Influenza A Viruses Lacking Sialidase Activity Can Undergo Multiple Cycles of Replication in Cell Culture, Eggs, or Mice

MARK T. HUGHES, 1,2 MIKHAIL MATROSOVICH, 3,4 M. ELIZABETH RODGERS, 3 MARTHA MCGREGOR, 3 AND YOSHIHIRO KAWAOKA 1*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706; 1 Department of Pathology, University of Tennessee—Memphis, Memphis, Tennessee 38163; 2 Department of Virology and Molecular Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105; and 3 M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, 142 782 Moscow, Russia

Received 29 November 1999/Accepted 3 March 2000

Influenza A viruses possess both hemagglutinin (HA), which is responsible for binding to the terminal sialic acid of sialyloligosaccharides on the cell surface, and neuraminidase (NA), which contains sialidase activity that removes sialic acid from sialyloligosaccharides. Interplay between HA receptor-binding and NA receptor-destroying sialidase activity appears to be important for replication of the virus. Previous studies by others have shown that influenza A viruses lacking sialidase activity can undergo multiple cycles of replication if sialidase activity is provided exogenously. To investigate the sialidase requirement of influenza viruses further, we generated a series of sialidase-deficient mutants. Although their growth was less efficient than that of the parental NA-dependent virus, these viruses underwent multiple cycles of replication in cell culture, eggs, and mice. To understand the molecular basis of this viral growth adaptation in the absence of sialidase activity, we investigated changes in the HA receptor-binding affinity of the sialidase-deficient mutants. The results show that mutations around the HA receptor-binding pocket reduce the virus’s affinity for cellular receptors, compensating for the loss of sialidase activity. Thus, sialidase activity is not absolutely required in the influenza A virus life cycle but appears to be necessary for efficient virus replication.

Influenza A viruses contain two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (14). The HA protein, a trimeric type I membrane protein, is responsible for virus binding to cell surface sialyloligosaccharide receptors and for mediating fusion between the viral envelope and cellular membranes. The NA possesses enzymatic activity that cleaves α-linked sialic acid from the receptor and the newly formed virions. Although viral sialidase activity was clearly dispensable for viral replication in this system, it was still uncertain whether such activity is needed to sustain efficient replication. In the experiments described here, we asked whether influenza virus can adapt to growth conditions lacking any sialidase activity, and if so, what is the molecular basis of such adaptation.

MATERIALS AND METHODS

Viruses and cells. The reassortant virus possessing the HA of A/NWS/33 and the NA of A/tern/Australia/G70c/75 (NWS-G70c) was obtained from the repository at St. Jude Children’s Research Hospital. Virus stock was grown either in 10-day-old embryonated chicken eggs or on Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin and 0.5 μg of trypsin per ml. For studies of receptor binding, all of the viruses were grown in MDCK cells and purified first by removing viral debris by low-speed centrifugation and then by pelleting through a 25% sucrose cushion. Purified viruses were suspended in 50% glycerol-0.1 M Tris buffer (pH 7.3) and stored at −20°C. MDCK cells were maintained in MEM supplemented with 5% newborn calf serum (Sigma, St. Louis, Mo.).

Production of the NA-expressing cell line 23-1i. MDCK cells were transfected with the use of Lipofectamine (Gibco-BRL, Gaithersburg, Md.) and the pDK775NA plasmid, which encodes the A/duck/Hong Kong/7/75 (DK/HK/75, H2N2) NA gene under the control of a chicken β-actin promoter, together with the puromycin resistance vector pPur (Invitrogen, Carlsbad, Calif.). A stable cell line expressing the DK/HK/75 NA gene was established by puromycin selection. The resultant cell line, 23-1i, was maintained in MEM supplemented with 5% fetal calf serum (SRH, Lenexa, Kans.) and 7.5 μg of puromycin sulfate (Sigma) per ml.

Generation of NA deletion mutant 23ANa. NWS-G70c virus was passaged 17 times on 23-1i cells in the presence of rabbit anti-N9 antiserum. The virus was then plaque purified (three successive rounds on 23-1i cells), and the resultant clone was designated NWS-G70c/23ANa (23ANa).

Production of sialidase-independent virus CK2-29. The NA deletion mutant 23ANa was passaged in liquid culture on MDCK cells in decreasing concentrations of exogenously added Clostridium perfringens sialidase (starting concentra-
tion, 30 mM/mL; Sigma). For each consecutive passage, the amount of added bacterial sialidase was reduced stepwise by approximately 0.5-log concentrations to a final concentration of 0.03 mM/mL by passage 12. Sixteen additional passages on MDCK cells were performed in the absence of any added bacterial sialidase. The resultant virus isolate was designated NWS-G70c/CK2-29 (CK2-29).

Passage of CK2-29 virus in embryonated chicken eggs. Undiluted CK2-29 was serially passaged five times in 10-day-old embryonated chicken eggs (1 mL of undiluted allantoic fluid per egg, five replicate samples) and incubated for 2 days at 35°C. Passages 6 and 7 were performed with 100 µL of undiluted allantoic fluid per egg, while passages 8 to 17 were performed with 100 µL of diluted allantoic fluid (1:10) per egg. Virus growth was monitored by hemagglutination of turkey erythrocytes and stained with anti-23-1i or anti-MDCK. Two independent egg-adapted viruses from separate replicates were biologically cloned in eggs by limiting dilution and are referred to as NWS-G70c/E17A (E17A) and NWS-G70c/E17E (E17E).

Passage of CK2-29 in BALB/c mice. BALB/c mice (6-week-old female) were intranasally infected with the CK2-29 virus concentration by ultracentrifugation (3.3 × 10^9 PFU/mouse). Mice were sacrificed on day 3 postinfection, and the lungs and nasal turbinates were harvested and homogenized in 1 mL of phosphate-buffered saline (PBS) containing antibiotics (1,000 U of penicillin and 10 µg of streptomycin per mL). For subsequent passage, 100 µL of the mixture of lung and nasal turbinate homogenates was used to infect two mice intranasally. In each passage, homogenates were grown on MDCK cells to determine the amount of virus present. After 18 passages, viral stock was prepared from mouse lung homogenates after a single passage on MDCK cells. This stock was designated NWS-G70c/M18B (M18B).

Sialidase activity assay. Viral sialidase activity was measured in virus suspensions containing 2 × 10^9 PFU and 2’-[(4-methylumbelliferyl)-o-N-acetyl neuraminic acid (Sigma) used as a substrate, as described previously (13). All reactions were done in triplicate.

Hemagglutination assay. Hemagglutinating activity was determined in a microplate format with 0.5% chicken red blood cells (RBCs) and PBS as a diluent. To avoid possible destruction of the sialyloligosaccharide receptors on chicken RBCs by the NA of parent virus, we included the neuraminidase inhibitor zanamivir (kindly provided by R. Bethell, Glaxo Wellcome, Hertfordshire, United Kingdom) (2 µM) in the reaction buffer. The hemagglutination reactions were performed in parallel on ice (0°C) and at 37°C.

Standardization of virus concentrations by ELISA for a receptor-binding affinity assay. The stock viruses in PBS (50 µL of serial twofold dilutions) were adsorbed in the wells of 96-well assay plates (Costar, Cambridge, Mass.) at 4°C overnight. The plates were washed with 0.1% Tween 80 solution in PBS. For detection by enzyme-linked immunosorbent assay (ELISA), 50 µL of anti-WSN virus rabbit antisera (diluted 1:1,000 in reaction buffer [RB]; 0.2% bovine serum albumin, 0.02% Tween 80 in PBS), was incubated in the wells for 1 h at 4°C. After washing, 50 µL of peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG; Sigma), diluted 1/5,000 in RB, was incubated in the wells for 1 h at 4°C. The plates were washed again, and bound peroxidase was visualized by incubation with 300 µL of standard 1:500 dilution of substrate solution for 30 min. The reaction was stopped by adding 50 µL of 5% sulfuric acid and quantified by measuring absorbancy at 490 nm. The dilutions of the viruses that produced an absorbancy of 0.4 were determined and served as a measure of virus concentration for the stock solutions (see Table 2).

Binding of fetuin-peroxidase conjugate. The viruses were adsorbed in the wells of 96-well assay plates (Costar, Cambridge, Mass.) at 4°C, and the binding of horseradish peroxidase-labeled fetuin to the solid-phase-adsorbed viruses was determined as previously described (9). To standardize the amounts of virus adsorbed onto 96-well assay plates, the binding of anti-WSN antibodies to virus-adsorbed plates on a replicate plate was assayed as described above.

Sequence analysis of NA and HA genes. Total viral RNA was obtained by digesting virus samples (250 µL each) with 100 µg of protease K in 10 mM Tris-CI (pH 7.5)–5 mM EDTA–0.5% sodium dodecyl sulfate–100 mM NaCl. For cDNA production, the oligonucleotide Uni-12, complementary to the conserved terminal nucleotides of influenza A virus gene segments, was used as a primer for the avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) reaction. The NA gene cDNA was specifically amplified during 30 rounds of PCR with the NA gene-specific primers N9KspUni12 (5’ cRNA sense primer; CTATATTAACCCTCACTAAAAGTAG) and WSN-E17A (3’ cRNA antisense primer; TTATTAACCCTCACTAAAAGTAG) and N9F3Un13 (3’ cRNA antisense primer; TTATTAACCCTCACTAAAAGTAG ATACAAGGGTGTTTTTTCT) and 10 U of Tag DNA polymerase (Promega). The resulting PCR product was separated by electrophoresis on 1% low-melting-temperature agarose (Gibco-BRL) and purified via Ultra-free-MC filtration (Millipore, Bedford, Mass.) per the manufacturer’s instructions. The resultant purified PCR product was then subcloned into the vector pCR2.1 (Invitrogen) and used as a template for automated fluorescent sequencing. The HA genes were cloned in a similar fashion using the HA gene-specific primers WSN-HA Forward (GATGCGATGACGCGCGAGAAAGTAG ATACAAGGGTGTTTTTTCT) and WSN-HA-Back (3’ cRNA antisense primer; CTCGAGATGAGTAGAAAACAGGTTTTTTCT). At least three independent cDNA clones were sequenced for each virus. When one of the three cDNA clones contained a different nucleotide at a given position, it was taken as evidence that an error had been introduced by the polymerase during PCR amplification.

RESULTS

Generation of a cell line expressing influenza virus NA. To facilitate generation of a sialidase-independent virus, we first produced a cell line that constitutively expressed an influenza virus NA capable of providing viral sialidase in trans. MDCK cells were transfected with pDK775NA, encoding the Dk/HK/75 NA (N2) protein. The resultant cell line, 23-1i, constitutively expressed Dk/HK/75 viral NA. All cells of the 23-1i line expressed N2 NA on their surface, as demonstrated by immunostaining (data not shown). The levels of sialidase activity expressed on 23-1i and MDCK cells infected with DK/HK/75 did not differ appreciably, as determined by a standard method (31) that used fetuin as a substrate (data not shown).

Generation of NA deletion mutant 23NA. To produce a mutant virus lacking sialidase activity, we passaged the NWS-G70c virus on 23-1i cells in the presence of rabbit anti-N9 antisera. Following 17 passages, the virus lacked detectable N9 NA expression, as determined by immunostaining of infected cells, and could no longer be neutralized by N9-specific antisera (data not shown). In contrast, the mutant virus gained sensitivity to neutralization by antibodies specific for the N2 NA supplied in trans by the 23-1i cell line (data not shown).

NA sequence of 23NA virus. To determine the molecular basis of resistance of 23NA to the N9 NA antisera, we sequenced the 23NA NA gene, discovering a large deletion of the NA open reading frame (bases 442 to 1170, CRNA orientation) as well as a point mutation, T110A, that created a stop codon at position 31 of the NA coding sequence (Fig. 1). These changes removed a large portion of the NA coding sequence, resulting in the expression of a small peptide that corresponded to the tail and the majority of the transmembrane domain of the NA protein (Fig. 1). Thus, passage of NWS-G70c in the presence of anti-N9 antisera and N2 NA supplied in trans yielded a virus similar to the previously described sialidase-deficient virus NWS-MviA (15).
To investigate whether the CK2-29 virus could be adapted to grow better in eggs, we passaged it 17 times in eggs as described in Materials and Methods. The virus titer in allantoic fluid gradually increased up to passage 5. Although we passaged the virus 12 additional times, we did not observe any further increase in virus titer. Two independently adapted clones, E17A and E17E, never reached the titers attained by the parental virus (NWS-G70c) in eggs (Table 1), but they still replicated substantially better than CK2-29.

To assess the ability of CK2-29 to grow in mice, we intranasally infected 6-week-old BALB/c mice with concentrated virus (3.3 × 10^9 PFU/mouse) in view of its limited growth in this animal. The initial infection produced titers approaching 4.6 × 10^8 PFU/g of tissue. After subsequent passage, the virus titers declined to approximately 10^5 to 10^7 PFU/g of tissue over 18 serial passages. The titer of the stock virus passed 18 times in mice (M15B) was substantially lower than the titers of other viruses (1.8 × 10^9 PFU/ml) (Table 1). CK2-29 also replicated poorly in eggs (5.9 × 10^5 EID_{50}/ml) (Table 1). PCR amplification of the NA gene of both egg-adapted viruses and the mouse-passaged CK2-29 virus indicated that all of the viruses maintained the truncated NA gene even after extensive passage. The PCR products produced were indistinguishable in

![Relative emission units (460nm)](http://jvi.asm.org/)

**FIG. 2.** Sialidase activity of the parental NWS-G70c virus, 23ΔNA, and the sialidase-independent CK2-29 mutant. For each sample, virus (2 × 10^9 PFU) was incubated for 1 h at 37°C in the presence of a fluorogenic sialidase substrate (4-methylumbelliferyl-α-D-neuraminic acid) in triplicate. The fluorescence of released 4-methylumbellif erone was determined with a fluorometer (Labsystems Fluoroskan II), with excitation at 355 nm and emission at 460 nm. Standard errors for the triplicate samples (at the 95% confidence interval) for NWS-G70c were too small to present in the graph. The detectable sialidase activity in the 23ΔNA NA deletion mutant results from sialidase activity of the N2 NA supplied in trans from 23-li cells.

### TABLE 1. Titers of the parental virus and its NA deletion variants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Description</th>
<th>Titer of stock examined in:</th>
<th>Relative infectivity&lt;sup&gt;a&lt;/sup&gt; (EID&lt;sub&gt;50&lt;/sub&gt;/PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MDCK cells (PFU/ml)</td>
<td>Eggs (EID&lt;sub&gt;50&lt;/sub&gt;/ml)</td>
</tr>
<tr>
<td>NWS-G70c</td>
<td>Parent</td>
<td>3.4 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>5.9 × 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>23ΔNA</td>
<td>NA deletion (sialidase dependent)</td>
<td>7.1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.0 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>CK2-29</td>
<td>NA deletion (sialidase independent, MDCK cell adapted)</td>
<td>6.9 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.1 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>E17A</td>
<td>NA deletion (sialidase independent in eggs)</td>
<td>1.0 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.3 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>E17E</td>
<td>NA deletion (sialidase independent in eggs)</td>
<td>4.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.8 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>M15B</td>
<td>NA deletion (sialidase independent passage in mice)</td>
<td>1.8 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.9 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio of the titer of virus stocks in eggs versus MDCK cells.

---

**Generation of sialidase-independent virus CK2-29.** To determine whether sialidase activity (including an exogenously added one) is an absolute requirement for influenza virus replication, we serially passaged the NA deletion mutant 23ΔNA in liquid culture on MDCK cells in decreasing concentrations of bacterial (*C. perfringens*) sialidase, obtaining sialidase-independent mutant virus CK2-29. Although this virus undergoes multiple rounds of replication in MDCK cells, even without exogenous sialidase, it grew to a lower titer than did the parent virus (Table 1) and produced smaller plaques (less than 1 mm in diameter) in the absence of bacterial sialidase than in its presence, suggesting that without sialidase activity, the virus cannot spread efficiently. During adaptation to growth in the presence of decreasing levels of sialidase activity, the HA titer of the virus gradually decreased. The CK2-29 virus, even when harvested from tissue culture wells displaying 100% cytopathic effect, did not hemagglutinate chicken RBCs. However, it retained this activity with turkey RBCs, which afford a more sensitive method of detecting influenza viruses. PCR amplification of the NA gene of the CK2-29 virus indicated that the virus retained the truncated NA gene. Analysis of the product revealed only two changes from that of 23ΔNA, a single-base deletion at residue 283 and a point mutation at residue 111, changing the TAG stop codon to a TAA stop codon. During adaptation to growth in mice, we intranasally infected 6-week-old BALB/c mice with concentrated virus (3.3 × 10^9 PFU/mouse) in view of its limited growth in this animal. The initial infection produced titers approaching 4.6 × 10^8 PFU/g of tissue. After subsequent passage, the virus titers declined to approximately 10^5 to 10^7 PFU/g of tissue over 18 serial passages. The titer of the stock virus passed 18 times in mice (M15B) was substantially lower than the titers of other viruses (1.8 × 10^9 PFU/ml) (Table 1). CK2-29 also replicated poorly in eggs (5.9 × 10^5 EID_{50}/ml) (Table 1). PCR amplification of the NA gene of both egg-adapted viruses and the mouse-passaged CK2-29 virus indicated that all of the viruses maintained the truncated NA gene even after extensive passage. The PCR products produced were indistinguishable in

---

**Growth of sialidase-independent CK2-29 in eggs and mice.** Increased functional sialidase activity is correlated with efficient replication of influenza virus in eggs (4). Moreover, adaptation to growth in mice or embryonated eggs results in changes in the viral genome owing to the selective pressures of these environments (22, 30). To determine the ability of a sialidase-independent virus to adapt to other environments, we assessed the growth of the CK2-29 mutant in both embryonated chicken eggs and mice.

We first determined the 50% egg infective dose (EID_{50}) of the CK2-29 stock in ovo. The virus replicated slightly less well in this medium than it did in MDCK cells (Table 1). By contrast, the NWS-G70c parent grew better in eggs than in MDCK cells, while the 23ΔNA virus grew poorly in eggs (Table 1). Thus, the loss of functional sialidase by 23ΔNA severely compromised its replication in eggs. However, adaptation to growth in MDCK cells restored the ability of the virus to grow in eggs.
size from that of CK2-29 by agarose gel electrophoresis (data not shown).

These results have demonstrated that viral sialidase activity is dispensable for multiple cycles of influenza virus replication in mice, although the replication is impaired.

**Receptor-binding properties.** To understand the molecular basis of viral adaptation to different environments in the absence of sialidase, we compared the receptor-binding properties of the viruses. Unlike the NA-deficient variants, the parent NWS-G70c virus contained enzymatically active NA that could affect the receptor-binding activity of this virus by partial desialylation of the receptors. To block this effect, we assayed the receptor-binding properties in the presence of the NA inhibitor zanamivir (GG167). Moreover, because the receptor-binding properties of the viruses can differ depending on the host-specific glycosylation of the HA (references 5 and 8 and references therein), we grew all viruses in MDCK cells for the binding studies.

First, we compared the ability of the viruses to agglutinate chicken RBCs. Virus concentrations in purified virus stock suspensions were determined by ELISA with polyclonal anti-WSN serum; specific hemagglutinating activity was calculated as the ratio of the hemagglutination titer at 4°C to the ELISA titer (Table 2, HA/ELISA). Thus, the lower the ratio, the lower the ability of the virus to agglutinate chicken RBCs. The specific hemagglutinating activity at 4°C, calculated as the ratio of the hemagglutination titer at 37°C to that at 4°C (Table 2, HA37/HA4), increased in the order parent virus > NA virus contained NA variant CK2-29 > E17A > E17E and M18B.

In addition to the specific hemagglutinating activity at 4°C, we determined the ratios of HA titers at two temperatures (Table 2, HA37/HA4). Given that a greater decrease in the HA titer at 37°C than at 4°C reflects a lower binding affinity of the virus (5), these experiments confirm that the affinity of the 23ΔNA variant is not lower than that of the parental virus. The CK2-29 variant bound much less avidly to chicken RBCs than did either the parental virus or the 23ΔNA variant. Both egg-adapted variants and the mouse-adapted virus had even lower affinities than that of the CK2-29 virus.

To further characterize the receptor-binding properties of the viruses, we determined their ability to bind the soluble sialylglycoprotein fetuin. After adsorption in the wells of plastic plates, the viruses were allowed to bind either peroxidase-labeled fetuin (Fig. 3A) or rabbit anti-WSN antibodies followed by peroxidase-conjugated anti-rabbit IgG antibodies (Fig. 3B). As shown in Fig. 3B, an experiment in which antibody binding was used to estimate virus density, all viruses except 23ΔNA were present in equal amounts on the plates. Therefore, substantial differences in the binding of fetuin-horseradish peroxidase conjugate by these viruses cannot be explained by their different concentrations on the solid phase but rather reflect their different abilities to bind fetuin. That is, CK2-29 bound fetuin much less avidly than did the parent virus; binding by the variant E17A was only slightly greater than background; while E17E and M18B binding failed to be detected with the assay used. The density of the 23ΔNA variant on the solid phase in our assay was always lower than that of other viruses, regardless of the stock preparation used. The reason for this discrepancy is not clear. Nonetheless, there was a clear correlation between the results of the hemagglutination (Table 2) and fetuin-binding (Fig. 3) assays. Both yielded the same rank order of viruses based on their relative affinity for sialic acid-containing receptors (Table 3): parental virus and 23ΔNA > CK2-29 > E17A > E17E and M18B.

**Mutations in the HAs of sialidase-independent viruses.** Because the function of the viral NA counters the activity of the HA protein, one could expect interplay between the HA’s sialic acid-binding activity and the NA’s sialidase activity, as has been shown recently (2, 10, 18, 27). Adaptation to growth in the absence of sialidase activity resulted in a concomitant decrease in the affinity of the HA protein for cellular receptors, as described above. To identify the HA alterations responsible for this decreased affinity, we sequenced the HA gene of the sialidase-independent mutants. The 23ΔNA virus contained two mutations, Ser to Arg at residue 193 (H3 numbering system according to the H1/H3 alignment of Nobusawa et al. [20]) and Val to Met at residue 205 (Table 3). Residue 193 is a component of the alpha-helix occupying the top of the receptor-binding pocket (32), and residue 205 lies on the far side of the globular HA protein head away from the receptor-binding site (Fig. 4). With one exception (E17E; M205V reversion), all NA-deficient variants obtained by passaging the 23ΔNA virus in different hosts retained these two mutations.

The HA of the sialidase-independent virus CK2-29 contains these two mutations as well as three additional ones: Val to Ala at residue 135, Ser to Asn at residue 145, and Arg to Lys at residue 220. All three residues are in close proximity to the receptor-binding pocket of the HA protein. All adapted variants of CK2-29 had additional mutations around the receptor-binding site (Table 3, also Fig. 4), consistent with their altered receptor-binding activity.

**DISCUSSION**

In this study, we have demonstrated that influenza A viruses can adapt to growth in tissue culture, embryonated eggs, and mice in the absence of a functional sialidase. The acquisition of sialidase independence resulted from mutations in the HA, which led to a decrease in the affinity of the virus for sialic

---

**TABLE 2.** Hemagglutination activity of parental virus and its NA deletion variants.

<table>
<thead>
<tr>
<th>Virus</th>
<th>ELISA titer</th>
<th>Hemagglutination titer at:</th>
<th>Specific hemagglutinating activity (HA/ELISA titer)</th>
<th>Ratio of hemagglutination titer at 37°C to that at 4°C (HA37/HA4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWS-G70c</td>
<td>6,000</td>
<td>1,600</td>
<td>1,200</td>
<td>0.3</td>
</tr>
<tr>
<td>23ΔNA</td>
<td>2,000</td>
<td>320</td>
<td>320</td>
<td>0.2</td>
</tr>
<tr>
<td>CK2-29</td>
<td>12,000</td>
<td>320*</td>
<td>40*</td>
<td>0.03</td>
</tr>
<tr>
<td>E17A</td>
<td>5,000</td>
<td>40*</td>
<td>&lt;10</td>
<td>0.01</td>
</tr>
<tr>
<td>E17E</td>
<td>5,200</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>M18B</td>
<td>6,600</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

* a Virus did not show complete agglutination.

* b NA, not available.
acid-containing receptors, thus restoring the balance between cell binding and viral release from cells. This finding is consistent with previous observations that an imbalance between NA activity and HA receptor-binding affinity, due to a deletion in the NA stalk (4) or reassortment between human and avian viruses (11), impairs the fitness of the virus. Similarly, resistance to the NA inhibitor zanamivir and related compounds accompanies changes in both the NA and HA proteins (10, 18, 27). Thus, mutations in the HA and NA appear to be capable of modulating the balance between HA receptor affinity and NA sialidase activity, depending on the growth environment.

Our sialidase-independent viruses represent an extreme example of how HA mutations can shift the normal HA-NA balance of influenza A viruses when no sialidase activity is present. The 23ΔNA virus does not grow on MDCK cells in tissue culture; however, its adapted variant (CK2-29) showed relatively good replication by the reduction of HA affinity. Interestingly, this tissue culture-adapted virus did not grow well in eggs and mice, suggesting that a decrease in the affinity of HA alone is not sufficient for sialidase-independent growth in eggs or mice. In other words, growth in these hosts (in vivo) is more dependent on functional NA than growth in tissue culture. This conclusion is reinforced by changes observed in the egg-adapted and mouse-adapted viruses; both acquired additional mutations in the HA and showed decreased affinity of HA by comparison with the tissue culture-adapted CK2-29 virus.

Adaptation of the sialidase-independent CK2-29 virus to eggs increased egg infectivity by more than 10-fold. Analysis of the receptor-binding activity suggested that the receptor affinity of egg-adapted and mouse-passaged viruses was reduced compared with that of their parent CK2-29 virus. Thus, for viruses lacking any sialidase activity, a reduction in HA receptor affinity seems to promote better growth in eggs. This interpretation agrees with the previous finding that higher functional sialidase activity is needed for increased virus replication in eggs (4). That is, to replicate efficiently in ovo, the virus must be efficiently released from cells through either an increase in sialidase activity or reduced receptor affinity.

Previously, Liu et al. (16) demonstrated the ability of the NA deletion mutant NWS-MviA to persist for 28 days in immunocompromised nu/nu mice. Our sialidase-independent CK2-29 virus can undergo multiple rounds of replication in BALB/c mice and be passaged more than 18 times, resulting in the introduction of two mutations in the HA which decrease the receptor-binding affinity of M18B virus compared with the parental CK2-29 virus, suggesting that they can compensate for

![FIG. 3. Fucin-binding affinity of NWS-G70c and its NA deletion variants. The viruses were adsorbed in the wells of plastic microplates and allowed to bind either peroxidase-labeled fetuin (Fet-HRP) (A) or rabbit anti-WSN antibodies, followed by peroxidase-conjugated anti-rabbit IgG antibodies (B). Levels of bound fetuin or antibody were then determined with the horseradish peroxidase substrate 3-phenylenediamine and quantified by measuring absorbance at 490 nm. The binding of antibodies served as a measure of virus density on the solid phase.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative receptor-binding affinity</th>
<th>Amino acid changes in the HA by H3 numbering (H1 numbering)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWS-G70c</td>
<td>++++++</td>
<td>S V</td>
</tr>
<tr>
<td>23ΔNA</td>
<td>++++++</td>
<td>A T V S H S R M R</td>
</tr>
<tr>
<td>CK2-29</td>
<td>++</td>
<td>A N</td>
</tr>
<tr>
<td>E17A</td>
<td>+</td>
<td>T A N</td>
</tr>
<tr>
<td>E17E</td>
<td>–</td>
<td>K A P N V K</td>
</tr>
<tr>
<td>M18B</td>
<td>–</td>
<td>A P O N K</td>
</tr>
</tbody>
</table>

* Based on hemagglutination data in Table 2. ++++++, highest affinity; specific hemagglutinating activity, >0.2; ++, moderate affinity; specific hemagglutinating activity, 0.03; +, weak affinity; specific hemagglutinating activity, 0.01; –, affinity below the sensitivity of the assay.

* Changes in the HAI sequences represent differences between the 23ΔNA and the other viruses, including the parental strain. Blank spaces indicate that the amino acid is identical to that in 23ΔNA.

* Substitution A96T creates a potential glycosylation site at Asn-96.
the lack of viral sialidase. However, we did not find any increase in virus titers in mouse lungs during passaging, so that the role of these mutations in virus replication in mice remains uncertain.

To understand the molecular mechanisms by which sialidase-deficient viruses decrease their binding affinity during adaptation to distinct environments, we analyzed the amino acid sequences of their HA proteins (Table 3). The original NA deletion mutant 23ΔNA acquired two mutations, S193R and V205M, by comparison with the parental virus. Changes at these HA positions were previously implicated in the altered receptor-binding activity of H3N2 human influenza viruses (6, 28, 29), suggesting that they may have the same effect in the 23ΔNA virus.

The MDCK-adapted, sialidase-independent isolate CK2-29 has three mutations (G135A, S145N, and R220K) with respect to its progenitor, 23ΔNA. The former two substitutions are shared by all sialidase-independent mutants and therefore appear to be critical for the reduction in their receptor-binding affinity. Amino acid 135 is located in the polypeptide chain from 134 to 139, which forms the “right” side of the receptor-binding pocket and is primarily responsible for interactions with sialic acid (residues 134, 135, 136, and 137 each participate in direct atomic interactions with the sialic acid [25, 32]). Therefore, even subtle changes in this region of the receptor-binding site may affect the affinity of the virus for the receptor determinant. Mutations at position 145 were previously shown to be associated with egg adaptation of H3 influenza viruses (22) and have also been identified in horse serum-resistant H3 variants (17). Thus, a mutation at this position alone could affect the receptor-binding activity of the virus. However, residues 135 and 145 are in direct contact (Fig. 4), so that mutations in both sites could act in concert, leading to greater effects on receptor binding than could be anticipated from a change in either site alone. Amino acid 220 is located on the boundary between the HA monomers in relatively close proximity to the amino acids that form the bottom left portion of the receptor-binding site, and the Arg-220 is conserved across all 15 influenza A virus HA subtypes (12, 20, 23). Mutations at this site (R220G or R220S) were found in serum-resistant variants of H3N2 human virus (24) and the H3 virus isolated from seals (3), suggesting that changes in position 220 may be involved in adaptation of the virus receptor-binding characteristics to new host environments. However, the effects of substitutions in position 220 on the receptor-binding properties of the virus have not been unambiguously demonstrated in either this or previous studies because the mutation was always accompanied by other substitutions in the HA.

The adaptation of CK2-29 to growth in eggs and mice led to a selection of variants with additional amino acid changes in the HA and a further decrease in affinity. In the mouse-adapted virus M18B there are two such substitutions, S140P and H141Q. These amino acids are located on the peptide loop (140 to 145) that forms the right bottom rim of the receptor-binding pocket and can potentially interact with the asialic part of the receptors. Interestingly, the S140P mutation was also independently selected in egg-adapted variant E17E. Another substitution in E17E that likely contributes to its decreased affinity, T132K, is located in the upper-right rim of the binding pocket. Finally, the E17A isolate contains a unique mutation,
A96T, that creates a potential glycosylation site, which is also present in H1 avian viruses and in most H1 human viruses. A bulky oligosaccharide moiety at this location could either sterically hinder the receptor-binding site or affect the position of polypeptide chain 220 to 228 and/or 134 to 139, which form the sialic acid-binding pocket. It is evident from these data that each variant contains multiple amino acid substitutions rather than single mutations, suggesting that NA deletion viruses are so severely impaired that a single substitution cannot compensate for the defect. 

As with the NWS-MViA virus (33), the CK2-29 virus, after extensive passage in tissue culture, eggs, or mice, retained a truncated NA gene with the capacity to direct synthesis of the cytoplasmic tail and some (our mutants) or all (NWS-MviA virus [33]) of the transmembrane domain and a short stalk. These findings suggest that the truncated NA protein may be important in the virus replication cycle, perhaps in virion morphogenesis and stability. Alternatively, the truncated NA RNA per se may be the critical element in events such as virion morphogenesis and ribonucleoprotein packaging. Recently, workers in our laboratory and others have established a new reverse-genetics system that allows influenza viruses to be generated entirely from cloned cDNA (7, 19). Using this system, one could directly address the potential role of the truncated NA gene, its gene product, or both in influenza virus replication.

ACKNOWLEDGMENTS

We thank Krisna Wells for excellent technical assistance and John Gilbert for editing the manuscript. Support for this work came from National Institute of Allergy and Infectious Diseases, Public Health Service, research grants.

REFERENCES