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Neuraminidase Hemadsorption Activity, Conserved in Avian Influenza A Viruses, Does Not Influence Viral Replication in Ducks

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The N1 and N9 neuraminidase (NA) subtypes of influenza A viruses exhibit significant hemadsorption activity that localizes to a site distinct from that of the enzymatic active site. To determine the conservation of hemadsorption activity among different NAs, we have examined most of the NA subtypes from avian, swine, equine, and human virus isolates. All subtypes of avian virus NAs examined and one equine virus N8 NA possessed high levels of hemadsorption activity. A swine virus N1 NA exhibited only weak hemadsorption activity, while in human virus N1 and N2 NAs, the activity was detected at a much lower level than in avian virus NAs. NAs which possessed hemadsorption activity for chicken erythrocytes (RBCs) were similarly able to adsorb human RBCs. However, none of the hemadsorption-positive NAs could bind equine, swine, or bovine RBCs, suggesting that RBCs from these species lack molecules, recognized by the NA hemadsorption site, present on human and chicken RBCs. Mutagenesis of the putative hemadsorption site of A/duck/Hong Kong/7/75 N2 NA abolished the high level of hemadsorption activity exhibited by the wild-type protein but also resulted in a 50% reduction of the NA enzymatic activity. A transfectant virus, generated by reverse genetics, containing this mutated NA replicated 10-fold less efficiently in chicken embryo fibroblast cultures than did a transfectant virus expressing the wild-type NA. However, both viruses replicated equally well in Peking ducks. Although conservation of NA hemadsorption activity among avian virus NAs suggests the maintenance of a required function of NA, loss of the activity does not preclude the replication of the virus in an avian host.

Influenza viruses rely on two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), to provide distinct activities important for virus replication. HA binds to terminal sialic acid groups on cell surface glycoconjugates for cell surface attachment. NA possesses an enzymatic activity that removes sialic acid from sialoglycoconjugates. This activity enables release of virions from infected cells and removes sialic acid from newly synthesized HA and NA molecules to prevent self-aggregation of virions (14).

While HA and NA have distinct activities in the infective process, it was found that the NA of A/tern/Australia/G70c/75 (H11N9) possesses a high level of hemadsorption activity in addition to its NA activity (10). Inhibition of the NA enzymatic activity by 2-deoxy-2,3-dehydro-*N*-acetyl-neuraminic acid has no effect on hemadsorption activity, indicating that these two activities are probably located at separate sites on the molecule (10). This was confirmed by examination of N9 variants with single-amino-acid substitutions that dramatically reduced or abolished the level of hemadsorption activity without affecting the NA activity (20). Mapping the locations of these amino acid substitutions on the crystallographic structure of N9 NA identified surface loops responsible for hemadsorption activity that formed a shallow depression on the surface, which was distinct from the deep depression that contained the NA enzymatic active site. Further, transfer of N9 amino acid residues 368 to 370 and residues 399 to 403, which participate in N9 hemadsorption activity, into the corresponding region of A/Tokyo/3/67 N2, which does not have hemadsorption activity, confers hemadsorption activity to the N2 molecule (13).

Identification of hemadsorption activity associated with the N1 NA subtype has also been reported (5). As for N9 NA, N1 NA hemadsorption of chicken or human erythrocytes (RBCs) occurs at 4°C but not at 37°C (1, 5, 10). However, once the N9 NA is bound to RBCs at 4°C the binding does persist at 37°C in phosphate-buffered saline (PBS), at pH 7.2, with little or no elution of the RBCs from the NA (1, 10). This observation and the conservation of NA hemadsorption activity among two NA subtypes suggest that there may be a biological role for hemadsorption activity in virus replication.

Like the HA molecule of the influenza virus, the hemadsorption sites of N1 and N9 NAs are believed to bind sialic acid. Pretreatment of RBCs with viral NA will prevent binding to HA but not to N9 NA, although the hemadsorption activity of N9 can be prevented by pretreating the RBCs with broad-specificity bacterial sialidases. Based on these results, it has been suggested that the NA hemadsorption site shows preference for an unidentified alternative species or linkage of sialic acid (1, 5).

We wanted to investigate the conservation of hemadsorption activity among NA subtypes of virus isolates from various species and to assess any possible biological role for the hemadsorption activity of NA. The hemadsorption activity of several avian, swine, and human virus NA molecules was characterized by using NA expressed on the surface of Cos-1 cells. Sequence comparisons between the hemadsorption-positive N2 NA of A/duck/Hong Kong/7/75 (H3N2) and N9 NA of A/tern/Australia/G70c/75 indicated conservation of residues critical to N9 hemadsorption activity. Based on this sequence analysis, the

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N2 NA gene was mutated in the region believed to be responsible for its hemadsorption activity. We report our findings on the altered hemadsorption activity of this mutant as well as on the growth characteristics, in chicken embryo fibroblasts (CEFs) and in ducks of a virus, generated by reverse genetics, that expresses the mutated N2.

MATERIALS AND METHODS

Viruses, cells, and antibodies. Influenza virus A/duck/Hong Kong/278/78 (H2N9) was used as a helper virus to rescue the N2 NA gene of A/duck/Hong Kong/7/75 (H3N2), by using reverse genetics (2, 3). Madin-Darby bovine kidney (MDBK) cells were cultured in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum (FCS). Madin-Darby canine kidney (MDCK) cells were cultured in MEM with 5% newborn calf serum. CEFs were prepared from 10-day-old chicken embryos, as described previously (17), and were cultured in MEM supplemented with 10% newborn calf serum. Cos-1 (simian virus 40 [SV40]-transformed African green monkey) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. For the selection of transfectant viruses, the N2- and N9-specific antibodies used were each made up of a pool of several monoclonal antibodies.

Cloning and expression of NA genes. The plasmid expression vector pCAGGS (12) was modified to contain a multiple-cloning site between the *Eco*RI and *Bgl*II sites of the rabbit β-globin gene. Unique restriction sites for the enzymes *Sac*I, *Cla*I, *Nsi*I, *Asp*718, *Sma*I, *Sph*I, *Xho*I, and *Nhe*I were inserted at this site to create pCAGGS/MCS. We selected at least one example of each NA subtype, except N4, from avian, swine, equine, and human virus isolates to examine for hemadsorption activity. Full-length cDNAs of the NA genes were cloned into the multiple-cloning site of pCAGGS/MCS, which is under the control of the chicken β-actin promoter. Each NA gene was expressed in Cos-1 cells, which express the SV40 large T antigen that enables the efficient replication of the plasmid that contains the SV40 origin of replication. Cells, at 70% confluency, were transfected with 2 μg of purified plasmid DNA per well of a six-well tissue culture plate by using Lipofectamine (Gibco). The cells and transfection mixture were incubated for 5 h at 37°C, and then the transfection medium was replaced with 2 ml of 5% FCS–OPTI-MEM (Life Technologies Inc.) per well. The cells were then incubated for 18 h at 37°C. Expression of NA was determined by measuring the enzymatic activity of the expressed protein. The cells were washed with calcium saline buffer (CaS) (6.8 mM $CaCl_2$, 154 mM NaCl, 19.5 mM H_3BO_3, 0.136 mM $Na_2B_4O_7$), scraped off the tissue culture well, and resuspended in a mixture of 50 μl of CaS and 50 μl of 1.25% fetuin in 0.2 M sodium phosphate buffer (pH 5.9). The cells were incubated for 12 h at 37°C, and the amount of liberated sialic acid was determined by the periodate-thiobarbituric acid assay (19). Alternatively, dilutions of the resuspended cells were prepared in CaS. In a 96-well plate, 5 μl of each dilution was incubated with 5 μl of 0.2 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma) in 0.2 M potassium phosphate (pH 5.9) for 30 min at 37°C. The reaction was stopped by the addition of 200 μl of 0.1 M glycine–25% ethanol (pH 10.7) per well. The fluorescence of released 4-methylumbelliferone was determined in a fluorometer (Labsystems Fluoroskan II) with excitation at 355 nm and emission at 460 nm.

Determination of hemadsorption activity. Six-well tissue culture plates containing Cos-1 cells expressing NA protein were washed with 0.01% gelatin–OPTI-MEM at 37°C. The cells were then incubated with 1% chicken RBCs in PBS at 4°C for 30 min. Selected NAs were also assayed for hemadsorption activity with human, swine, equine, and bovine RBCs. Unbound RBCs were removed by washing with 0.01% gelatin–OPTI-MEM followed by one wash with PBS. The cells were then fixed with 10% phosphate-buffered formalin for 2 h at 4°C. Following one wash with methanol, the cells were air dried and then stained with 1 ml of a 1:20 dilution of Giemsa stain (Sigma) for 15 min at 4°C.

To determine the effect of inhibiting NA enzymatic activity on hemadsorption activity, cells were incubated with 0.5 μM 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid (GG167) (Glaxo) for 2 h at 37°C prior to the addition of RBCs.

Site-directed mutagenesis of N2 NA. The full-length N2 NA gene of A/duck/Hong Kong/7/75, including its 5′ and 3′ untranslated regions, was cloned into the pUC19 *Eco*RI site as previously described (8). A unique *Sap*I restriction enzyme site was introduced at the 5′ end of the coding sequence without disrupting the sequence of the untranslated region, and the T3 RNA polymerase promoter sequence was added at the 3′ end of the N2 gene to generate plasmid pUCT3D 775NASap. Based on sequence comparison of the N2 protein and that of N9 of A/tern/Australia/G70c/75 in a region important for the hemadsorption activity of N9 (residues 367 to 371) (20), conserved residue serine 370 (N2 numbering) was selected for mutagenesis to leucine. Mutagenesis of the construct at nucleotides 1127 and 1128 (Ser-370 to Leu) was done by using the oligonucleotide 5′-AATCAGCAAAGATCTACGCTCAGGTTATGA-3′. Mutagenesis was performed according to the procedure of the Clontech Transformer site-directed mutagenesis kit (catalog no. K1600-1) with the kit selection Trans Oligo *Nde*I/*Nco*I to select plasmid pUCT3D775NASap-370SL. To generate a plasmid for expression of the mutated N2 NA gene, the *Nsi*I/*Asp*718 restriction enzyme digestion fragment was subcloned into the pCAGGS/MCS vector that contains the wild-type N2 NA gene to generate plasmid pCAUCT3D775NASap-370.

Generation of viruses containing wild-type and mutant N2 genes by reverse genetics. The nucleoprotein and polymerase proteins were purified from egg-grown A/Puerto Rico/8/34 (H1N1) as described previously (15). Plasmids pUCT3D775NASap and pUCT3D775NASap-370SL were digested with *Sap*I, and their ends were filled in with Klenow fragment as described previously (3). To generate an NA-ribonucleoprotein complex, the plasmids were transcribed in vitro with T3 RNA polymerase in the presence of nucleoprotein and polymerase. The NA-ribonucleoprotein complex was then transfected into 80% confluent MDBK cells infected 1 h earlier with A/duck/Hong Kong/278/78 (H2N9) at a multiplicity of infection of 1. Eighteen hours after transfection, the culture supernatants were collected and clarified by centrifugation (10,000 × *g*, 30 s). Transfectant viruses were selected by using N2-specific antibodies to trap N2-expressing virions, and N9-specific antibodies were used to suppress growth of the helper virus, according to the procedure previously described (7). The N2-expressing virions were then plaque purified three times on MDCK cells, and a final tissue culture stock of the cloned virus was grown on MDCK cells and used to inoculate 11-day-old embryonated chicken eggs. Allantoic fluid stocks of viruses Dk78/75N2 (containing the wild-type N2 gene) and Dk78/370SL (containing the mutated gene) were stored at −70°C. The sequence of the N2 gene of each virus was confirmed by sequencing of the gene from PCR-amplified cDNAs, produced from viral RNA isolated from each virus, with an automated sequencer (Applied Biosystems Inc., Foster City, Calif.).

Comparison of transfectant virus NA enzymatic activities. Tenfold serial dilutions of allantoic fluid stock of each transfectant virus were blotted onto Hybond nitrocellulose blotting membrane (Amersham) by using the Bio-Dot SF microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. The blot was blocked overnight in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 1% bovine serum albumin [BSA]) and then probed with a 1:500 dilution of the N2-specific antibody pool for 3 h at 20°C. After the blot was washed, the bound primary antibody was detected by chemiluminescence by using the ECL Western blotting kit (Amersham) according to the manufacturer's protocol. The optical densities of the bands were determined with a Sparc station 2 densitometer (Sun Systems, Mountain View, Calif.) with Bioimaging Visage 110 software (Millipore, Bedford, Mass.). With equal amounts of NA protein based on densitometry results, the NA enzymatic activities of the transfectant viruses were determined by using the substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, as described above.

Titration of transfectant viruses in Peking ducks. The median egg infectious dose (EID_{50}) titers of the allantoic fluid stocks of viruses Dk78/75N2 and Dk78/370SL were determined by titration in 11-day old embryonated chicken eggs. Three 3-month-old Peking ducks (Ridgeway Hatcheries) were orally inoculated with serial dilutions of equal EID_{50} titers of each transfectant virus (1 ml/duck). Three days postinoculation, the ducks were sacrificed and their colons were removed. The tissue was homogenized, resuspended in PBS, and titrated in 11-day-old embryonated chicken eggs to determine the extent of replication of the virus in the duck intestine. The sequence of the N2 gene of virus isolated from one duck inoculated with Dk78/370SL was determined by sequencing of the entire gene from PCR-amplified cDNA, as described above.

Growth of transfectant viruses on CEFs. The growth of the two transfectant viruses was examined by using CEFs as an avian cell model. To determine the median tissue culture infective dose ($TCID_{50}$) of each virus, 10-fold serial dilutions of the transfectant viruses were titrated in triplicate wells of CEFs in 24-well tissue culture plates in the presence of MEM supplemented with 0.3% BSA and 0.5 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin per ml. Replication of virus was determined 2 to 3 days postinfection by hemagglutination with 0.5% chicken RBCs. Based on the $TCID_{50}$ of each virus, one well of CEFs in a six-well plate was incubated with each virus at an estimated multiplicity of infection of 0.25 for 1 h at 37°C. Cells were then washed three times to remove excess virus. Virus that was not internalized or removed by washing was neutralized by incubation with a 1:100 dilution of H2-specific polyclonal antiserum for 30 min at 37°C. After the antibody was removed, the cells were overlaid with 3 ml of MEM–0.3% BSA containing 0.5 μg of TPCK-treated trypsin per ml and incubated at 37°C. Virus replication was monitored at selected time intervals by titration of the supernatant on CEFs. Titers are reported as the $TCID_{50}$ calculated from duplicate assays.

Characterization of thermostabilities of transfectant viruses. The thermostabilities of transfectant viruses Dk78/75N2 and Dk78/370SL were compared. The viruses were incubated at 37 and 45°C, and aliquots were removed at intervals of 4, 8, and 12 h and titrated on CEFs. The viability of each virus at each time point was reported as the $TCID_{50}$.

NA amino acid sequence analysis. The amino acid sequences of NAs were compared among viruses in the regions of residues 367 to 371 and residues 399 to 403, previously shown to be responsible for N9 hemadsorption activity (20). The amino acid sequence of each NA from position 364 to 375 and position 394 to 410 (N2 numbering) was aligned by using the Wisconsin Package of the Genetics Computer Group, Inc. At least one example of each NA subtype was examined, with either sequences of NAs examined in this study or published NA sequences being used.

TABLE 1. Relative enzymatic and hemadsorption activities of Cos-1 cell-expressed NAs

Construct	Virus	Host	Subtype	Enzymatic activity[a]	Hemadsorption activity[b]
pCTMN8	A/turkey/Missouri/81	Avian	H1N1	++++	++++
pCDAN26	A/duck/Australia/80	Avian	H1N1	+++	++
pCD775NA	A/duck/Hong Kong/7/75	Avian	H3N2	+++	+++
pCDHK342N2122	A/duck/Hong Kong/342/78	Avian	H5N2	++++	++++
pCG2838NA18	A/gull/Delaware/2838/87	Avian	H7N2	+++	+++
pCNA88	A/guinea fowl/New York/3070/91	Avian	H2N2	++++	++++
pCKWANA46	A/widgeon/Alberta/284/77	Avian	H7N3	++++	++++
pCTENA85	A/turkey/England/63	Avian	H7N5	+	++
pCPDANA67	A/pintail duck/Alberta/712/80	Avian	H3N6	++	++
pCCVNA19	A/chicken/Victoria/85	Avian	H7N7	++++	+++
pCDHNA116	A/duck/Hokkaido/8/80	Avian	H3N8	+++	++
pCAGGSN9NA	A/tern/Australia/G70c/75	Avian	H11N9	+++	++++
pCETNNA18	A/equine/Tennessee/5/86	Equine	H3N8	++++	++++
pCSITNA15	A/swine/Italy/437/76	Swine	H1N1	++++	++
pCR8NA51	A/Puerto Rico/8/34	Human	H1N1	+++	+
pCROMENA1	A/Rome/49	Human	H1N1	++++	+
pCUd72NA48	A/Udorn/307/72	Human	H3N2	++++	+
pCLANA55	A/Los Angeles/2/87	Human	H3N2	++++	+

[a] The relative enzymatic activities of Cos-1 cell-expressed NAs were quantified based upon color development, measured at 549 nm, by the periodate-thiobarbituric acid assay (19) for sialic acid released from fetuin following overnight incubation with the cells. Maximal activity (++++) corresponds to an optical density at 549 nm (OD_{549}) of ≥2.1; +++ corresponds to an OD_{549} of 1.1 to 2.0, ++ corresponds to an OD_{549} of 0.6 to 1.0, and + corresponds to an OD_{549} of 0.2 to 0.5.

[b] Results are reported for hemadsorption of chicken RBCs. Representative micrographs of the relative hemadsorption activities for the Cos-1 cell-expressed NAs are shown in Fig. 1. ++++, 100% maximal activity; +++, 60 to 80% maximal activity; ++, 5 to 10% maximal activity; +, <1% maximal activity.

RESULTS

Hemadsorption among NA subtypes from avian, swine, equine, and human influenza A viruses. To examine the conservation of hemadsorption activity among a wide range of NA subtypes from several host species, we expressed cloned NA genes from a plasmid expression vector in Cos-1 cells. The hemadsorption activity of the NA protein expressed on the cell surface was determined by incubating the cells with chicken RBCs at 4°C. The enzymatic and hemadsorption activities of each NA were determined and are indicated relative to the activities of A/tern/Australia/G70c/75 (Table 1). The relative levels of hemadsorption activity indicated in Table 1 are shown in Fig. 1 for comparison. In all cases, the expressed NA had detectable levels of hemadsorption activity. Different virus isolates from the same host species showed little variation in their hemadsorption activities, while the differences between isolates from different species, particularly between avian and human viruses, were more pronounced. All of the avian virus

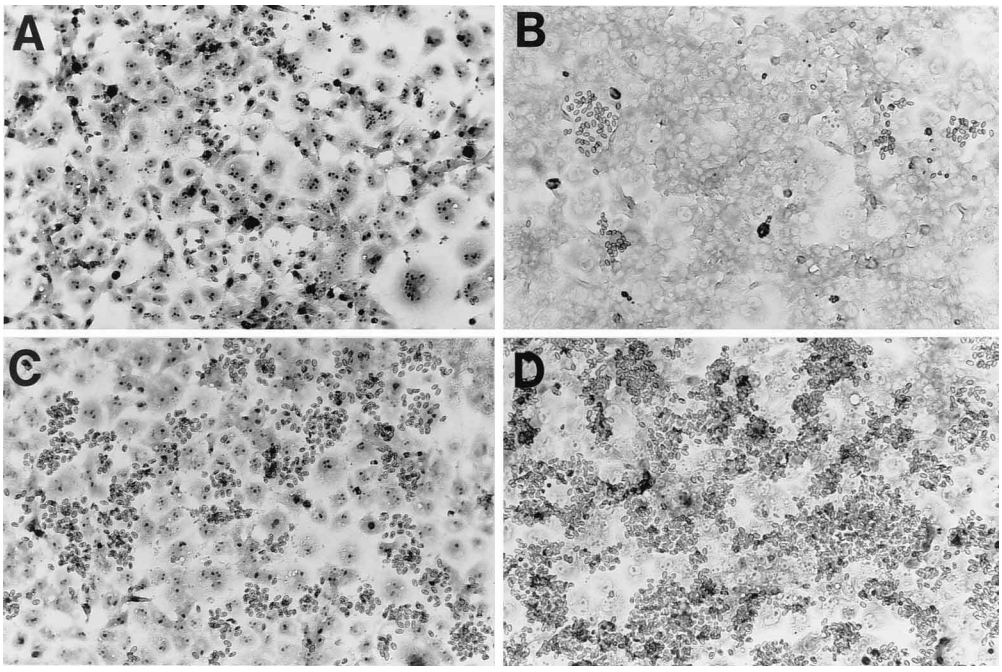

FIG. 1. Relative NA hemadsorption activities of Cos-1 cell-expressed NA proteins. The hemadsorption activity of the expressed NA was determined by incubation of the cells with chicken RBCs at 4°C. Shown are the relative hemadsorption activities from the lowest to highest level observed: <1% maximal activity (A), 5 to 10% maximal activity (B), 60 to 80% maximal activity (C), and 100% maximal activity (D). The relative activities were estimated based upon comparison of bound RBCs to Cos-1 cells observed in five separate fields.

TABLE 2. NA hemadsorption activity with RBCs from various animal species

Construct (virus)	Hemadsorption activity with the following RBC type[a]:				
	Chicken	Human	Equine	Bovine	Swine
pCSITNA15 (A/swine/Italy/437/76)	++	++	−	−	−
pCETNNA18 (A/equine/Tennessee/5/86)	++++	++++	−	−	−
pCLANA55 (A/Los Angeles/2/87)	+	+	−	−	−
pCAGGSN9NA (A/tern/Australia/G70c/75)	++++	++++	−	−	−
pCAGGS/MCS (negative control)	−	−	−	−	−

[a] Representative micrographs of the relative hemadsorption activities (defined in Table 1, footnote *b*) for the Cos-1 cell-expressed NAs are shown in Fig. 1. −, no hemadsorption activity.

NAs examined had a level of hemadsorption activity similar to or slightly lower than that of A/tern/Australia/G70c/75. Although there was some variation in the levels of hemadsorption activity among the avian virus NAs, standardization of the hemadsorption activity relative to the measured enzymatic activity, used as an indicator of the level of cell surface expression of the NA protein, indicated that all avian virus NAs have high hemadsorption activity. This is in contrast to the human virus NA-transfected cells, which all had high levels of enzymatic activity but only barely detectable levels of hemadsorption activity. Among the NAs tested from other host species, the single equine virus N8 had high hemadsorption activity, similar to that of the avian viruses. The single swine virus N1 tested had high enzymatic activity and moderate hemadsorption activity, placing it between those of the avian and human viruses. Hemadsorption activity appears to be a conserved property of avian virus NA molecules and perhaps equine virus NA molecules. It is present only at low levels in viruses isolated from human hosts.

Hemadsorption of human, swine, equine, and bovine RBCs to selected NAs. Examples of NAs that do and do not adsorb chicken RBCs were examined for their ability to bind RBCs from other animal species. This analysis allowed us to determine whether hemadsorption activity can be seen in NAs that do not bind chicken RBCs but might react differently to the RBCs of other host species. In addition, it provided some information on the range of sialic acid species and linkages that are efficiently recognized by the hemadsorption binding site. Sialic acid expression on horse RBCs is nearly 100% *N*-glycolylneuraminic acid, most of which is bound to galactose through an α2,3 linkage (NeuGcα2,3Gal) (9, 16). Similarly, about 90% of sialic acid on bovine RBCs is *N*-glycolylneuraminic acid, primarily of the form NeuGcα2,3Gal (9, 16). Swine RBCs express a more heterogeneous population of sialic acid, with about 66% *N*-glycolyl neuraminic acid, most of which is bound to galactose by an α2,6 linkage (4, 9). The remaining sialic acid is primarily *N*-acetylneuraminic acid with unknown linkage to the adjacent sugar group (4, 9). Constructs expressing the NAs of A/tern/Australia/G70c/75 (N9), A/Los Angeles/2/87 (N2), A/equine/Tennessee/5/85 (N8), and A/swine/Italy/437/76 (N1) were tested for their abilities to adsorb bovine, swine, equine, and human RBCs in addition to chicken RBCs as previously examined. The N9 NA of A/tern/Australia/G70c/75 and N8 NA of A/equine/Tennessee/5/86 strongly adsorbed human and chicken RBCs (Table 2). The N2 NA of A/swine/Italy/437/76 weakly bound human and chicken RBCs, while the N2 NA of A/Los/Angeles/2/87 had barely detectable affinity for these RBCs. None of the constructs were able to bind the equine, bovine, or swine RBCs, indicating that these RBCs do not possess the molecule(s) that is recognized by the hemadsorption site of the NAs.

Effect of GG167 on hemadsorption activity. Incubation of Cos-1 cell-expressed N9 NA with 0.5 μM GG167 prior to incubation with chicken RBCs inhibited the enzymatic activity of the expressed NA when 4-methylumbelliferyl-α-*N*-acetylneuraminic acid was used as a substrate (data not shown). However, the hemadsorption activity of the NAs remained unaffected (data not shown). This result supports previous studies of the N1 and N9 NA activities that showed that the hemadsorption and enzymatic activities are probably located at separate sites (5, 10).

Expression of wild-type and mutant N2 in Cos-1 cells. Wild aquatic birds are believed to be the natural reservoir of all influenza viruses (for reviews, see references 6 and 11). For this reason, we examined the role that the NA hemadsorption activity, which we have shown is conserved among avian viruses, plays in the replication of an avian virus in its natural host. The duck virus A/duck/Hong Kong/7/75 (H3N2) was selected as a model for studying the effect of loss of NA hemadsorption activity in Peking ducks. The first step of this study was to abolish the hemadsorption activity of the NA and to characterize this mutant. A sequence comparison of the N2 NA with the regions of the N9 NA of A/tern/Australia/G70c/75 identified several conserved residues, which are important for the hemadsorption activity of N9 (20). Serine 370 of the N2 NA was selected for mutagenesis to leucine because the corresponding mutation in N9 NA completely abolished its hemadsorption activity (20). In addition, a leucine is present in the N2 NA sequences of several naturally occurring virus isolates, suggesting it would not have a detrimental effect on the enzymatic activity. A double-nucleotide mutation was introduced in the codon for serine 370 to prevent reversion of the sequence. The hemadsorption activities of the wild-type and mutant N2 genes, expressed in Cos-1 cells, are shown in Fig. 2A and B, respectively. The mutation at residue 370 completely abolished the hemadsorption activity of N2. Cells expressing the wild-type or mutant N2 NA had similar levels of cell surface-expressed NA enzymatic activity, which indicates that the mutation at residue 370 did not prevent cell surface expression or destroy the enzymatic activity of the N2 NA.

Comparison of NA enzymatic activities of transfectant viruses. Two transfectant viruses, Dk78/75N2 and Dk78/370SL, containing the wild-type and mutated N2 genes, respectively, were generated. The remainder of the genes of both viruses were derived from the avian helper virus A/duck/Hong Kong/278/78 (H2N9). To determine whether the mutation at residue 370 in Dk78/370SL affected the enzymatic activity of the N2 NA, the NA protein levels in allantoic fluid stocks of the two viruses were first quantified by chemiluminescent detection of N2-specific antibodies attached to membrane-bound virus. The enzymatic activities of equal amounts of N2 protein from each virus were then measured with the substrate 2′-(4-methylum-

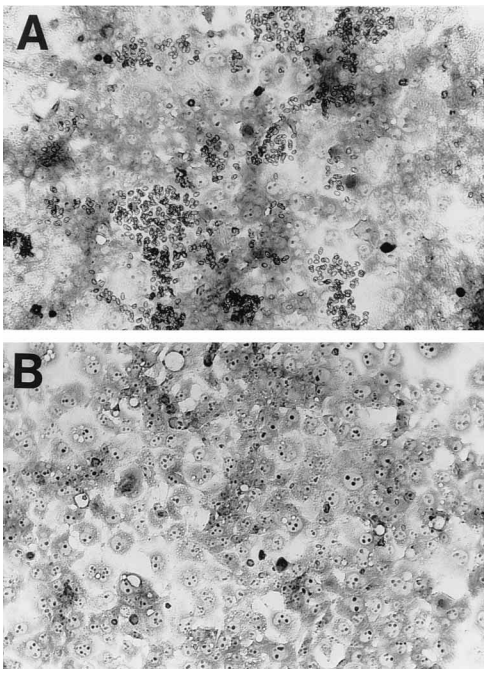

FIG. 2. Loss of hemadsorption activity of mutant N2 (Ser-370-to-Leu) NA of Dk/HK/7/75. Cos-1 cells expressing the wild-type N2 NA (A) and mutated N2 NA (B) were incubated with chicken RBCs at 4°C to compare their relative levels of hemadsorption activity.

belliferyl)-α-D-*N*-acetylneuraminic acid. The NA enzymatic activity of the mutant virus was reduced to about 50% of the wild-type virus N2 activity (Fig. 3). It is not clear whether the lower NA activity is a direct consequence of loss of NA hemadsorption activity or due to perturbation of the NA structure as a result of the mutation at residue 370.

One-step growth curve of transfectant viruses on CEFs. Because NA hemadsorption activity is conserved among the avian virus NAs, the growth of these viruses on avian cells, namely, CEFs, was examined over a 12-h period (Fig. 4). Dk78/370SL replicated less efficiently than Dk78/75N2 at 37°C over the period examined. At 12 h there was an approximately 10-fold difference in the yields of the two viruses. However, viral cultures which were allowed to grow for longer periods reached the same final titer of progeny virus in the supernatant (data not shown). The observed difference in growth of these viruses over short periods may reflect the lower NA enzymatic activity of Dk78/370SL, which slows the release of progeny virions from infected cells and thereby reduces infectious virions in the culture supernatant.

Relative thermostabilities of transfectant viruses. The effect on virus stability of the loss of NA hemadsorption activity was determined by incubating the transfectant viruses Dk78/75N2 and Dk78/370SL at 37 and 45°C for 12 h. The $TCID_{50}$ titer of each virus stock was determined at selected time points by titration of the viruses on CEFs. Both viruses showed similar losses of viability at both temperatures, with a 10-fold and 100-fold reduction in titer at 37 and 45°C, respectively (data not shown). The results suggest that loss of NA hemadsorption activity does not have a deleterious effect on virus stability.

Replication of transfectant viruses in Peking ducks. We determined whether loss of NA hemadsorption activity affected the ability of an avian virus to replicate in an avian host. Serial dilutions of equal titers of the viruses were orally inoculated into Peking ducks. Virus replication was determined 3 days postinoculation by titrating virus in the intestine of each duck. The duck infectious doses of Dk78/75N2 and Dk78/370SL were 5.6 and 5.8 EID_{50}s, respectively. Thus, both viruses replicated efficiently in duck intestine. Loss of hemadsorption activity and reduction of NA enzymatic activity did not significantly affect the ability of the N2 NA to support virus replication in duck intestine. Complete sequencing of the N2 gene of the virus isolated from one duck inoculated with the mutant

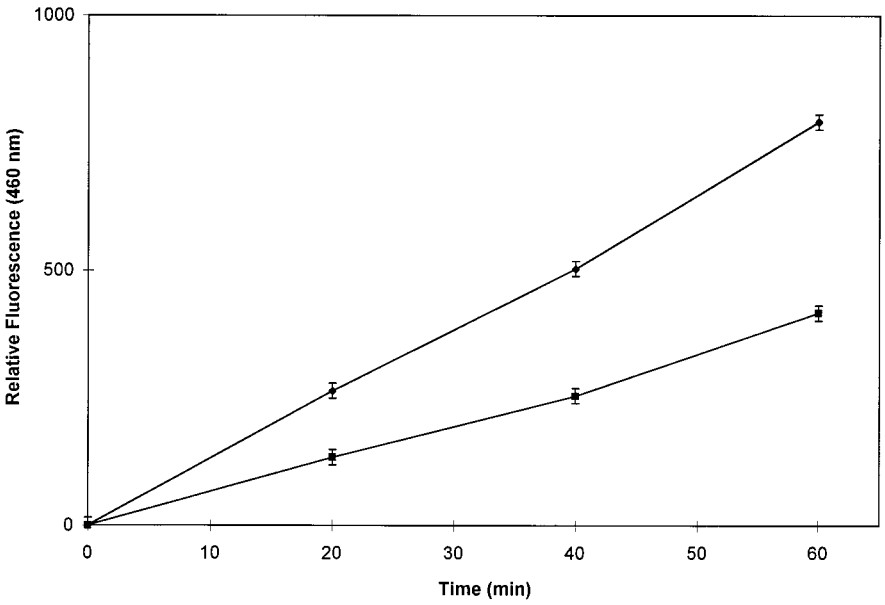

FIG. 3. NA activities of the transfectant viruses. The N2 NA protein levels of transfectant viruses Dk78/75N2, with the wild-type NA, and Dk78/370SL, with the mutated NA, were standardized by immunoblot analysis and densitometric quantitation of NA bands. The enzymatic activities of equal amounts of N2 NA of Dk78/75N2 (◆) and Dk78/370SL (■) were then measured. Virus was incubated with the substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid at 37°C, and the fluorescence of released 4-methylumbelliferone was quantitated with excitation at 355 nm and emission at 460 nm. Error bars, standard errors of the means.

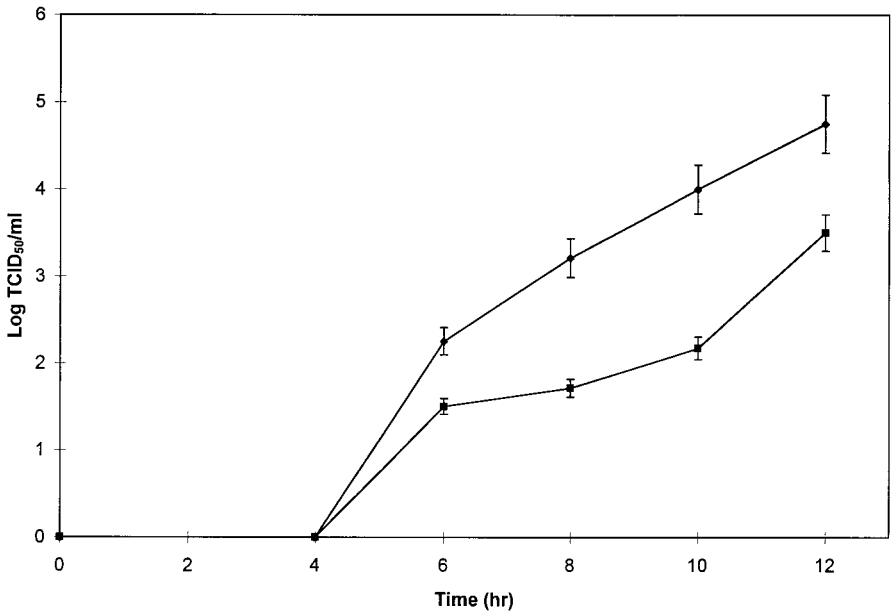

FIG. 4. Growth curve of the transfectant viruses. Equal doses of viruses Dk78/75N2, with the wild-type NA (◆), and Dk78/370SL, with the mutated NA (■), were used to infect CEFs at 37°C. Aliquots of tissue culture supernatant were assayed by titration on CEFs at selected times to determine the extent of replication. The results shown are the average values obtained from duplicate assays. Error bars, standard errors of the means.

virus showed that no amino acid sequence changes in the protein had occurred and the mutation introduced at residue 370 was unchanged.

DISCUSSION

NA hemadsorption activity was first identified for N9 of A/tern/Australia/G70c/75 (10) and more recently for N1 of A/FPV/Rostock/34 (5). However, little is known about the occurrence of a similar activity among other NA subtypes or about the role that this activity may play in replication of the virus. We have shown that a high level of NA hemadsorption activity, comparable to that of the N9 of A/tern/Australia/G70c/75, is a conserved property among avian viruses of most NA subtypes. A similar level of activity was found for a single equine virus, A/equine/Tennessee/5/86 (H3N8), while the N1 NA of the single swine isolate, A/swine/Italy/437/76 (H1N1), had significant but lower hemadsorption activity. The hemadsorption activities of the N1 and N2 NAs of human virus isolates, on the other hand, while still detectable, were present at only very low levels. The NA from a human H2N2 virus, isolated in 1957, does not possess detectable hemadsorption activity (10), suggesting that, although the human virus N2 NA originated from an avian source, loss of the N2 NA hemadsorption activity occurred rapidly after its introduction into the human virus population. This may reflect the need to lose a property that is detrimental to virus replication in humans, or perhaps NA hemadsorption activity is not required for replication in humans and is coincidentally lost during adaptation to support virus growth in a new host or due to antigenic drift. We cannot rule out the possibility that NA hemadsorption activity is required only at reduced levels for virus growth in human and swine hosts. Alternatively, it is possible that the nature of the hemadsorption binding site has been altered in viruses isolated from these hosts to better support interaction with a different host cell molecule and that the binding activity of this site can no longer be detected by binding of chicken RBCs. Nevertheless, our results indicate that NA hemadsorption activity is conserved in avian viruses and possibly in equine viruses and suggest that it may play a role in the maintenance of virus in these hosts.

The activity, molecular determinants, and possible receptors of the N1 and N9 NA hemadsorption activities have been examined (1, 5, 10, 13, 20). However, these studies did not address the role that NA hemadsorption might play in virus replication. To systematically examine this question, we generated two transfectant viruses by reverse genetics. Dk78/75N2 expressed the wild-type N2 gene of avian virus A/duck/Hong Kong/7/75 (H3N2) in a background of genes derived from avian virus A/duck/Hong Kong/278/78 (H2N9). Dk78/370SL is identical to Dk78/75N2 except for a serine-to-leucine substitution at position 370 of the N2 gene, which results in the loss of NA hemadsorption activity. A reduction in the ability of Dk78/370SL to replicate in CEFs, compared to that of Dk78/75N2, was observed. The reduction in enzymatic activity of the mutant N2 compared to the wild-type protein may be sufficient to account for this reduction in growth in vitro. This result could be interpreted to mean that NA hemadsorption activity can influence NA enzymatic activity even though the two activities are located at separate sites of the protein. However, the generation of N1 NA mutants which have markedly reduced hemadsorption activity but have enzymatic activity nearly identical to that of wild-type N1 has been reported (5), suggesting that NA hemadsorption activity probably does not influence NA enzymatic activity. The reduction in NA enzymatic activity of Dk78/370SL may result from the structural perturbation of the molecule as a result of the mutation, perhaps altering the conformation of the active site. The crystallographic structure of the N2 NA of A/Tokyo/3/67 shows that the side chain of Arg-371 interacts with sialic acid bound in the enzyme active site (18). A mutation at position 370 could affect the orientation of Arg-371, perhaps affecting the interaction of its side chain with sialic acid and causing a decrease in enzymatic activity like that seen for the N2 NA of Dk78/370SL. Perturbation of the NA structure by the substitution at position 370

could also cause a decrease in the thermostability of the mutant NA compared to the wild-type NA, resulting in the mutant's apparently lower enzymatic activity.

Although NA hemadsorption activity is highly conserved among avian viruses, we did not observe any effect on the ability of NA to support virus replication in duck intestine or virus stability when we eliminated the hemadsorption activity. The mutation did cause a decrease in enzymatic activity but did not affect the growth of Dk78/370SL in ducks compared to that of Dk78/75N2. This result could mean that NA hemadsorption activity does not have a role in virus replication. An alternate explanation, however, which may explain the discrepancy between the conservation of hemadsorption activity among avian virus NAs and the apparent lack of function in the replication of the virus in ducks, is that NA adsorption to RBCs may not accurately represent the biological activity of the NA hemadsorption site. Binding of RBCs by NA occurs only at low temperatures, reflecting the low affinity of the NA hemadsorption site for the molecular species recognized on RBCs. We suggest that this site may actually bind a molecule in the avian host with high affinity at physiological temperatures as a required function for efficient growth in this host. While we successfully eliminated the ability of the N2 NA to bind RBCs at 4°C by a single amino acid change, it is possible that we did not abolish binding of NA to this putative receptor in ducks, due to its higher affinity for the hemadsorption site. Therefore, the NA hemadsorption site may still be able to perform a required function in ducks and we would not observe a significant effect on the ability of the NA to support growth in ducks.

NA hemadsorption to human RBCs was similar to that seen with chicken RBCs, but even the N9 NA of A/tern/Australia/G70c/75 was unable to adsorb RBCs from pigs, horses, or cows (Table 2). Sialic acid is believed to be the molecule recognized by the NA hemadsorption site (1, 5). *N*-Glycolylneuraminic acid, bound to galactose through either an α2,3 or an α2,6 linkage, is expressed at high levels on the RBCs of these three animals but is not recognized with sufficient affinity for detectable hemadsorption to occur (Table 2). About 35% of the sialic acid expressed on swine RBCs is *N*-acetylneuraminic acid (4), and it also unable to function as the NA hemadsorption receptor. Clearly, chicken and human RBCs express at least one other species or linkage of sialic acid that is not found on bovine, swine, or equine RBCs and is preferentially recognized by the NA hemadsorption site.

As in N1 and N9 NAs, serine 370 is crucial for expression of hemadsorption activity of the N2 NA (5, 20). Even though swine and human virus NAs have very reduced levels of hemadsorption activity, serine 370 is conserved in many of their NAs and is clearly not the only residue that is critical for hemadsorption activity (Table 3). Changes at one or more other residues required for high expression of hemadsorption activity could account for the reduced activities of human and swine NAs. Among the other residues of N9 needed for full expression of hemadsorption activity are serine 367, alanine 369, serine 372, and asparagine 400 (according to N2 numbering) (20). Of the NAs we examined for hemadsorption activity (Table 1), we found that a serine is conserved at positions 367 and 372 of every NA that displayed high hemadsorption activity (Table 3). Serine is also conserved in hemadsorption-positive NAs at position 370, although glycine is also permitted at this position in the N3 NA subtype. In addition, the NAs with high hemadsorption activity all had either asparagine, leucine, or isoleucine at position 400. Although alanine 369 is critical to N9 NA hemadsorption (20) and its being replaced by aspartic acid abolishes the activity, aspartic acid is found at this position in several of the hemadsorption-positive NAs of other subtypes (Table 3). It appears that the precise sequence requirements for NA hemadsorption activity among different NAs may be subtype or even isolate specific.

TABLE 3. Alignment of NA sequences containing residues responsible for N9 NA hemadsorption activity

Virus isolate	NA subtype	NA sequence residues[a]	
		364–375	394–408
A/turkey/Missouri/81	N1	RTK**S**I**S**S**R**S**G**FE	KQDIIA**L**TDWSGYSG
A/swine/Italy/437/76	N1	RTK**S**I**S**S**R**S**G**FE	KQDIVA**L**TDWSGYSG
A/Puerto Rico/8/34	N1	RTK**S**H**S**S**R**H**G**FE	RQDVVA**M**TDWSGYSG
A/duck/Australia/80	N1	RTK**S**T**S**S**R**S**G**FE	KQDIVA**I**TDWSGYSG
A/Udorn/307/72	N2	RTI**S**E**D**S**R**S**G**YE	RQVIVD**S**DNRSGYSG
A/Los Angeles/2/87	N2	RTI**G**E**E**L**R**S**G**YE	RQVIVD**S**GNRSGYSG
A/gull/Delaware/2838/87	N2	RTI**S**K**D**S**R**S**G**YE	RQVIVD**N**NNWSGYSG
A/duck/Hong Kong/7/75	N2	RTI**S**K**D**S**R**S**G**YE	RQVIVD**N**NNWSGYSG
A/duck/Hong Kong/342/78	N2	RTI**S**K**D**S**R**S**G**YE	RQIIVD**N**NNWSGYSG
A/widgeon/Alberta/284/77	N3	RTV**S**T**S**G**R**S**G**FE	TQTLVS**N**NDWSGYSG
A/equine/New Market/79	N3	RTI**S**R**T**S**R**L**G**FE	KQVIVD**N**LNWSGYSG
A/duck/Potsdam/1689/85	N3	RTI**S**T**S**G**R**S**G**FE	TQTLVS**N**NDWSGYSG
A/turkey/Ontario/618/68	N4	RTK**S**L**E**S**R**S**G**FE	VQDIID**N**NNWSGYSG
A/shearwater/Australia/72	N5	RTI**S**V**S**S**R**S**G**FE	KVEVLN**N**KNWSGYSG
A/pintail duck/Alberta/712/80	N6	RTI**S**K**D**S**R**S**G**YE	HQVIVN**N**QNWSGYSG
A/FPV/Weybridge	N7	RTI**S**P**R**S**R**S**G**FE	RQEIVD**N**SNWSGYSG
A/equine/Cor/16/74	N7	RTI**S**P**R**L**R**S**G**FE	RQEIVS**N**DNWSGYSG
A/equine/Tennessee/5/86	N8	RTI**S**R**T**S**R**S**G**FE	RQVIID**N**LNWSGYSG
A/duck/Hokkaido/8/80	N8	RTI**S**R**T**S**R**S**G**FE	KQVVVD**N**LNWSGYSG
A/tern/Australia/G70c/75	N9	RTI**S**I**A**S**R**S**G**YE	GQTIVL**N**TDWSGYSG

[a] N2 numbering is indicated. Boldface lettering indicates residues aligned with those residues of N9 NA that are critical for hemadsorption activity (20).

In summary, our data demonstrates the conservation of high levels of NA hemadsorption activity in avian influenza A viruses and perhaps equine viruses. This conservation of NA hemadsorption activity among many isolates of avian viruses representing most subtypes of NA suggests that it is maintained for a specific role in virus replication. However, loss of NA hemadsorption activity, due to the introduction of a mutation in the hemadsorption binding site, did not preclude the ability of the mutated NA to support virus replication in an avian host. NA hemadsorption activity, as detected by the ability of NA to bind chicken or human RBCs at 4°C, is not required for viral replication in an avian host. However, these results do not eliminate the possibility that the low-affinity binding of RBCs by NA does not represent the affinity of the NA hemadsorption site for a putative biologically relevant receptor in the avian host.

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REFERENCES

1. **Air, G., and G. Laver.** 1995. Red cells bound to influenza virus N9 NA are not released by the N9 NA activity. Virology **211:**278–284.
2. **Enami, M., W. Luytjes, M. Krystal, and P. Palese.** 1990. Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA **87:**3802–3805.
3. **Enami, M., and P. Palese.** 1991. High-efficiency formation of influenza virus transfectants. J. Virol. **65:**2711–2713.

4. **Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley.** 1962. The contribution of sialic acid to the surface charge of the erythrocyte. J. Biol. Chem. **237:**1992–2000.
5. **Hausmann, J., E. Kretzchmar, W. Garten, and H.-D. Klenk.** 1995. N1 neuraminidase of influenza virus A/FPV/Rostock/34 has haemadsorbing activity. J. Gen. Virol. **76:**1719–1728.
6. **Hinshaw, V. S., and R. G. Webster.** 1982. The natural history of influenza A viruses, p. 79–104. *In* A. S. Beare (ed.), Basic and applied influenza research. CRC Press, Boca Raton, Fla.
7. **Horimoto, T., and Y. Kawaoka.** 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. **68:**3120–3128.
8. **Huddleston, J. A., and G. G. Brownlee.** 1982. The sequence of the nucleoprotein gene of human influenza A virus strain, A/NT/60/68. Nucleic Acids Res. **10:**1029–1037.
9. **Ito, T., Y. Suzuki, L. Mitnaul, A. Vines, H. Kida, and Y. Kawaoka.** 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227:**493–499.
10. **Laver, W. G., P. M. Colman, R. G. Webster, V. S. Hinshaw, and G. M. Air.** 1984. Influenza virus neuraminidase with hemagglutinin activity. Virology **137:**314–323.
11. **Murphy, B. R., and R. G. Webster.** 1996. Orthomyxoviruses, p. 1397–1445. *In* B. N. Fields, D. M. Knipe, P. M. Howley et al. (ed.), Fields virology. Lippincott-Raven Publishers, Philadelphia, Pa.
12. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–200.
13. **Nuss, J. M., and G. M. Air.** 1991. Transfer of hemagglutinin activity of influenza virus neuraminidase subtype N9 into an N2 neuraminidase background. Virology **183:**496–504.
14. **Palese, P., K. Tobita, M. Ueda, and R. W. Compans.** 1974. Characterization of temperature-sensitive influenza virus mutants defective in neuraminidase. Virology **61:**397–410.
15. **Parvin, J. D., P. Palese, A. Honda, A. Ishihama, and M. Krystal.** 1989. Promoter analysis of influenza virus RNA polymerase. J. Virol. **63:**5142–5152.
16. **Suzuki, Y., M. Matsunaga, and M. Matsumoto.** 1985. *N*-Acetylneuraminyllactosylceramide, GM3-NeuAc, a new influenza A virus receptor which mediates the adsorption-fusion process of viral infection. J. Biol. Chem. **260:**1362–1365.
17. **Taylor, J., G. Weinberg, Y. Kawaoka, R. G. Webster, and E. Palata.** 1988. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine **6:**504–508.
18. **Varghese, J. N., J. L. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt, and P. M. Colman.** 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins **14:**327–332.
19. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234:**1971–1975.
20. **Webster, R. G., G. M. Air, D. W. Metzger, P. M. Colman, J. N. Varghese, A. T. Baker, and W. G. Laver.** 1987. Antigenic structure and variation in an influenza virus N9 neuraminidase. J. Virol. **61:**2910–2916.