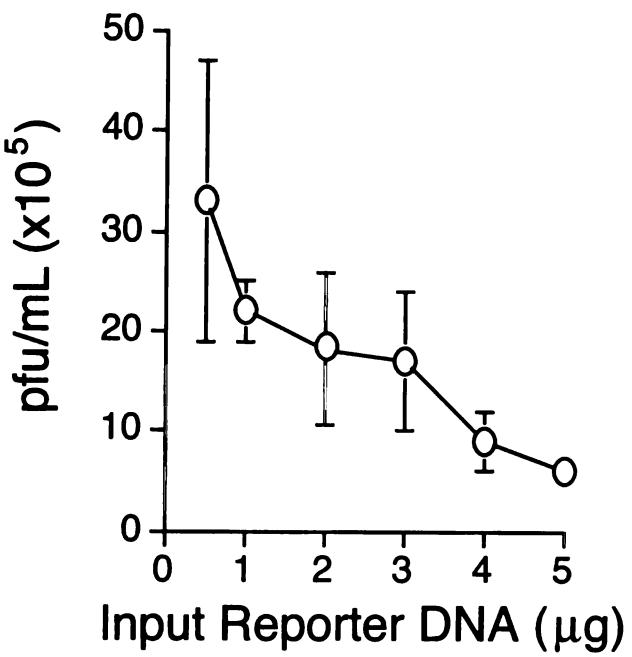
A)


EGFP-N1
pHH21
NP-Y (µg)
0.5
1.0
2.0
3.0
4.0
5.0
NP-Y (µg)
0.5
1.0
2.0
3.0
4.0
5.0
mRNA
cRNA
+ wt NP (1ug)
- wt NP

B)


Transductants/20K cells
1200
1000
800
600
400
200
0
0
1
2
3
4
5
Input Reporter DNA (µg)

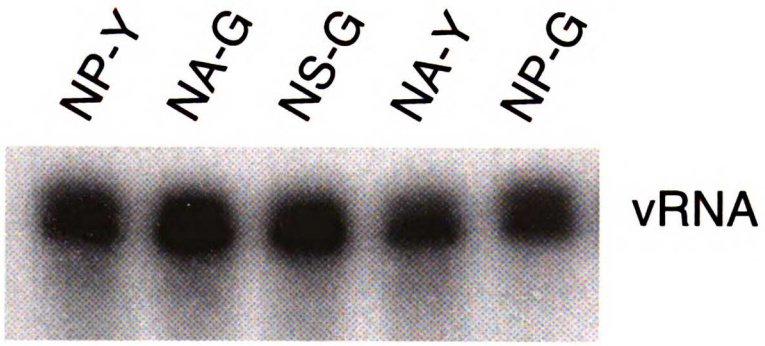
C)



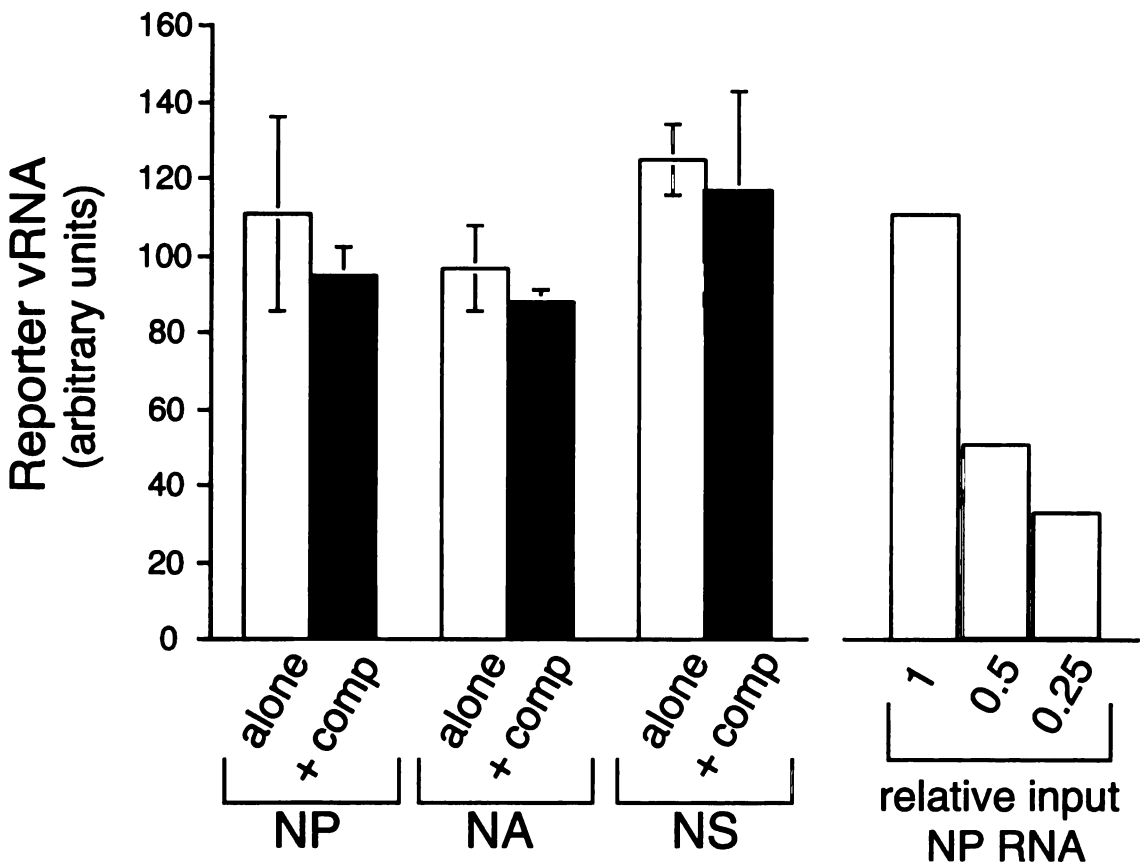
packaging of the wild-type vRNAs needed for transduction or infectivity. Consistent with that interpretation, we found that the plaque-forming titers of these same supernatants likewise tended to decline as reporter input increased (Figure 4C). Because our planned experiments involved cotransfecting various pairs of reporters into 293T cells, we next tested whether these vectors interfered with each other at the level of vRNA expression. Using an RPA probe that detected negative-sense sequences common to both the GFP and YFP coding region, we first measured the levels of vRNA produced by 1 μg of each of the five reporters transfected individually, and found that they were similar (Figure 5A). We then compared the effects of transfecting 1 μg of each reporter either alone or in combination with 1 μg of a second "competitor" vRNA vector in which UTRs from the NP or NA chromosomes flanked the coding sequence of the red fluorescence protein (RFP), which could not be detected by the RPA probe. In repeated transfections, we tested all possible pairwise combinations of the five reporters with the two competitor vectors, and assayed reporter vRNA by RPA. Because we observed no significant differences in the effects of the two competitors, or between homologous reporters, the data were pooled. As depicted in Figure 5B (at right), serial dilution of the input RNA confirmed that the RPA could readily detect a 50% decrease in reporter vRNA expression in the 293T cells. Nevertheless, we found no evidence that expression of any reporter vRNA was affected by cotransfecting a second vRNA vector, regardless of whether the two were homologous or non-homologous (Figure 5A, at left). This was consistent with our earlier finding that omitting the wild-type NP vRNA

Figure 5. Reporter vRNAs are comparably and independently expressed in 293T cells. **(A)** RPA of reporter-specific negative-sense RNA (vRNA) in 293T cells transfected 48 hr previously with 1 μg of each indicated reporter plasmid along with the 17-plasmid assembly system. **(B)** Reporter expression in 293T cells 48 hr after transfection with 1 μg of each indicated reporter type either alone (alone) or in combination with an NA- or NP-based competitor plasmid encoding RFP (+ comp). Levels of vRNA were assayed by RPA, using a probe that detects GFP and YFP but not RFP sequences, and quantified by phosphorimaging. Each of the five reporters was tested alone (in quadruplicate) and with each of the two competitors in duplicate; data shown are mean ± SEM, pooled for homologous reporters. At right, titration of one representative cellular RNA sample to determine sensitivity of the assay.

A)



B)


Reporter vRNA
(arbitrary units)
160
140
120
100
80
60
40
20
0
alone
+ comp
alone
+ comp
alone
+ comp
NP
NA
NS
1
0.5
0.25
relative input
NP RNA

vector from the standard transfection did not appreciably affect expression of NP-Y reporter RNA (see Figure 4A, at right).

The foregoing results together indicated that, when transfected into 293T cells, all five reporters generated comparable amounts of genetically-tagged vRNA that could be packaged and transduced. When the total amount of reporter plasmid introduced was on the order of 1-2 μg, these vRNAs appeared to be expressed independently, in that expression of any one reporter did not detectably influence the expression of other reporters in the 293T cells, regardless of whether they carried the same or different UTRs. The quantity of virions produced by the standard transfection of these cells, moreover, had a finite capacity to transduce any single reporter vRNA. This capacity was 70-80% saturated by 1 μg and fully saturated by 2 μg of input reporter plasmid (Figure 4B), and presumably reflected the finite quantity of any single reporter vRNA that could be packaged into transduction-competent virions.

The saturable nature of this phenomenon offered the chance to ask whether non-homologous reporters – i.e., those with dissimilar UTRs -- competed for a common niche (as would be predicted by the random packaging model) or instead for independent, chromosome-specific niches (as expected for specific packaging) within the assembled virions. We addressed this question using a combinatorial approach, by comparing the transduction efficiency of each reporter when transfected either singly or together with any other reporters that carried the alternative fluorescence marker. In simultaneous transfections on a single day, all possible binary combinations of reporters were tested, which

ensured that every YFP reporter was individually paired with every GFP, and also that each reporter was paired with one homologous reporter as well as with one or two non-homologous reporters. By repeating this entire experiment three times, we obtained a dataset of triplicate measurements for each of the twelve possible reporter combinations, and also for 1-μg and 2-μg transfections of each reporter alone. As in our preliminary studies (Figure 4B), we found that 1 μg of any single reporter yielded 70-80% as many transductants as 2 μg (data not shown); mean values for the three experiments ranged from 72% for NPG to 79% for NAG. This confirmed that packaging of each of the five vectors was near saturation in these studies. We then analyzed this dataset with regard to two separate parameters for which the random and specific packaging models gave divergent predictions.

In the first analysis, we compared the total yield of transductants produced by cotransfecting pairs of homologous or nonhomologous reporters. Under the random packaging model, where all reporters compete equally for a common niche, the yield of transductants from 1 μg of any two reporters is expected to approximate the yield from 2 μg of either reporter alone, since 2 μg is sufficient to saturate the niche. (To the extent that the apparent niches for two reporters are not identical, the predicted value for the pair would lie in the range between their individual yields.) Under specific packaging, this same prediction applies only to homologous vRNAs, whereas the yield from any pair of non-homologous reporters, by contrast, is expected to equal the sum of the yields from 1 μg of each reporter alone. Table 2 presents the relevant data from our study, with actual

Table 2. Cotransfection of Homologous and Non-Homologous Reporter Pairs in Near-Saturating Amounts.

Reporter Pairs	Expt.	Total Transductant Yield [1] Predicted: Random Model [3]	Predicted: Specific Model [4]	Actual [2]
Homologous				
NP-Y + NP-G	1	2331-2334	same	2280
	2	1545-2479	same	1769
	3	1103-1300	same	1206
NA-Y + NA-G	1	1013-1308	same	1360
	2	742-1977	same	1077
	3	589-1033	same	1229
Nonhomologous				
NP-Y + NA-G	1	1013-2334	2509	1835
	2	1545-1977	2717	1966
	3	589-1103	1270	1125
NP-Y + NS-G	1	848-2334	2349	1930
	2	1486-1672	2708	1589
	3	642-1103	1156	849
NP-G + NA-Y	1	1308-2331	3033	2198
	2	742-2479	2071	1400
	3	1033-1300	1576	999
NA-Y + NS-G	1	848-1308	1741	1312
	2	742-1672	2076	1050
	3	642-1033	989	847

[1] Includes yellow, green and dual-colored MDCK transductants, per 20,000 cells.

[2] Yield obtained by cotransfecting 1 μg each of the 2 plasmids indicated.

[3] Predictions under the random model were calculated as the range between the the yields obtained by transfecting 2 μg of either the GFP (underlined) or YFP plasmid alone in the same experiment. For statistical analysis, sign test was performed with respect to the center (mean) of the range.

[4] For nonhomologous pairs, predictions under the specific model represent the sum of the yields obtained by transfecting 1 μg of each plasmid alone in the same experiment. For homologous pairs, the predictions are the same as under the random model.

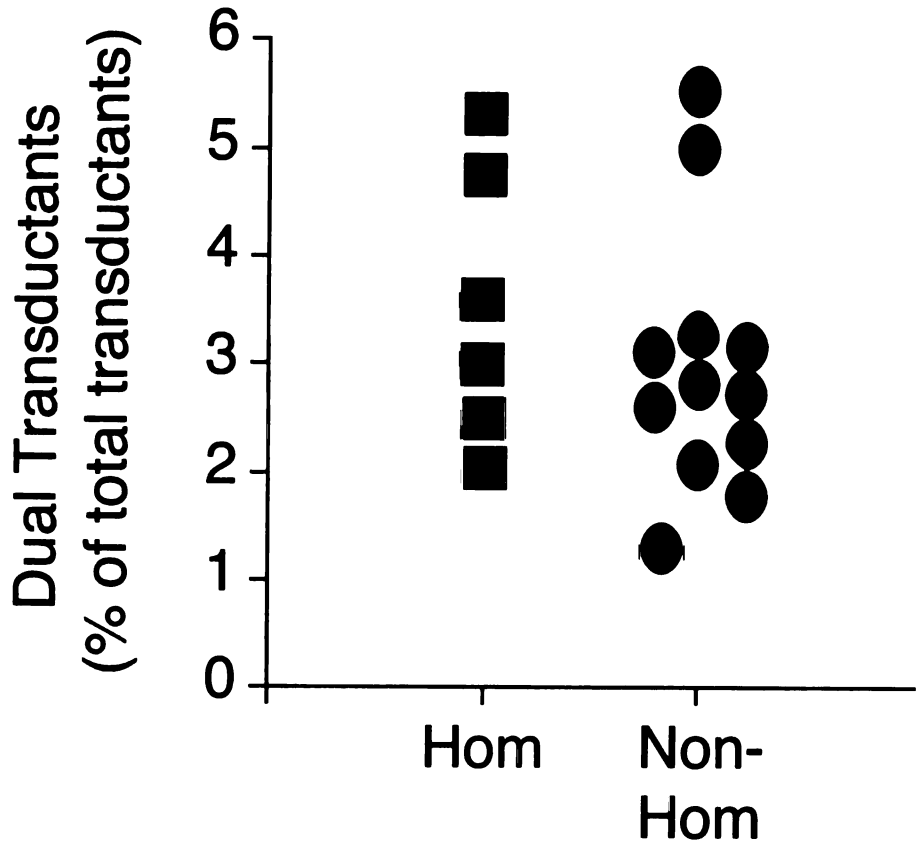
transductant yields listed alongside those predicted by the random and specific models. Among the 18 transfections listed, which include both homologous and nonhomologous vRNA pairings, 12 gave transduction yields that fell within the range predicted under the random model; of the remainder, 4 exceeded this range and 2 fell below it. Based on those results, the random model could not be excluded on statistical grounds, either for the entire group ($p = 0.12$ by sign test) or for either the homologous or nonhomologous groups individually ($p = 0.31$ and 0.12, respectively). Of the nonhomologous vRNA pairs, however, all 12 produced transductant yields lower than that predicted for specific packaging ($p < 0.001$). The results of this analysis are therefore statistically compatible with the random but not with the specific model.

Our second analysis focused on virions that co-packaged and transduced two reporter vRNAs simultaneously, producing single infected MDCK cells that expressed both yellow and green fluorescence. We had previously observed a small percentage of such dual transducers among virions carrying homologous pairs of NP or NA reporters (Figure 3B); the low MOI used in these studies made it unlikely that these were an artifact of dual infection. Neither the random nor the specific packaging model can be used to predict the absolute frequency of dual transductions *a priori*. Under the specific model at saturating conditions, however, copackaging would be expected to occur more frequently with nonhomologous than with homologous vRNAs, since only the latter would compete for packaging into a common niche. Random packaging, on the other hand, predicts equal frequencies with both sets of vRNAs. The actual

frequencies of dual transductions from our dataset are depicted in Figure 6. The frequencies observed for homologous and nonhomologous reporters were statistically indistinguishable ($p = 0.30$ by Student's T-test), though there was a nonsignificant trend toward lower frequencies with nonhomologous (mean 2.9% ± 0.37 SEM) as compared to homologous (3.6% ± 0.56) reporter pairs for the study as a whole.

Together, these results indicate that vRNAs may compete for packaging into influenza virions but that this competition is not vRNA-specific. Certain limitations of our study must be noted. First, the virions we examined were produced using a plasmid-directed assembly system. Although this system yields authentic, replication-competent virus, the first round of assembly and packaging events which we studied involve vRNAs and proteins expressed from heterologous promoters, and may not accurately mimic those of a natural infection. While previous studies of influenza packaging have often focused on viral supernatants, which contain a preponderance of nonviable particles, ours scored only particles that could support at least one round of infection and transduction, and hence had some level of biological activity. The proportion of those particles that were fully infectious and replication-competent, however, is unknown and likely to be small (data not shown). We do not know whether a single transduced copy of reporter vRNA can confer detectable fluorescence; if multiple copies must be transduced by one virion, specific packaging of the first reporter might be obscured in our analysis by subsequent copies packaged randomly. Our analysis, moreover, considered only three of the eight vRNAs

Figure 6. Frequency of dual-transductants. Dual-transductant MDCK cells are expressed as a percentage of total transductants for combinations of homologous (Hom) and nonhomologous (Non-Hom) reporters. Data are pooled from three independent experiments.


Dual Transductants
(% of total transductants)
6
5
4
3
2
1
0
Hom
Non-
Hom

and only the extreme alternatives of random or specific packaging. We cannot exclude more sophisticated models in which particular vRNAs might be packaged in a hierarchical, length-dependent, or interdependent manner.

Our results nevertheless add to the preponderance of evidence that influenza packages its chromosomes nonspecifically. The recent finding that critical packaging signals map to within the 3' UTR sequences common to all influenza vRNAs lends particularly strong support to this view (127). Though seemingly inefficient, a random packaging mechanism could in theory suffice if each influenza virion incorporates, on average, more than eight vRNAs. Other authors have calculated, for example, that random packaging of 10 to 12 vRNAs per particle would enable 2.8-9.3% of virions to acquire the full complement of eight (24, 115); those values are compatible with the estimated 5-10% ratio of infectious to noninfectious particles found in actual influenza populations (19, 59).

Two separate groups have isolated and characterized influenza strains carrying at least nine or ten vRNAs, respectively (24, 114), and the maximum number that can be accomodated stably is unknown. In this regard, we note that when we formed viruses in the presence of both GFP- and YFP-tagged versions of a given vRNA at equal concentrations, roughly 3-5% of transduction-competent virions incorporated both reporters (Figures 3 and 6). Assuming that the two reporters were packaged equivalently, this implies that an additional 3-5% of virions carry two (or more) copies of each reporter alone, so that a total of 9-15% of the population carries at least two reporter vRNAs. Under a random

packaging scheme, the probability (p) that a given virion will encapsidate r copies of any given vRNA can be calculated using the equation:

$$p(r) = \left[\frac{b!}{(b-r)!\, r!} \right] (1/k)^r \, (k-1/k)^{b-r}$$

where b = the number of vRNAs acquired randomly by each virion, and k = the number of unique vRNA species available for packaging. In our experiments, k = 9, as each virus selects from among eight wild-type vRNAs and one pair of homologous reporter vRNAs that are assumed to be equivalent. Under these conditions, 5-7 chromosomes must be packaged randomly into each virion in order to ensure that 10-15% will incorporate 2 or more copies of the reporter. (The calculated probabilities are 6.4%, 9.8%, 13.5%, and 17.8% for b = 4, 5, 6, and 7, respectively.) Our assay scored only transduction-competent virions, however, all of which presumably must contain either three or four specific wild-type vRNAs (i.e., those that encode the three components of the viral polymerase and possibly NP), as these are likely required for efficient transcription and expression of the reporter (21, 45, 51, 63, 78). By virtue of the scoring process, these four would not be considered part of the randomly-packaged pool, but rather must be added to the critical value of 5-7 chromosomes determined above. Our results are therefore most compatible with the view that, on average, each influenza A virion packages 9-11 vRNAs, and does so in a nonspecific manner.

Chapter 3

O-Linked Sialomucins Do Not Function As Receptors for Infection By Influenza Viruses

Influenza virus infection is initiated by binding of the viral hemagglutinin (HA) protein to specific sugar residues on the host cell surface. HA is believed to bind primarily or exclusively onto derivatives of sialic acid, a monosaccharide that occurs commonly at the termini of carbohydrate sidechains in glycoproteins from a wide range of organisms and cell types (44). As a result, removing sialic acid residues from the cell surface (for example, by treating cells with the enzyme neuraminidase) drastically reduces susceptibility to influenza infection (107). The HA protein functions as a trimer of identical polypeptide chains, each of which includes a discrete binding pocket that can accommodate a single sialic acid residue (135). The particular chemical form of sialic acid that is recognized varies among viral strains: HA proteins from influenza A or B viruses bind selectively to N-acetyl-neuraminic acid (i.e., to unmodified sialic acid) whereas those from influenza C recognize an acetylated derivative, N-acetyl-9-O-acetyl-neuraminic acid (41, 106). Certain human or avian strains of influenza A can also discriminate the geometry of the linkage between sialic acid and its neighboring monosaccharide unit, binding preferentially to α2,6 or α2,3 disaccharides, respectively (20, 47, 105, 108, 124, 125). One tissue-specific sialoglycoprotein, the mannose receptor, appears to be especially important as a receptor for influenza A infection in macrophages, though not in other cell types (102). Apart from this,

however, there is little to suggest that HA binding to sialic acid is influenced either by the vicinal sugars in a carbohydrate sidechain or by the glycoprotein backbone to which it is attached.

Because the affinity of HA binding to any individual sialylated determinant is relatively weak, efficient viral binding requires a multivalent interaction between the virion and its target cell (113). This is achieved in part by the trivalent structure of HA and by the presence of multiple HA trimers on each virion. The possible importance of multivalent cellular ligands for HA, on the other hand, has not been extensively explored. Of particular interest in this regard are the sialomucin-type surface glycoproteins, a diverse group distinguishable from other surface proteins in having an extremely high density of heavily sialylated O-linked carbohydrate sidechains attached to a single polypeptide backbone (reviewed in (16)). The most heavily O-glycosylated regions of these proteins occur within an extracellular region termed the mucin domain. Although the proportion of surface carbohydrates on mammalian cells that can be accounted for by sialomucins is not known, and is likely to vary among cell types, these proteins have a prominent role in such carbohydrate-dependent functions as cell-cell attachment, leukocyte homing, and signal transduction (16, 50, 100, 112). An example is CD34, a transmembrane sialomucin that is expressed selectively on hematopoietic stem and progenitor cells, vascular endothelial cells, and embryonic fibroblasts (53). The 258-amino-acid extracellular domain of CD34 protein includes a mucin domain and as many as 75 potential sites for O-linked glycosylation. By presenting a very dense

spatial cluster of accessible sialylated ligands, mucins such as CD34 could, in principle, offer ideal targets for multivalent HA binding, and hence might function preferentially as attachment sites for influenza A viruses.

In this report, we test the hypothesis that surface sialomucins make an important quantitative contribution to influenza infection in mammalian cells. In particular, we examine the effects of overexpressing one specific sialomucin (CD34), or of depleting surface sialomucins in general, on initial virus-cell binding, on the transcription and replication of influenza RNAs, and on the ultimate release of new viral particles from infected cells. To deplete sialomucins, we take advantage of the properties of the bacterial enzyme O-sialoglycoprotein endopeptidase (OSGE), which provides the only known means of selectively degrading cell-surface sialomucins as a class (1, 122, 123). Surprisingly, we find that although these molecules are critical for bulk viral attachment to erythrocytes, they make no appreciable contribution to the efficiency, kinetics, or yield of infection by influenza A or C virus.

Materials and Methods:

Cells and viruses. Chinese hamster ovary cells that had been stably transfected (8) with expression vectors for the glycosylating enzymes fucosyltransferase VII and Core 2 β1→6 *N*-acetylglucosaminyltransferase (here called CHO cells) and a derivative of those cells that stably expressed human CD34 under the control of a CMV promoter (called CHO-CD34 cells) were grown in Dulbecco's H-21 medium supplemented with penicillin-streptomycin (pen-strep), L-glutamine, and 10% heat-inactivated fetal calf serum (FCS). Uninfected MDCK cells were grown in Eagle's minimal essential medium supplemented with pen-strep, L-glutamine, non-essential amino acids (NEAA; Gibco/BRL), and 10% heat-inactivated FCS; after infection, they were maintained in L-15 medium supplemented with pen-strep, L-glutamine, NEAA, 15 mM HEPES (pH 7.5), 0.75 g/L $NaHCO_3$, and 0.125% (w/v) bovine serum albumin (BSA). After infection of cells with influenza C, the above growth media were supplemented with 5 μg/ml N-acetyl trypsin. The influenza viruses used in this study were A/WSN/33 (H1N1) (influenza A), a gift from P. Palese, and C/Ann Arbor/1/50 (influenza C), a gift from A. Pekosz. Plaque-purified influenza A viral stocks were MDCK-cell supernatants grown at 37°C and harvested 2-3 days after infection when complete cytopathic effect (CPE) was observed. Influenza C viral stocks were MDCK-cell supernatants grown at 33°C and harvested approximately 7 days after infection when CPE became evident. Infectious titers of these stocks, determined by plaque-forming unit (pfu) assay on MDCK

monolayers, were 8 x 10^8 pfu/ml for influenza A and 2 x 10^6 pfu/ml for influenza C.

Infections. For infection, cells were first washed once with PBS+CM, then incubated with the indicated viral innocula in serum-free L-15 medium for 1 hr (influenza A) or in the same medium supplemented with 5 µg/ml N-acetyl trypsin for 2 hr (influenza C) at 37°C; they were then washed once with PBS+CM and refed with the appropriate growth media indicated above. Where indicated, cells growing in 35-mm dishes were pretreated with 100 µl of 0.72 mg/ml OSGE or 6 U/ml NA in PBS, or with PBS alone, for 90 min at 37° prior to infection.

Quantitative analysis of influenza RNAs. CHO or MDCK cells were harvested at the indicated times following infection, and cellular RNA was isolated by homogenizing the cells in 1 ml TRIZOL reagent (Gibco-BRL) according to the manufacturer's directions. Influenza RNAs were then quantified using a ribonuclease-protection assay (RPA). A 264-bp SacI/BamHI fragment from the NA gene of influenza A/WSN/33 was isolated from the vector pPolI-NA-RT (94) and subcloned into the corresponding polylinker sites in pBluescript II KS+ (Stratagene). This plasmid then was linearized with either SacI or KpnI, and in vitro transcription was performed with ∝[^{32}P]-UTP and either T3 or T7 RNA polymerase, to generate RPA probes complementary to either positive- or negative-sense NA RNAs, respectively, from influenza A. An RPA primer complementary to negative-sense RNA from influenza C was prepared by RT-PCR using primers 5′ CCCCCCAAGCTTTAATTGACAA TAACATTTGTGATC 3′ and 5′ CCGCCGGAATTCTGCAGATGGCGATCCCAGAG 3′ to amplify

residues 1717-1920 of the hemagglutinin esterase fusion gene of influenza C/AA/1/50. The PCR product was digested with EcoRI and Hind3 and then cloned into the polylinker of pBluescript II KS+. This plasmid was linearized with EcoRI, and in vitro transcribed using T3 RNA polymerase as described. The RPA III kit (Ambion) was used to perform RPA with 5-10 µg of input RNA and 80,000 cpm of probe per reaction.

Transient expression of CD34 in MDCK cells. MDCK cells at 50% confluency were transfected with 1 or 2 µg of plasmid expressing a full-length human CD34 cDNA under the control of the CMV promoter, or with the plasmid pBS KS+ as a sham control. Trans IT-LT1 (Panvera, Madison, WI) transfection reagent was used (at 2 µl/µg of DNA) essentially according to manufacturer's instructions. Cells were harvested 48 hrs after transfection, treated with PBS+CM or with 100 µl 0.72 mg/ml OSGE for 90 min at 37°C, and then analyzed by FACS as described below.

Hemagglutination Assay. Two-fold serial dilutions of viral stocks were made in PBS, and 50 µL of each dilution was placed in one well of a round-bottom 96-well plate. 50 µL (0.5% v/v) chick red blood cells (RBC) was then added to each well and incubated at 4°C until agglutination was complete.

FACS Analysis. 1 x 10^5 CHO , CHO-CD34 , or MDCK cells were stained with 40 µl of phycoerythrin-conjugated monoclonal mouse Qbend10 anti-human-CD34 antibody (Immunotech) for 1 hr at 4°C. FACS analysis was then conducted on a FACScan (Beckman Coulter).

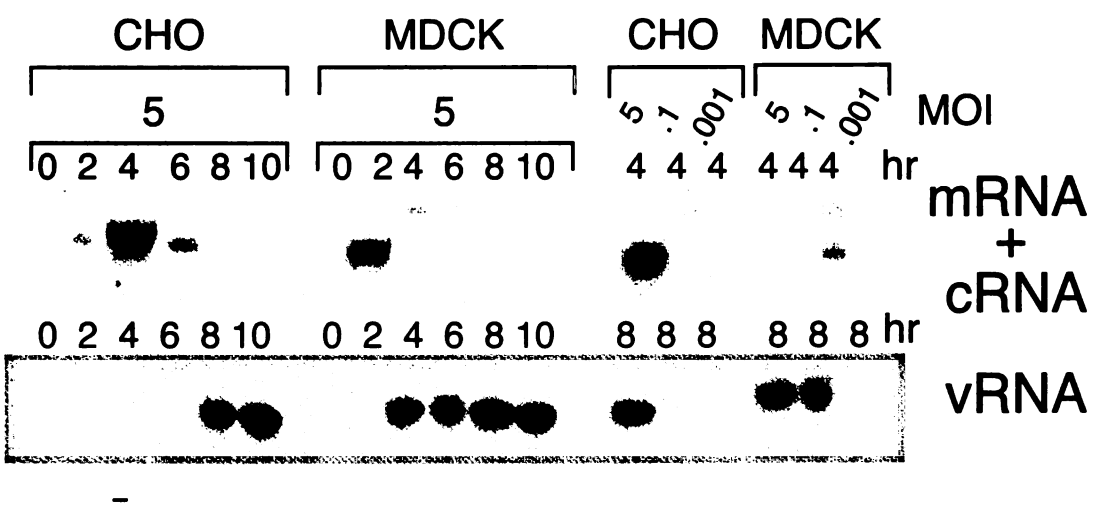
Results

To evaluate the role of sialomucins in productive infection, we studied influenza viral replication in vitro using the well-characterized host cell lines MDCK and CHO. Each can support productive influenza A infection, but the yield of new infectious virions is substantially higher in MDCK, which consistently generates infectious titers two orders of magnitude higher than those obtained with CHO cells in standard 48-hour cultures (Table 3). For our studies, these two cell lines thus defined a useful range of high or low infectibility, respectively, though the basis of this disparity was unknown.

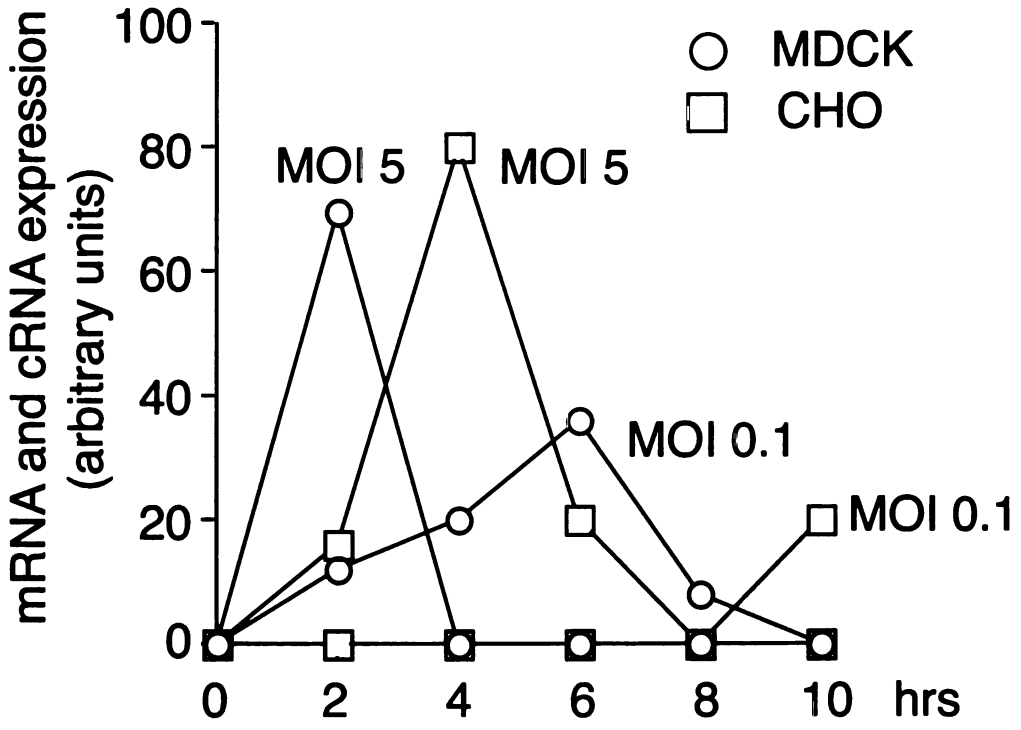
To restrict our analysis to the earliest post-entry events, we developed a quantitative RNase-protection assay (RPA) to monitor the synthesis of specific viral RNA products during the first hours after infection. Host cells were infected synchronously at various multiplicities-of-infection (MOIs) with a 1-hr pulsed exposure to influenza A virus, then were washed and cultured for up to 10 hr. Total cellular RNA was isolated at 2-hr intervals post-infection, and was probed by RPA for negative- or positive-sense RNA products derived from the viral neuraminidase gene. Typical results are shown in Figure 7. In each cell line, as expected, RNA expression was found to occur in two successive phases: an initial period of positive-sense (cRNA and mRNA) transcription, which then provides templates for the subsequent phase of replication of new negative-sense chromosomes (vRNA). These two phases appeared non-overlapping in that, in each host cell, positive-sense RNAs disappeared abruptly before negative-sense RNAs became detectable. Interestingly, we found that the peak RNA

Figure 7. Dynamics of influenza A viral RNA expression in CHO and MDCK cells. **(A)** Expression of positive-stranded (mRNA and cRNA) and negative-stranded (vRNA) RNAs from the viral neuraminidase gene, as a function of cell-type, time, and multiplicity of infection (MOI). Cells were infected with a 1-hr pulsed innoculum at the indicated MOIs and were harvested at the indicted times post-infection for RNA analysis by RNase-protection assay (RPA) using strand-specific probes. Each lane was prepared using 10 μg of total cellular RNA. Timecourses shown were performed at MOI = 5 over a 10-hr period (at left); peak positive- and negative-strand RNA levels were determined at 4 hr and 8 hr post-infection, respectively, at MOIs of 0.001 to 5 (at right). Timecourses of positive-stranded **(B)** and negative-stranded **(C)** RNA expression are shown graphically for each cell type at MOIs of 0.1 and 5. Each panel depicts one of three replicate experiments.

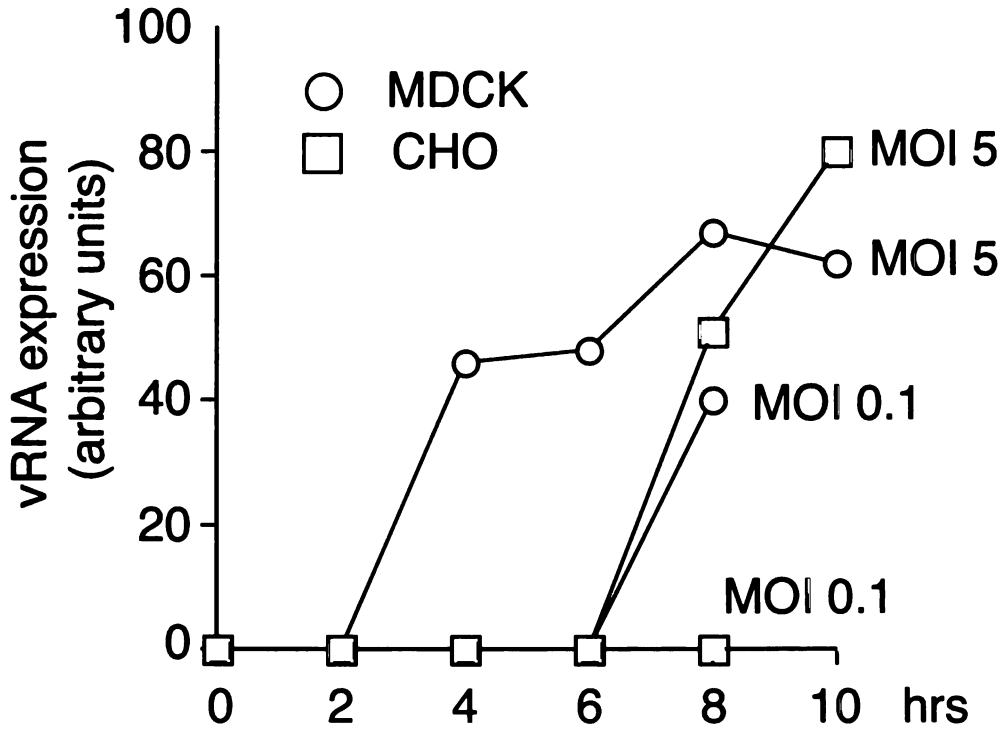
A)


CHO
MDCK
CHO
MDCK
5
5
5 .1 .001
5 .1 .001
MOI
0 2 4 6 8 10
0 2 4 6 8 10
4 4 4
4 4 4
hr
mRNA
+
cRNA
0 2 4 6 8 10
0 2 4 6 8 10
8 8 8
8 8 8
hr
vRNA
-

B)


mRNA and cRNA expression
(arbitrary units)
100
80
60
40
20
0
0
2
4
6
8
10
hrs
MDCK
CHO
MOI 5
MOI 5
MOI 0.1
MOI 0.1

C)

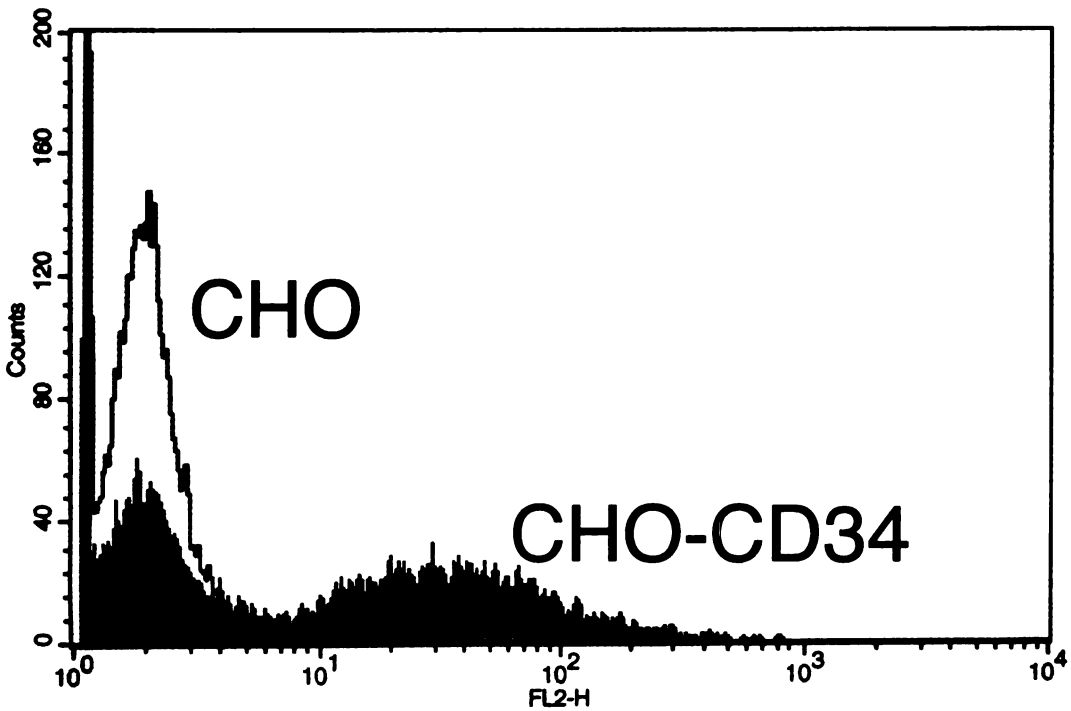

100
80
60
40
20
0
vRNA expression
(arbitrary units)
MDCK
CHO
MOI 5
MOI 5
MOI 0.1
MOI 0.1
0
2
4
6
8
10
hrs

concentrations achieved in each phase did not differ greatly between cell lines under any of the conditions we tested. Instead, at any given MOI, MDCK cells exhibited a significantly earlier and shorter transcription phase than CHO cells, and new vRNA synthesis began sooner (at 4 hr vs 8 hr post-infection) and persisted longer in MDCK than in CHO cells during the time period examined. For each cell line, the time-to-onset of both the transcription and replication phases was inversely related to MOI, but both occurred much sooner after infection for MDCK than for CHO over the entire range of MOIs tested (Figure 7). These differences in kinetics of RNA expression likely account, at least in part, for the higher yield of infectious particles from MDCK as compared to CHO cells.

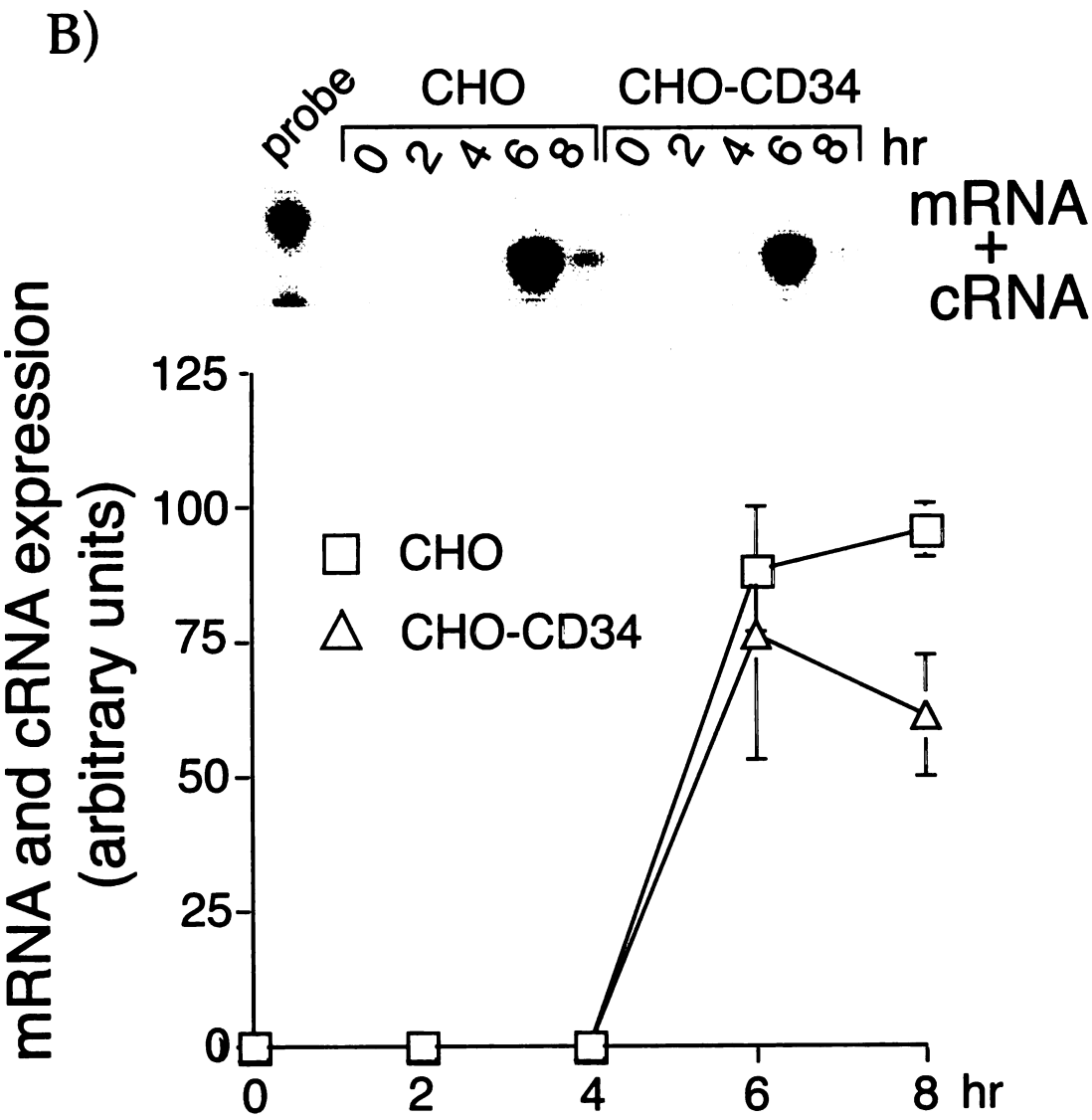
We then used the RPA to determine whether overexpression of CD34, a representative mucin-type glycoprotein, could enhance the initial steps in influenza A infection. For this purpose, we employed a clonal derivative of CHO, called CHO-CD34 (a kind gift of S. Hemmerick) that stably expresses the human CD34 coding sequence under the control of the CMV promoter. Flow cytometric analysis after staining with an anti-CD34 antibody (Figure 8A) confirmed that roughly half the cells in an unsynchronized CHO-CD34 population display CD34 on their surfaces at a given time, whereas the parental CHO cells show no detectable CD34 expression. Though these results do not reveal the absolute amount of CD34 expressed on CHO-CD34 cells, the difference in immunofluorescence intensity is substantial (approximately 10-fold) between CD34-positive and –negative cells. Nevertheless, when measured at

Figure 8. Infection of CHO and CHO-CD34 cells with influenza A virus. **(A)** Comparison of surface CD34 expression in uninfected CHO (open curve) and CHO-CD34 cells (filled curve) as determined by immunofluorescent flow cytometry. Timecourses of positive-stranded **(B)** and negative-stranded **(C)** viral RNA expression in each cell line at indicated times following a 1-hr pulsed infection with influenza A at MOI = 1. Each panel depicts the same data as an autoradiogram (above) and graphically (below), from one representative experiment of three performed at various MOIs. Probe = RPA probe alone, prior to RNase digestion.

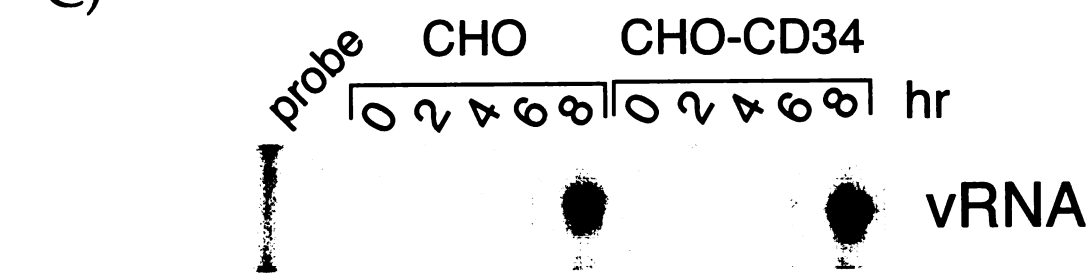
A)

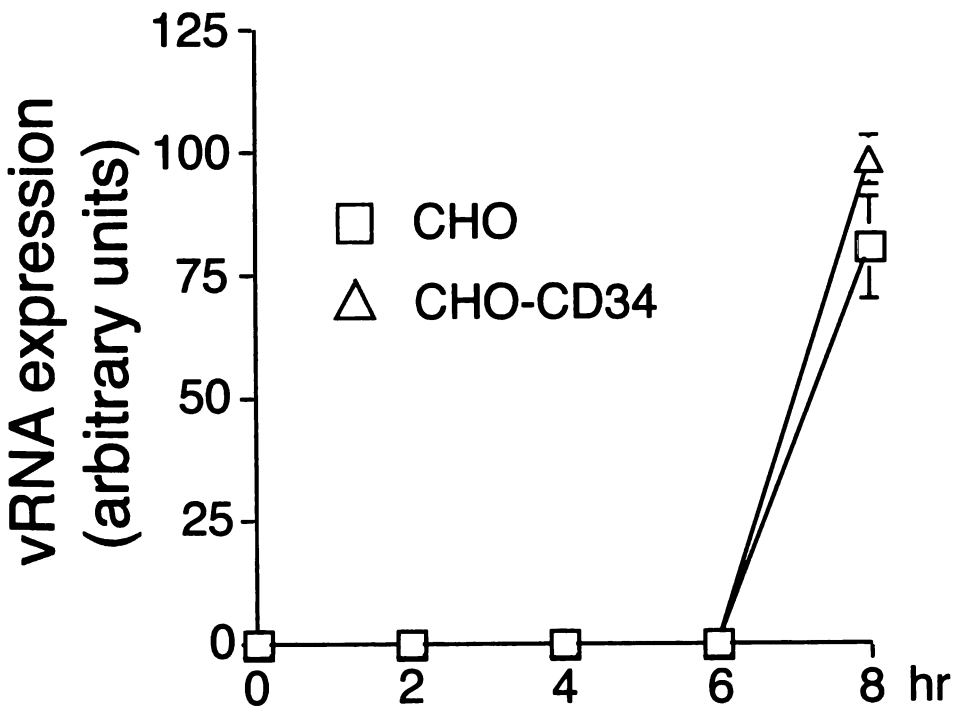


B)


probe
CHO
CHO-CD34
0 2 4 6 8 0 2 4 6 8 hr
mRNA
+
cRNA
mRNA and cRNA expression
(arbitrary units)
125
100
75
50
25
0
CHO
CHO-CD34
0
2
4
6
8 hr

C)


probe
CHO
CHO-CD34
0 2 4 6 8 0 2 4 6 8
hr
vRNA

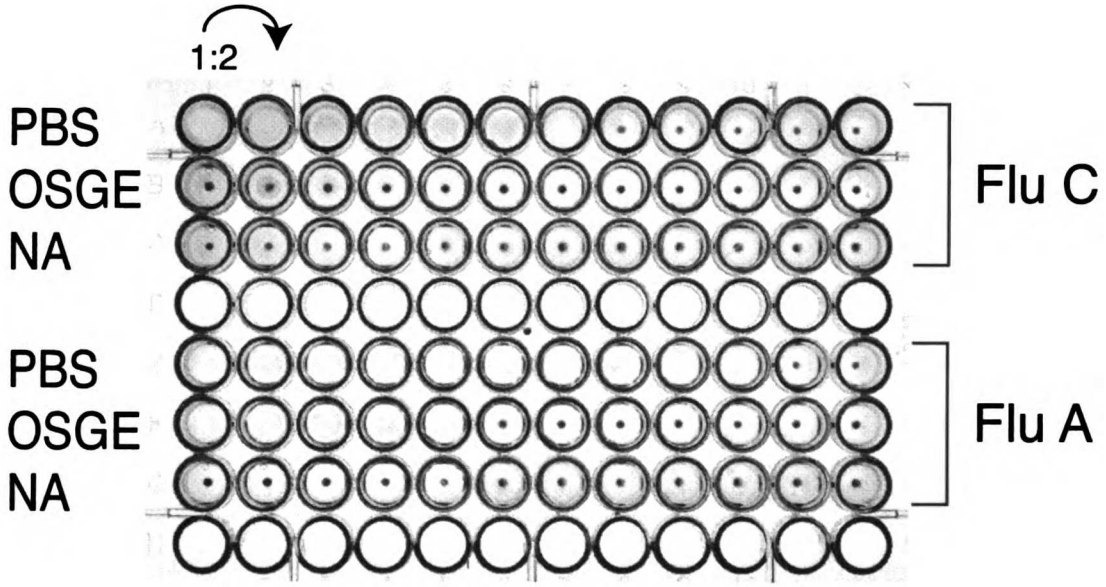

vRNA expression
(arbitrary units)
125
100
75
50
25
0
CHO
CHO-CD34
0
2
4
6
8 hr

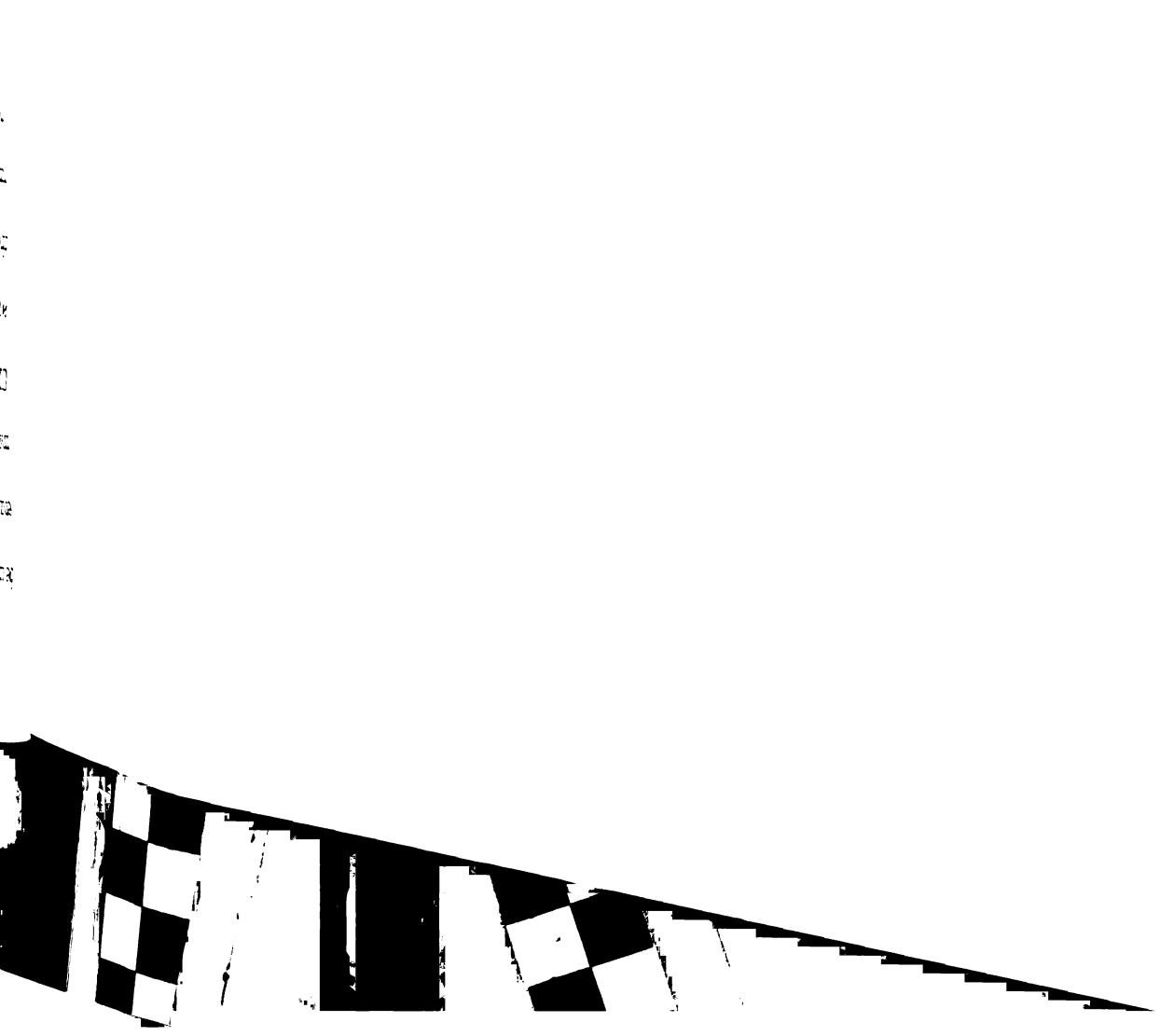
the level of viral RNA synthesis, the infectibility of these two populations was indistinguishable (Fig. 8B and 8C): we found no evidence that CD34 overexpression affected either the timing or the absolute amounts of viral transcription or replication in CHO cells over the range of MOIs (0.1-1.0) we tested.

We then tested whether influenza viral attachment or infection could be inhibited by eliminating sialomucins from the host-cell surface, using the enzyme OSGE. This protease, derived from *Pasteurella haemolytica*, has the useful property of selectively cleaving the polypeptide backbones of diverse mucin domains, and so provides a broadly-specific means of depleting cell-surface mucins (1, 122, 123). To determine the effect of this enzyme on virus binding, we pretreated intact chick erythrocytes with various concentrations of OSGE or of bacterial neuraminidase (NA) in vitro, and then measured their ability to bind influenza A or C viruses using a standard hemagglutination assay. As illustrated in Figure 9, we found that maximally effective pretreatment with OSGE inhibited hemagglutination by approximately 64-fold for influenza A, and by at least 128-fold for influenza C. Though OSGE was notably less efficacious than NA in this regard, the result suggested that more than 98% of hemagglutinating activity in both influenza A and C depends upon OSGE-sensitive (and so, presumptively, mucin-like) molecules on the erythrocyte surface.

The hemagglutination assay, however, measures bulk viral binding but not viral entry or other activities that define a functional virus receptor. We therefore asked whether infection of any of our previously-tested cell lines could

Figure 9. Hemagglutination of 0.5% (v/v) chick red blood cells by two-fold serial dilutions of influenza C (above) or influenza A (below). The red blood cells were pretreated for 1.5 hr at 37°C with maximally inhibitory concentrations of OSGE or NA in PBS+CM, or with PBS+CM alone.


1:2
PBS
OSGE
NA
PBS
OSGE
NA
Flu C
Flu A

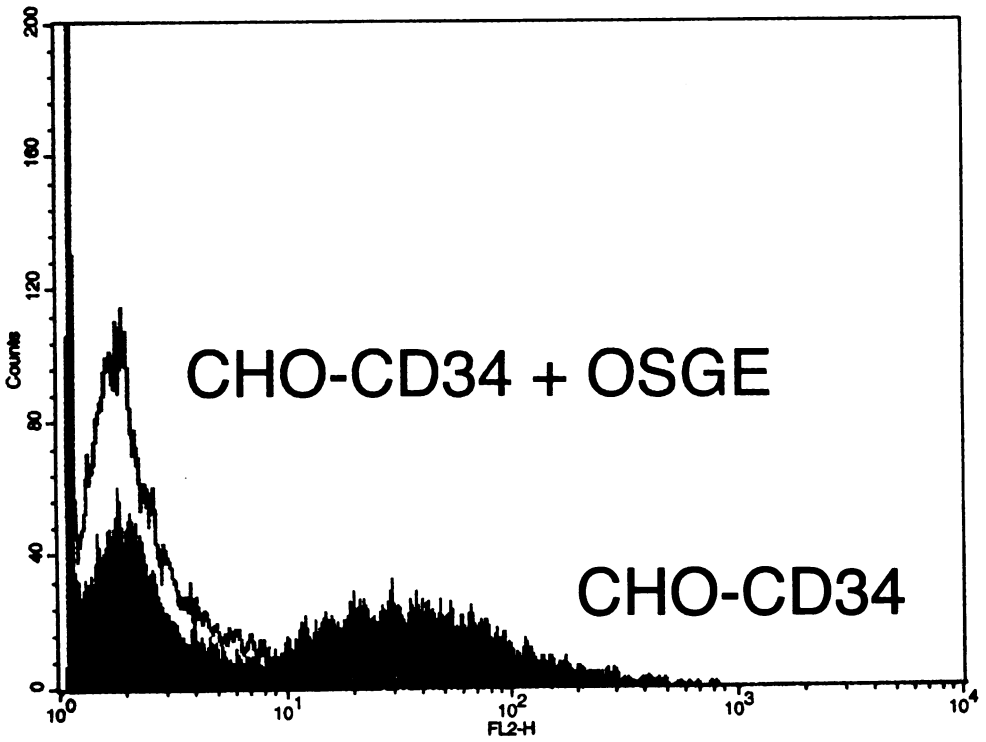


be inhibited by pretreatment with OSGE. To determine the efficacy of the enzyme in removing surface sialomucins from these cells, we began by monitoring the disappearance of surface CD34-immunoreactivity from CHO-CD34 cells following OSGE digestion. As shown in Figure 10A, the conditions we used were sufficient to eliminate essentially all immunoreactive surface CD34 from the transfected cells, so that their flow cytometric profile after enzyme-treatment was identical to that of parental CHO cells (compare Figures 8A and 10A). This confirms that OSGE-treatment drastically diminishes surface CD34 expression, and it suggests, in light of the broad substrate-specificity of OSGE, that other sialomucins are eliminated to a comparable degree. Sequential measurements confirmed that CD34 epitope expression remained undetectable for at least one hour following OSGE treatment (data not shown), the time required for a standard pulsed infection with influenza A. When these cells were assayed by RPA, however, we found that the pattern of influenza RNA expression during the 8 hr following infection was essentially identical to that seen in untreated CHO-CD34 or parental CHO cells (Fig. 10B and 10C), implying that OSGE treatment had not altered these cells' ability to support influenza A viral entry or RNA replication.

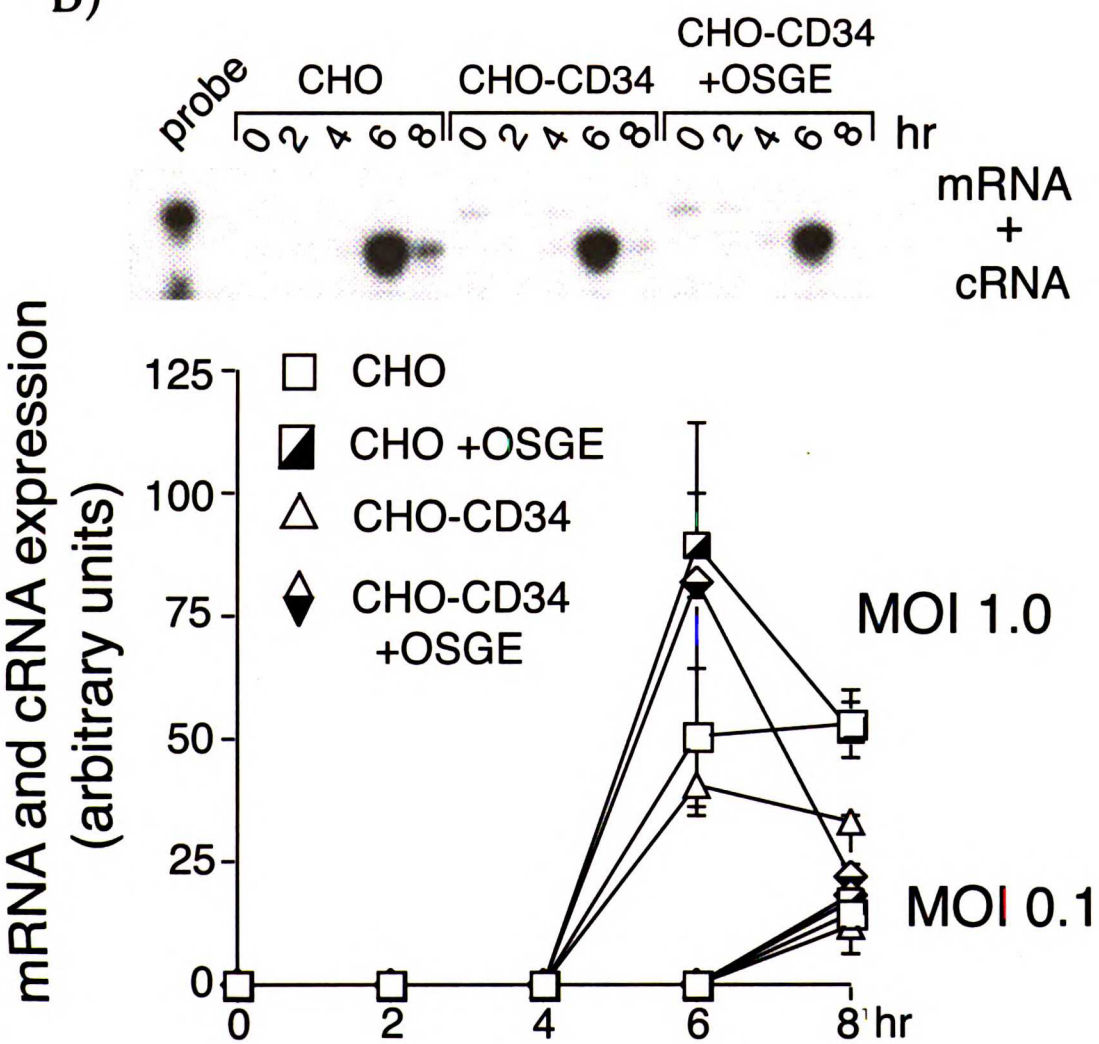
Identical results were observed following OSGE treatment of the more highly infectible cell line MDCK. We first verified again that pretreatment with OSGE greatly reduced surface expression of mucins, using CD34 expression as a marker. MDCK cells were transiently transfected with a plasmid expressing CD34, then treated with OSGE and tested for surface CD34 expression by FACS.

Figure 10. Lack of effect of CD34 expression or of OSGE digestion on influenza A infection in CHO cells. **(A)** Flow cytometric analysis of surface CD34 expression on CHO-CD34 cells before (filled curve) and after (open curve) OSGE digestion. Timecourses of positive-stranded **(B)** and negative-stranded **(C)** viral RNA expression in CHO and CHO-CD34 cells with and without OSGE-pretreatment. Viral RNA was assayed at the indicated times following a 1-hr pulsed infection with influenza A. Each panel includes an autoradiogram from infections at MOI = 1 (above), as well as graphical depiction of infections at MOI = 0.1 and 1.0 (below). Representative data from three independent experiments. Probe = RPA probe alone, prior to RNase digestion.

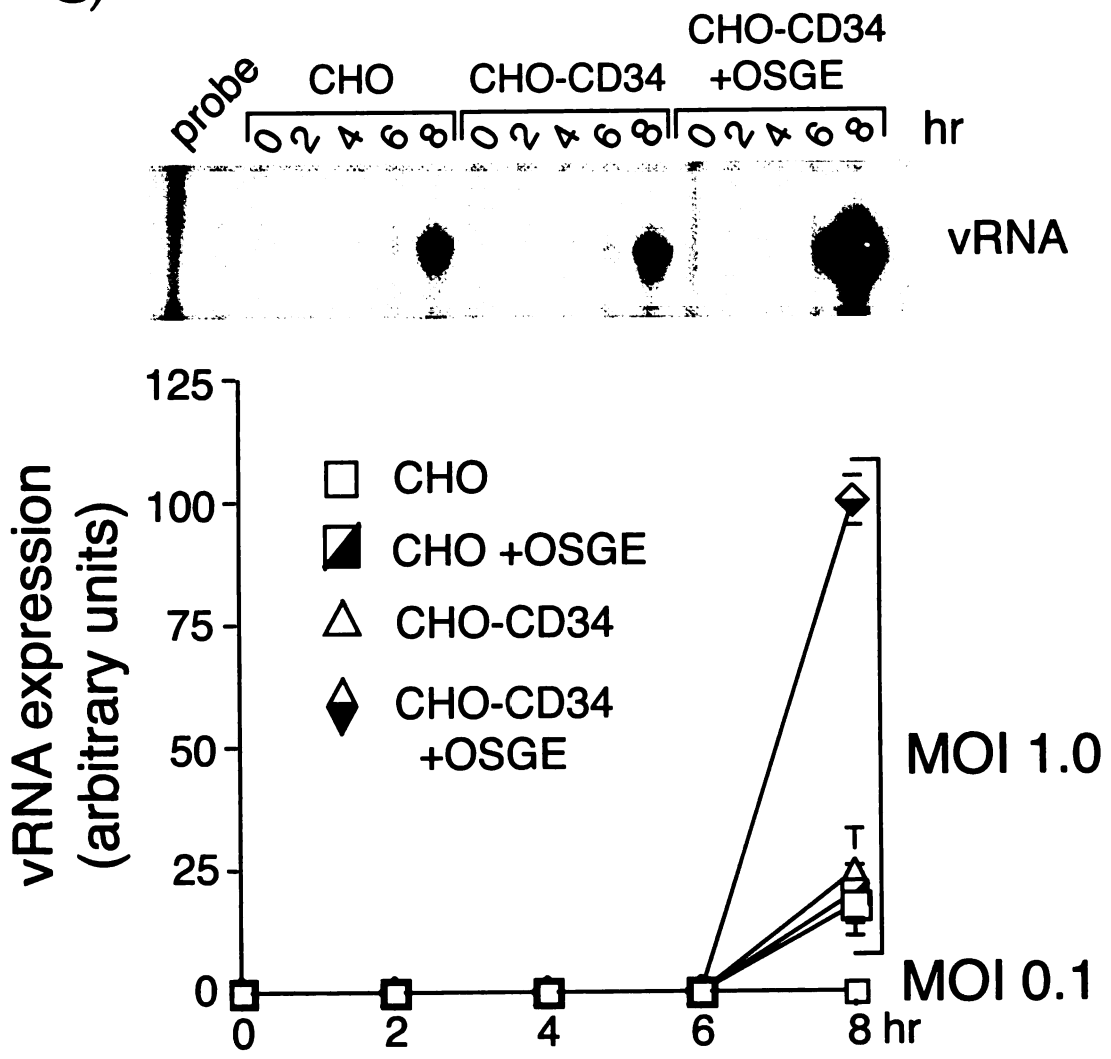
A)


CHO-CD34 + OSGE
CHO-CD34
Counts
FL2-H
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

B)


probe
CHO
CHO-CD34
CHO-CD34 +OSGE
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 hr
mRNA + cRNA
mRNA and cRNA expression (arbitrary units)
125
100
75
50
25
0
CHO
CHO +OSGE
CHO-CD34
CHO-CD34 +OSGE
MOI 1.0
MOI 0.1
0
2
4
6
8
hr

C)

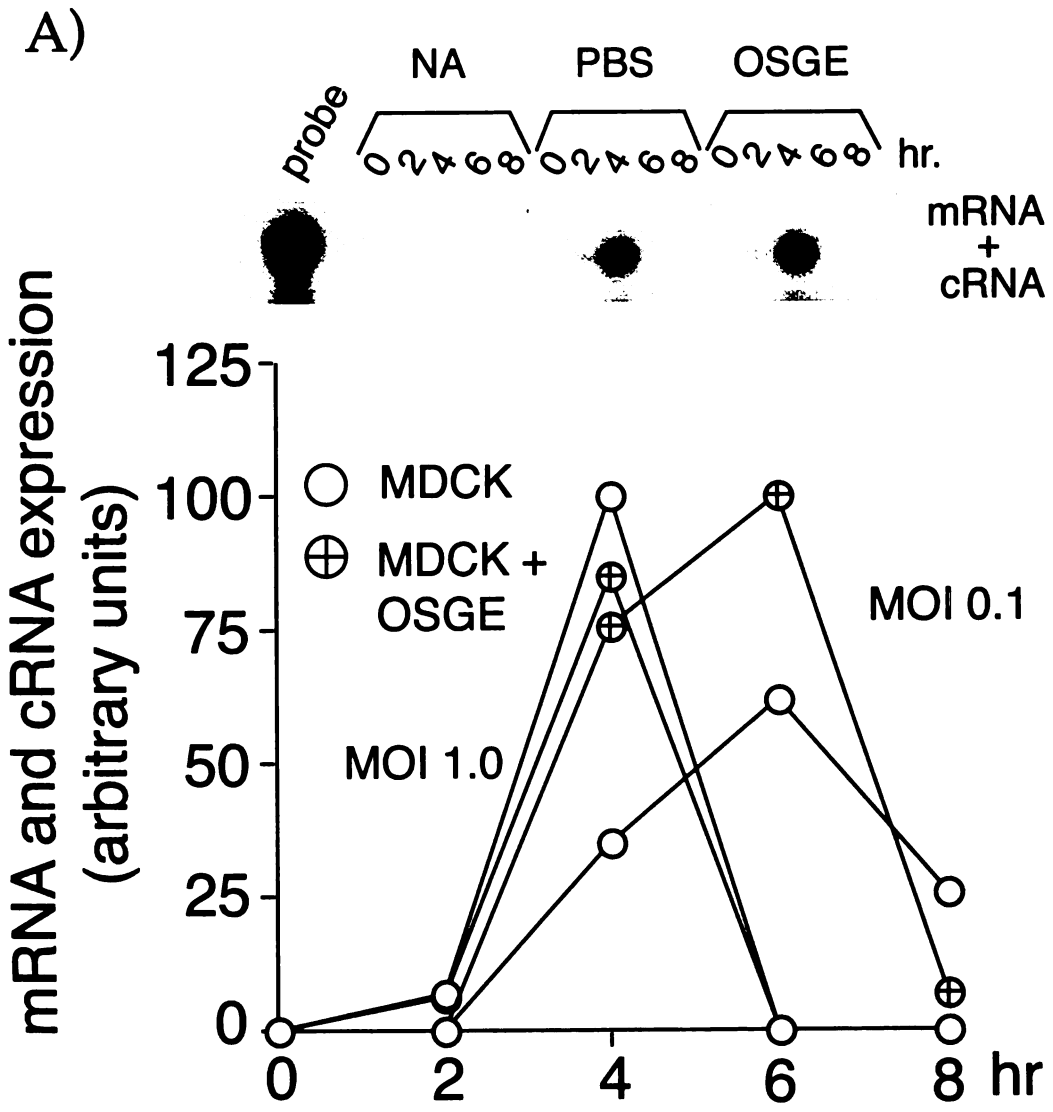

probe
CHO
CHO-CD34
CHO-CD34 +OSGE
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8
hr
vRNA
vRNA expression (arbitrary units)
125
100
75
50
25
0
CHO
CHO +OSGE
CHO-CD34
CHO-CD34 +OSGE
MOI 1.0
MOI 0.1
0
2
4
6
8 hr

In this transient assay, only 10-20% of transfected MDCK cells showed detectable CD34 expression before enzyme treatment, but OSGE digestion reduced this expression to undetectable levels indistinguishable from those of mock-transfected cells (data not shown). This suggests that other, endogenous sialomucins were similarly depleted from all cells in the treated population. Using the RPA, we then confirmed that pretreatment with neuraminidase could completely prevent influenza A infection of MDCK, resulting in the complete abrogation of both positive-sense and negative-sense RNA production (Figure 11). Treatment with OSGE, by contrast, had no discernible effect (Figure 11).

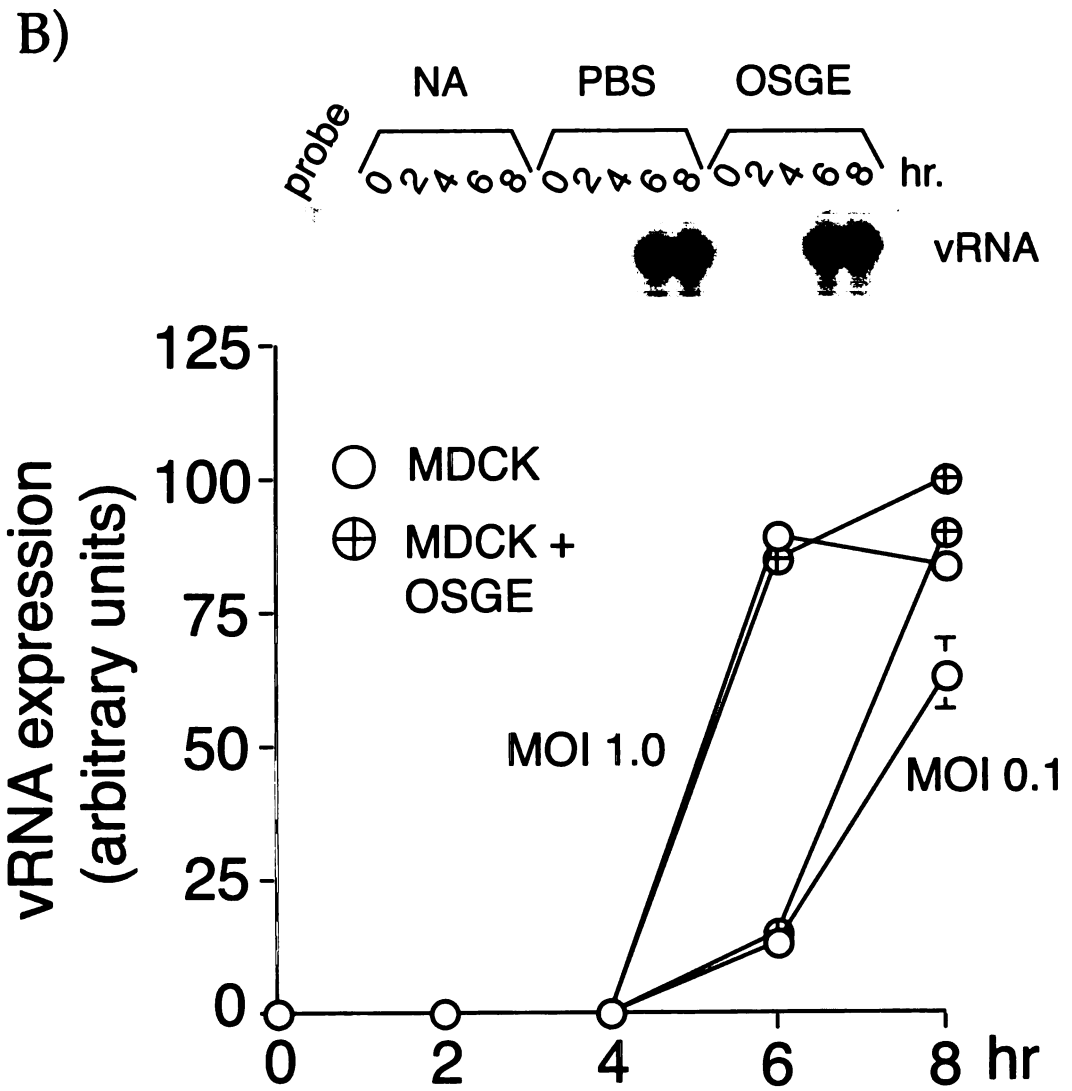
As another test of the foregoing results, we assayed the infectious plaque-forming titers of influenza A particles produced by CHO and MDCK cells with or without OSGE pretreatment. As shown in Table 3, these studies confirmed the disparate yields of virus produced by the two cell types at each of the two MOIs tested (0.1 and 1.0 pfu/cell), but revealed no evidence that replication was inhibited after OSGE treatment. Indeed, in most instances, treatment resulted in modestly (generally less than 4-fold) increased infectious titers in the supernatant, perhaps reflecting an improved efficiency of influenza A particle release from these partially desialylated cells.

Finally, we also examined the effects of OSGE digestion on influenza C infection in MDCK cells. Earlier reports indicate that an OSGE-sensitive sialomucin, termed gp40, serves as the principal binding site for influenza C on these cells (141, 142), perhaps indicating that it is a preferential substrate for 9-O-acetylation of sialate residues, which is required for influenza C binding. The

Figure 11. Influenza A infection of MDCK cells is inhibited by pretreatment with NA but not with OSGE. MDCK cells underwent maximally efficacious pretreatment with either NA or OSGE, or with PBM+CM alone, prior to a 1-hr pulsed infection with influenza A. Cells were harvested at indicated times post-infection and assayed for positive-stranded **(A)** and negative-stranded **(B)** viral RNA. Each panel depicts an autoradiogram from infections at MOI = 1 (above), as well as graphical depiction of infections at MOI = 1.0 and 0.1 (below). Representative data from three experiments at various MOIs. Probe = RPA probe alone, prior to RNase digestion. Efficacy of the OSGE-pretreatment was confirmed using MDCK cells that had been transiently transfected with CD34 (data not shown).


A)
NA
PBS
OSGE
probe
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 hr.
mRNA
+
cRNA
mRNA and cRNA expression
(arbitrary units)
125
100
75
50
25
0
MDCK
MDCK +
OSGE
MOI 0.1
MOI 1.0
0
2
4
6
8 hr

B)


NA
PBS
OSGE
probe
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 hr.
vRNA
125
100
75
50
25
0
vRNA expression (arbitrary units)
MDCK
MDCK + OSGE
MOI 1.0
MOI 0.1
0
2
4
6
8 hr

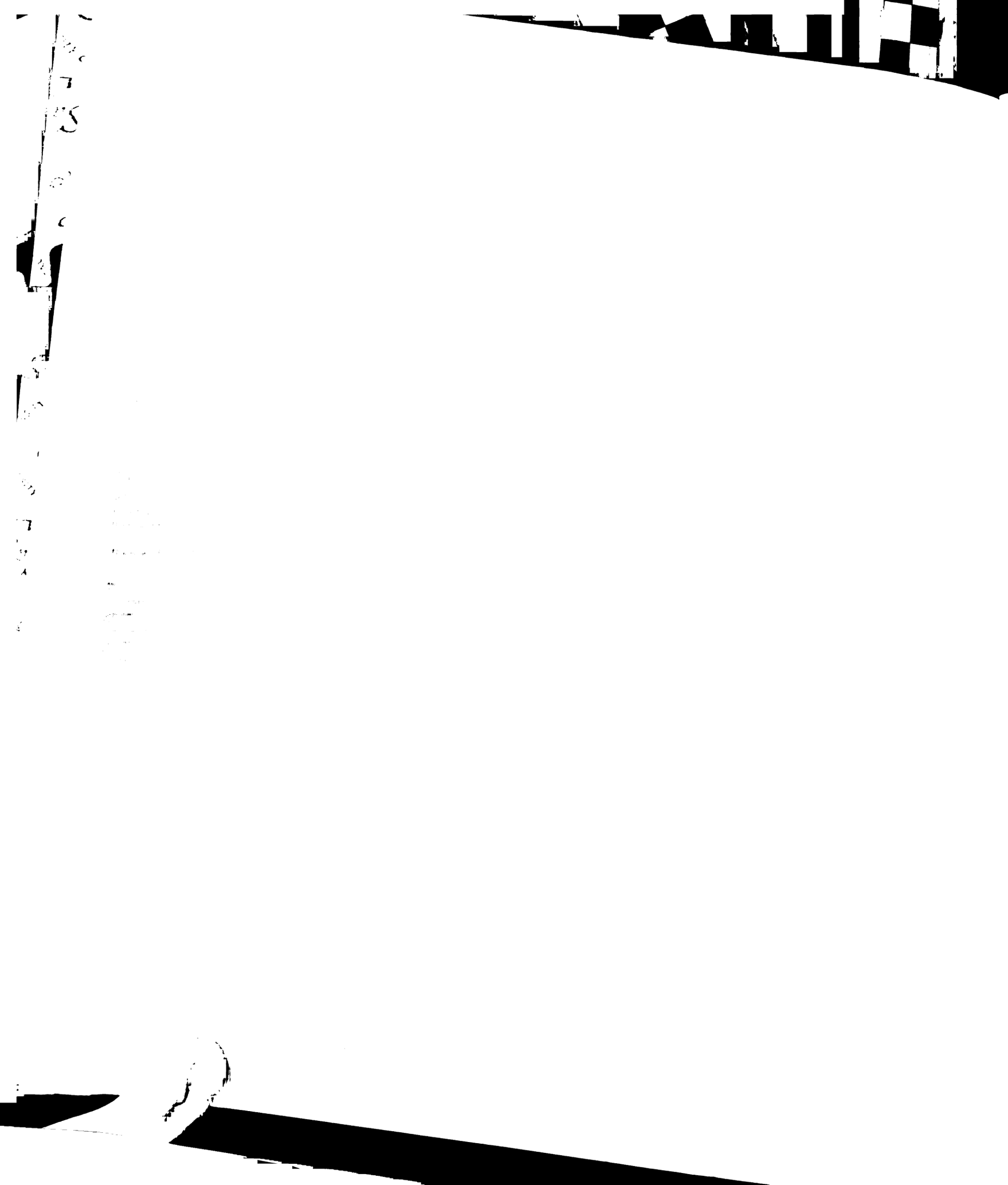


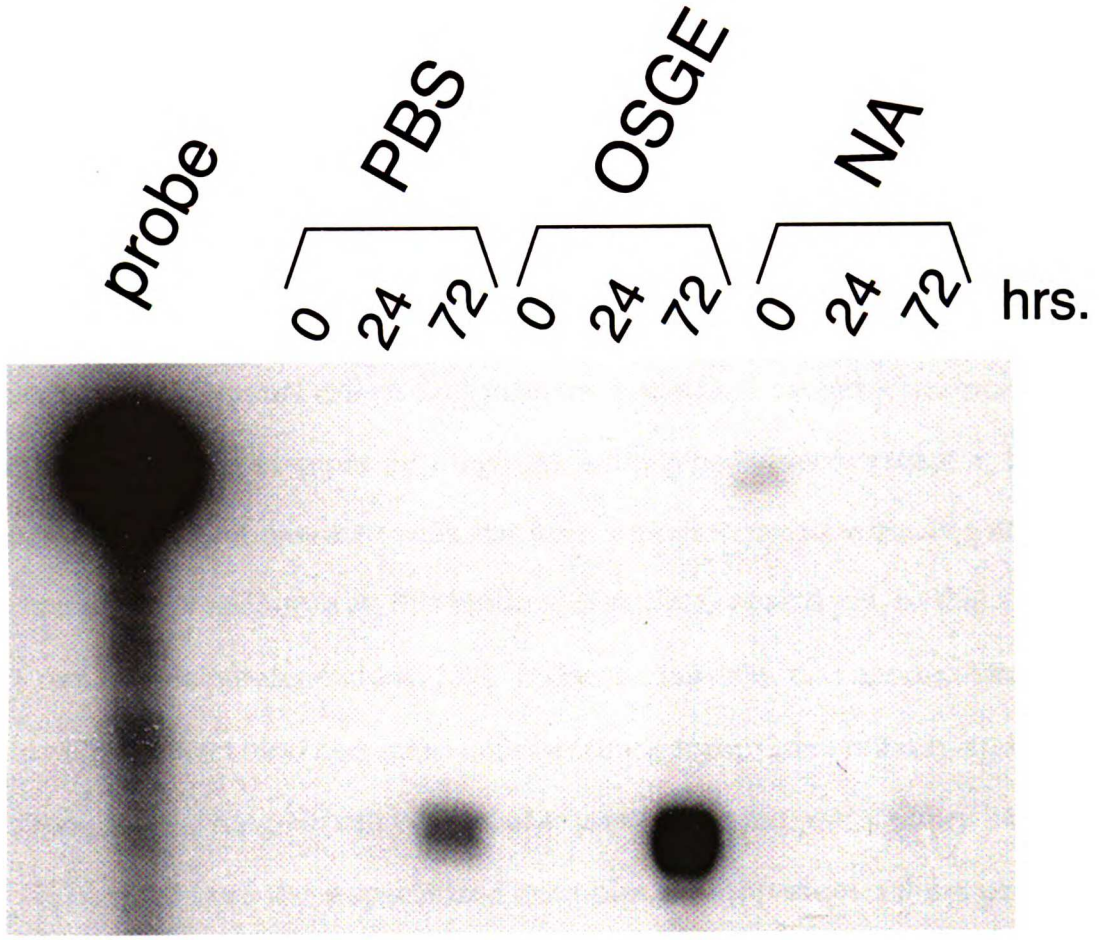
TABLE 3. Titres from influenza A infected CHO and MDCK cells [1]

		Titers (x 10^5 pfu/mL)			
		24 hr		48 hr	
		PBS	OSGE	PBS	OSGE
MOI 0.1					
	CHO	7.4	28	1.5	10
	CHO + CD34	9.7	30	1.4	24
	MDCK	1900	4000	2000	3200
MOI 1.0					
	CHO	38	51	32	85
	CHO +CD34	32	42	7.5	120
	MDCK	3300	2700	1600	2300

[1] One representative experiment of three

role of this mucin in viral infection, however, has not been documented directly. We therefore pretreated MDCK cells with either NA or OSGE, and then monitored the early timecourse of influenza C infection by RPA, using a probe specific for the vRNA that encodes the hemagglutinin esterase fusion (HEF) protein of this virus. As shown in Figure 12, even at its optimal growth temperature of 33°C, influenza C replicates much more slowly than influenza A, with vRNAs becoming detectable only 24-72 hr after a pulsed infection. As with influenza A, however, we found that, whereas NA-pretreatment completely abolished infection, treatment with OSGE did not. Instead, OSGE consistently produced an increase (ranging from 2- to 5-fold in triplicate experiments) in vRNA expression at 72 hr post-infection. Consistent with that finding, influenza C viral titers, as estimated by hemagglutination, were also increased to a comparable degree by OSGE-pretreatment, but were undetectable in supernatants from NA-pretreated cells (data not shown).

Figure 12. Influenza C infection of MDCK cells is inhibited by pretreatment with NA but not with OSGE. Expression of vRNA derived from the viral hemagglutinin esterase fusion gene was assayed by RPA at the indicated times following a 2-hr pulsed infection with influenza C, in following optimally efficacious pretreatment with OSGE or NA, or with PBS+CM. Probe = probe alone, prior to RNase digestion. One representative experiment of three.


probe
PBS
OSGE
NA
0
24
72
0
24
72
0
24
72
hrs.

Discussion

Though it is well established that influenza viruses bind to sialylated determinants on their host cells and that this binding is essential for infection, little is known about the particular types of sialylated cell-surface molecules that may function as viral receptors. To gain entry to a cell, an influenza virus must not only attach stably to its surface, but then be endocytosed, enter the endosomal pathway, and remain membrane-bound in the acidic interior of the endosome, so that membrane fusion can occur (reviewed in (61) and refs. therein). Few individual cell-surface proteins could be expected to support all of these functions. For example, although the lectin-type mannose receptor, found on macrophages and dendritic cells, has been shown to mediate binding and internalization of influenza A, this binding dissociates at acid pH, so that viral entry remains sialate-dependent (102). Influenza particles that are decorated with antibodies can bind and infect cells bearing appropriate antibody-specific Fc receptors, but the range of cell types that express those receptors is very limited (89, 126). Apart from these specialized examples, no individual cellular protein or collection of proteins has yet been shown to function as a key biological receptor for influenza virions.

The sialomucins would appear to offer several useful properties in this regard. By presenting an exceptionally high density of sialylated sidechains, each of which is a known, acid-stable ligand for the influenza A hemagglutinin (HA), a single sialomucin protein could support multivalent binding that would compensate for the low affinity and rapid dissociation of individual HA-sialate

contacts. Projecting from the cell surface on an elongated and rigid polypeptide backbone, the mucin domains of these proteins are highly accessible and commonly exploited for cell-cell binding in the hematopoietic system (53). Certain sialomucins, including CD34, are known to undergo ligand-dependent endocytosis (54) and have intracellular signal-transduction domains (25) that could conceivably help signal virus-cell contacts. Moreover, glycophorin, the major HA-binding protein on erythrocytes, is itself a sialomucin (128).

For all of those reasons, we hypothesized that one or more sialomucins might have a preeminent role in influenza infection of mammalian cells. Our results, however, indicate that this is not the case. Utilizing the enzyme OSGE as a broadly-specific means of depleting cell-surface sialomucins, we found that these proteins are indeed responsible for at least 98% of the hemagglutinating activity of influenza A and C viruses, implying that they are a major site of viral attachment on erythrocytes. Nevertheless, in studies of two different cell lines (CHO and MDCK), we found no evidence that infection with these viruses could be either increased by overexpressing CD34 or decreased by enzymatically depleting sialomucins from the surface of the host cell. This was true over a range of MOIs, and regardless of whether infection was assayed at the level of viral RNA expression or by the production of new viral particles.

Our studies has certain limitations. Foremost is the assumption that all sialomucins are OSGE-sensitive and subject to digestion on cell surfaces. Although the conditions we used for OSGE digestion were sufficient to suppress hemagglutination by nearly two orders of magnitude and to eliminate essentially

all immunoreactive CD34 from the target cells for a period of at least one hour, we cannot be certain that all other sialomucins were affected to a similar degree. It is possible that influenza receptor activity resides in a subset of mucins that are OSGE-resistant for reasons of structure or accessibility. Similarly, as the absolute quantities of sialomucins that are normally expressed on MDCK and CHO cells are unknown, it is not clear whether the stable or transient overexpression of CD34 on these cells significantly increased their overall abundance. On the other hand, we are not aware of any preferable alternatives to these approaches for manipulating expression of sialomucins as a class.

Our results indicate that sialomucins are a quantitatively major site for influenza viral binding to erythrocytes but do not contribute to viral entry in CHO or MDCK cells. This is consistent with the findings of Zimmer et al., who reported that a unique OSGE-sensitive glycoprotein, termed gp40, is the predominant site for influenza C viral binding on MDCK cells (141, 142). No direct evidence of a role for gp40 in influenza C infection has been reported, however, and our results fail to indicate such a role, though it remains possible that MDCK substrains differ in this regard. Our findings are also compatible with earlier reports that influenza viruses bind avidly to secreted mucins in the respiratory tract, which presumably serve a protective function (136). Indeed, lacking any intrinsic receptor activity themselves, membrane-bound sialomucins might be proposed to function as a protective, high-capacity "sink" that could sequester influenza viruses at the cell surface to minimize both entry and release. The loss of such a protective barrier might account for the enhanced virion

production (and, more variably, increased vRNA expression) we observed when infecting OSGE-treated cells, especially at lower MOI (see Table 3 and Figs. 10C and 12). It is also possible that enzymatically cleaving the extracellular domains of mucins which, like CD34, have signaling domains can trigger intracellular events that promote vRNA synthesis and subsequent stages of influenza viral replication. Through either of the mechanisms, sialomucins might indirectly modulate influenza infectivity, while the identity of the influenza viral receptors remains unknown.

Chapter 4

Summary and Implications

We have presented data which elucidate a fundamental aspect of influenza particle assembly: specifically, the packaging of viral genomic RNA segments. The mechanism by which Influenza virus packages a full complement of 8 viral RNAs to create an infectious particle has been debated for some time. It is clear that influenza can discriminate its own genomic RNAs from that of the host cell, as influenza virions have not been found to package cellular RNAs (76). It has been tempting to postulate a similar specificity in packaging the individual viral chromosomes, so that each could be assembled into a virion in a selective and specific manner. As discussed in Chapter 2, there has been limited experimental evidence to support such a specific model and in a mutant viral system, which may confound the observed results(23, 83-85, 91).

The influenza packaging problem has eluded resolution in part because typical routes to map viral packaging signals (ie. mutational approaches) are complicated by the fact that the promoter sequences that direct viral replication and transcription appear to overlap sequences important for packaging (34, 64, 127). Our lab, along many others, has attempted to use alternative reverse-genetic approaches to tackle this problem. Particularly, my own early efforts employed the methods of Pleschka et al. (94) and Mena et al. (79) both of which proved inefficient for our purposes. The former utilizes a helper virus system, which is inefficient at producing recombinant viruses and so necessitates a

stringent genetic selection system, whereas the latter, though plasmid-based, necessitates the use of vaccinia virus for vRNA synthesis once the plasmids are introduced into recipient cells (79, 94). In our hands, the use of vaccinia produced unacceptable levels of host cell death, moreover, the resulting virus-like particles were not replication-competent and so required use of a helper virus. In addition, these particles do not contain the entire complement of chromosomes and may have packaging characteristics that do not mimic those of authentic influenza. The advent of an entirely plasmid-based system, developed by Kawaoka and colleagues, that produces infectious, replication- competent Influenza virus particles has proved to be extremely effective for investigating the packaging problem in our laboratory (87).

The data I report in Chapter 2 strongly support the view that influenza vRNAs are packaged in a segment-nonspecific manner. Our results confirm that influenza virus will package a heterologous gene, flanked by viral sequences on the 3′ and 5′ termini (69). Since these sequences are the minimal amount required for replication and packaging, one might conclude that the so-called packaging signal is contained in these regions. Therefore, to test the model of specific packaging, we looked at these regions for sequences, which differ among chromosomes and might allow for specific recognition and packaging of each viral RNA. The untranslated region, which allows for replication and packaging of a viral segment, is typically described as having two domains. The distal 12 and 13 nucleotides of the 3′ and 5′ ends are conserved among all 8 chromosomes,

whereas the proximal sequences differ among chromosomes, but are highly conserved among strains (22, 121).

We wanted to determine if the nonconserved untranslated nucleotide sequences of the viral RNA are sufficient to direct segment-specific packaging of a viral RNA. Therefore, we created genetically tagged chromosomes, which mimic viral RNAs in the manner in which they are replicated and packaged. By constructing RNAs which are identical in every respect other than their viral noncoding sequences, we hoped to determine whether these sequences are sufficient to direct packaging of viral RNAs in a segment-specific manner. Our results confirm that virus-like chromosomes do indeed compete for packaging, in that there appears to be a maximal level of packaging which is saturable even if more virus-like RNA is available for packaging. However, the virus-like RNAs do not compete in a segment-specific way. Instead, competition for packaging occurs equivalently between segments with either homologous or non-homologous UTR sequences (Chapter 2).

Although this does not seem to be the most efficient method of packaging for the virus it is consistent with the majority of other experimental evidence in this area. Enami et al. isolated a temperature-sensitive mutant virus, which seems to carries an extra NS1 gene segment (24). According to their data, it appears possible for the virus to package and stably carry more than the necessary eight chromosomes. Scholtissek et al. Isolated and characterized a heterozygous virus which carried two copies of segments 3 and 6 derived from

two different parental viruses, which demonstrated that the virus can package and tranduce at least 10 viral segments (114).

This provides a mechanism by which Influenza could randomly package its genome yet increase its chances of assembling a particle with all of the necessary genetic material. In fact, theoretical calculations of the probability of packaging all eight necessary chromosomes have shown that, if the virus regularly packages 12 segments, it could randomly package the eight necessary segments 10% of the time, which is the observed particle to plaque forming unit ratio seen experimentally (24, 82). Sekellick et al. have also shown interesting, though indirect, data that bear on the question of segment packaging frequency. They have documented a population of viruses that have transient resistance to antiviral interferon activity in the infected cell. Statistical analysis of this population has provided indirect evidence that the average virion within it has packaged multiple copies of the NS gene, resulting in increased levels of NS1 protein during infection and a dose-dependent increase in resistance to Interferon. Based on their own calculation and those of Enami et al. (24), they predicted that, if influenza can randomly package twelve viral chromosomes, the probability of packaging one, two or three copies of any individual vRNA is 0.6, 0.3 and 0.08, respectively. These numbers mirror the relative percentages of the population that are resistance to low, medium and high levels of cellular interferon. Based on our observations that the percentage of co-expressing (GFP and YFP) transduction events is approximately 3% of the total population of transductants and the assumption that equal numbers of dual packaging events

(GFP-GFP and YFP-YFP) are also occurring at the same frequency, we would estimate that the number of RNA segments packaged by each virion is 12. Thus our results lend credence to the now growing field of evidence that influenze packages its viral genome in a non-sequence specific manner.

This evidence is further supported by mutational evidence mapping the packaging signal of vRNAs. The recent paper by Tchatalbachev et al. has mapped the nucleotide and, presumably, structural difference, which allows vRNA to be packaged over cRNA. The packaging signal appears to be a bulge in the 5′ end of the vRNA, an unpaired Adenine at position 10 next to the hairpin loop of the promoter. Because this structure is common to all vRNAs, and this one structural change allows cRNA to be packaged instead of vRNA, it is apparent that the packaging signal is one that is common to all vRNAs, thus would allow them to be packaged in a segment non-specific manner(127).

The idea that influenza packages its genome in a segment non-specific manner seems counter-productive to the virus life-cycle. Calculations of the probability of packaging have shown that even if the virus contains 12 niches for vRNAs, it still only achieves a 10% likelihood of collecting the 8 it needs to be infectious (24). So, the question remains as to why the virus has evolved this seemingly inefficient assembly system. One possibility stems from evidence in Sekellick et al. that packaging multiple copies of the NS gene enhances interferon resistance of the virus. Multiple copies of the NS gene presumably produce an increased concentration of NS1 protein in the cell, which is thought to be important to combat the antiviral effects of interferon (115). By having the ability

to package multiple copies of any one gene, the virus can, with each replicative cycle, test for advantages that may be conferred by carrying multiple copies of any gene. In terms of viral replication, carrying >one copy of any RNA would give that segment a replication advantage and thus increase its chances of being packaged once again. Thus would occur again and again until the copies of one gene were at such an advantage for packaging that other necessary RNAs were out-competed and the virus was rendered uninfectious. Thus influenza has an instantaneous method for evolving characteristics which might enhance its ability to survive and replicate in the host.

The ability to randomly package multiple viral RNAs might also lend a selective advantage to the virus by enhancing its genetic diversity. Influenza is known to exhibit a high frequency of mutations. This reflects the infidelity of the RNA-dependant RNA polymerase, which tends to make mistakes during transcription (119). This results in mutations of the genetic sequence from that of the parent strand and the possibility for an altered protein as a result, which may or may not benefit the virus. Recently, after many years of speculation, controversial results have been published about the possibility of genetic recombination occurring in influenza virus (33). It has been shown experimentally that viruses can re-assort viral segments between like strains which enhances genetic diversity; however, evidence of recombination during viral replication has not been reported until recently. In the paper by Gibbs et al. (33) it is demonstrated that nucleotide sequences of influenza hemagglutinin RNA sequence from human and swine lineages occur within one segment of one

RNA. The phylogenetic trees from these divergent strains of virus show that it is unlikely that these sequences would occur together within one RNA from normal genetic mutation alone. If correct, this recombination would have even greater implications in the context of a virus that can randomly package its genome and package more than one copy of many of its segments.

One could envision that multiple viruses might infect one mixing vessel, such as a pig, at once. In this setting each virus could re-assort and recombine its genome with that of the other virus, thus resulting in a highly recombinant strain (47). However, if the virus was also able to package multiple copies of some of its genes, it would amplify its genetic diversity even more. For example, one virus might package two different copies of the HA gene into one virion. In the second round of infection, both HA proteins would be expressed and incorporated into virions. Thus the progeny, which expressed two types of HA molecules, would have an advantage in evading the immune system. A simple example such as this illustrates that the use of a random method of packaging might not necessarily be the selective disadvantage that we tend to think it is and instead may be a simple, yet efficient, way of maintaining genetic diversity and evolving to be a better adapted virus in the context of the host.

The second facet of our research on influenza virus has focused on how this virus recognizes the host cell. This line of research has roots from the very beginning of characterization of influenza virus. It was originally found that influenza had the characteristic of agglutinating red blood cells (ie. red blood cells would form a lattice up the sides of a round-well dish as opposed to falling

to the center of the bottom (26, 73). This is thought to be due to viruses crosslinking multiple red blood cells and keeping them in position. It was then discovered that red blood cells treated with bacterial neuraminidase, an enzyme which removes sialic acid from the cell surface, lost their ability to be agglutinated by influenza (107). These early results provided the first evidence that sialic acid was the principal binding site for influenza virus on mammalian cells.

Until recently, specifics of the viral receptor still remained undefined. et al. and others have shown using lectin-based characterization of sialic acid species on tracheal epithelium of human, swine, ducks and horses that they differ in important ways. Sialic acid residues on human tracheal epithelium have glycoprotein chains that link sialic acid to its neighboring galactose in an α 2,6 linkage, whereas avian species have an α 2,3 linkage. Human and avian influenza strains preferentially bind the α 2,6 and α 2,3 forms, respectively. Swine epithelium express a mix of both linkages, which provides insight as to how this species may serve as a mixing vessel for both human and avian strains of influenza viruses. Specificity for each of these linkage structures has been characterized genetically in the viral hemagglutinin (HA). Recognition seems to depend on a single amino acid residue in the distal tip, the binding site, of the HA protein. In fact, mutating residue 226 of hemagglutinin from strain H1 changing it from a Gln to Leu amino acid, changes the specificity of the protein from NeuAc∝2, 3Gal to NeuAc∝2, 6Gal (20, 47, 105, 107, 124, 125).

Thus the recognition of sialic acid species appears to be highly specific. However, very little is known about types of protein or lipid backbones which might serve as optimal receptors for influenza viruses. Recently, mannose receptors have been implicated as being the primary receptor for influenza infection in macrophages (102). It is hypothesized that the interaction of the virus with the mannose receptor is mediated primarily by binding to terminal sialic acids in the glycosylated chains of the protein, but also may be enhanced by interactions of glycosylation sites on viral HA and NA that may interact with mannose receptor lectins (102). Thus, characteristics other than expression of sialic acid residues on glycosylated cell surface proteins may influence which proteins influenza uses as its primary receptor for infection.

Ablan et al. have tried to further define classes of glycoproteins which influenza may exploit as functional receptors. With the idea that influenza may specifically target glycolipids as receptors as opposed to glycoproteins, they compared a mouse fibroblast cell line that is devoid of cell surface glycolipids to a daughter cell line which expresses glycolipids to determine relative infectivity. In their hands, the expression of surface glycoplipids does not effect infectivity or fusion with influenza virus. Therefore, they conclude that glycolipids do not function as the major cell surface receptor in this cell line (2).

Specific characteristics of cell surface proteins may serve to make them better targets for influenza viruses. As sugar-protein interactions are known to be relatively weak, interactions between multiple HA molecules and receptors may become important to increase binding affinity. Zimmer et al. found that

influenza C virus appears to bind specifically to one particular cell surface protein in Madin-Darby Canine Kidney cells (MDCK). By subjecting the cells to digestion with a variety of proteases prior to binding studies, they were able to define the putative receptor as O-sialoglycoprotein endopeptidase (OSGE) sensitive, thus characterizing the protein as a O-linked sialomucin (141). Sialomucins typically have a high level of glycosylation as clusters of serine and threonine residues in the protein backbone, which terminate in sialic acid residues (16). Therefore, it would be possible for these proteins to support a polyvalent interaction between multiple hemagglutinin proteins and sialic acid residues and increase the possibility of achieving a productive interaction and infection.

By contrast, the high avidity of mucin binding to influenza HA may also serve a protective function for the host during infection. Wu et al. have characterized glycoproteins secreted from hamster tracheal cells as being mucin-like, with up to 12% of their amino acid and carbohydrate composition being sialic acid. They reported that secreted mucins from hamster tracheal cells have the ability to competitively inhibit influenza virus agglutination of red blood cells in vitro (136). This evidence, along with the reports from Zimmer et al. give evidence as to the highly effective nature of mucin binding by influenza virus and suggested multiple ways in which this interaction might be important to the biology of the virus (141, 142).

Therefore, it seemed logical to us that other strains of influenza might also utilize a similar receptor type, such as a sialomucin, for infection. In order to

determine if sialomucins, as a generic class of surface molecules, function as highly effective influenza receptors. In an attempt to answer this question, we asked whether over-expressing CD34, as sialomucin family molecule, on a moderately infectible cell line would increase infectivity of influenza A. Our results showed, by RNA expression patterns, that expression of CD34 on the surface of these cells did not increase infectivity. However, because we lacked an adequate means of measuring proportion of total sialiate on the cell surface contributed by sialomucins, we didn't have a clear idea if CD34 over-expression, as judged by immunostaining and FACS analysis, actually increased the number of mucin family molecules on the cell surface significantly above endogenous expression. Therefore, we utilized O-sialoglycoprotein endopeptidase (OSGE), which specifically cleaves O-linked cell surface sialomucin molecules (1, 122, 123). We were able to remove all surface sialomucin molecules, using CD34 removal as a marker, and to determine if endogenous mucin contribute significantly to influenza A infection in CHO, CHO-CD34 or MDCK cells. Using both RNA expression and viral titres to monitor viral replication, we found that surface mucin expression does not significantly impair infection by influenza A virus in any of the tested cell lines. Interestingly, at certain MOIs, we observed an increase in viral vRNA expression when cells were pre-treated with OSGE.

Because this was counter to the results described in Zimmer et al., we decided to test whether influenza C virus differs from influenza A in that it utilizes a mucin as its primary receptor (141). Therefore, we tested whether OSGE treatment of MDCK cells, by cleaving off gp40 and other sialomucins,

would decrease infectivity by influenza C. Our results show that OSGE treatment does not decrease infectivity of influenza C virus as judged either by RNA expression patterns or by hemagglutination assay. Interestingly, we also saw the increase in vRNA signal that was observed in influenza A infections.

These results led us to believe that either 1) influenza C virus, which binds strongly to gp40 in an in vitro virus binding assay, does not utilize this as its main functional receptor to infect MDCK cells, or 2) the sub-line of MDCK cells that we were working with did not express the gp40 sialomucin molecules predicted to function as the main receptor for influenza A in this cell line. Therefore, we tested if our MDCK cells expressed gp40 by using the antibody created by Zimmer et al. to detect gp40 expression (142). We first biotinylated surface proteins, treated with or without OSGE, and then immunoprecipitated with gp40 antibody. Precipitated proteins were separated on SDS-PAGE and probed with streptavidin-HRP. Additionally, we also separated total cell lysate, pre-treated with OSGE on SDS-PAGE and probed a Western with anti-gp40 and goat-anti mouse HRP. We were unable to detect gp40 surface expression in either assay and OSGE-treatment of cells did not change protein expression patterns on either gel. Therefore, we cannot determine if our MDCK cell line expresses the putative influenza C receptor gp40. However, we have shown that OSGE treatment in MDCK cells does not negatively affect infection; therefore, other sialomucin family members do not function as major receptors for influenza C in this cell line. Our results suggest that neither influenza A and C

utilizes surface sialomucin glycoproteins, on CHO and MDCK cells, as their primary functional receptors.

Our observation that OSGE treatment of cells prior to infection with influenza A or C virus increases vRNA expression levels is interesting as it is counter-intuitive to our prediction of a decrease in signal due to reduced infectivity of the virus. It is possible that the OSGE treatment somehow activates the cell, which results in an increase in transcription rates by the influenza polymerase. Many mucin family molecules, including CD34, have cytoplasmic domains that may be function as signaling domains. Two forms of CD34 are expressed in both murine and human cells, a full-length protein and a truncated form, the latter lacking all except 16 amino acids of the C-terminal domain and also lacking a potential target site for protein kinase C. There is preliminary evidence for a role of CD34 in prevention of terminal differentiation of myeloid cells. When myeloid leukemia cells were induced to differentiate in the presence of either the full-length or the C-terminal truncated form of CD34 they displayed a different differentiation program, providing indirect evidence for the importance of the C-terminal domain for cell signalling (25). However, it is not known if the cytoplasmic domain has signaling properties, such as the activation of Pol II transcription, which might increase viral RNA expression in the host cell, and which would thereby provide a mechanism by which cell surface molecules might influence infection other than virus binding at the cell surface.

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