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Virulence Factors of Influenza A Viruses: WSN Virus Neuraminidase Required for Plaque Production in MDBK Cells

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The genetic basis for the distinctive capacity of influenza A/WSN/33 (H0N1) virus (WSN virus) to produce plaques on bovine kidney (MDBK) cells was found to be related to virus neuraminidase. Recombinant viruses that derived only the neuraminidase of WSN virus were capable of producing plaques, whereas recombinant viruses identical to WSN except for neuraminidase did not produce plaques. With viruses that do not contain WSN neuraminidase, infectivity of virus yields from MDBK cells was increased approximately 1,000-fold after in vitro treatment with trypsin. In contrast, no significant increase in infectivity was observed after trypsin treatment of viruses containing WSN neuraminidase. In addition, polyacrylamide gel analysis of proteins of WSN virus obtained after infection of MDBK cells demonstrated that hemagglutinin was present in the cleaved form (HA1 + HA2), whereas only uncleaved hemagglutinin was obtained with a recombinant virus that derived all of its genes from WSN virus except its neuraminidase. These data are in accord with the hypothesis that neuraminidase may facilitate production of infectious particles by removing sialic acid residues and exposing appropriate cleavage sites on hemagglutinin.

In previous communications we reported that the eight RNA segments of different strains of influenza A virus have different patterns of migration on polyacrylamide urea gels and that by analysis of RNAs of recombinant viruses it was possible to determine from which parent each gene was derived (12, 15, 16, 20, 23). These methods were utilized to establish a genetic map of influenza A viruses (12, 16, 20) and provided a tool that could be used to correlate strain-related differences in virulence with particular genes.

One intriguing model that seemed uniquely appropriate in this respect is the MDBK (bovine kidney) cell system, in which only one human influenza virus strain (influenza A/WSN/33 virus) is capable of multicycle replication (2; unpublished observations). This continuous cell line has been used extensively for biochemical studies of influenza A/WSN/33 (H0N1) virus (WSN virus) but was not suitable for similar studies of other human influenza viruses. Consequently, we set out to determine which gene(s) of WSN virus is/are required for productive infection in these cells.

Previously we had obtained one recombinant containing only the hemagglutinin and neuraminidase of WSN virus, which produced plaques in MDBK cells (19). In the present experiments we attempted to determine whether WSN virus hemagglutinin and/or neuraminidase are the only WSN virus genes required for multicycle replication in MDBK cells. Our approach was to isolate and determine the gene derivation of recombinant viruses of WSN virus and other human isolates and then to examine the capacity of each to produce plaques on MDBK cells. The results demonstrate that WSN neuraminidase is required for plaque production in these cells.

In addition, we have explored the mechanism of this specificity by examining the effects of trypsin on viral infectivity and by comparing the cleavage of hemagglutinin in viruses that do or do not contain WSN neuraminidase. The results indicate that the requirement for WSN neuraminidase is probably related to removal of sialic acid from hemagglutinin, facilitating subsequent proteolytic cleavage to HA1 and HA2.

MATERIALS AND METHODS

Viruses. The strain of influenza A/Hong Kong/8/68 (H3N2) virus (HK virus) is identical to the one described previously (15, 16); WSN virus was obtained from an earlier strain (15) after passage in

eggs; RV 10 was previously obtained (19) by recombination of ts12 of the WSN virus mutant set (24, 25) and influenza A/FM/1/47 (H1N1) virus (FM1 virus). RV 10 has previously been shown to derive its hemagglutinin and neuraminidase from WSN virus and all other genes from FM1 virus (19).

By using these viruses as parents, the following recombinants were isolated after mixed infection of canine kidney (MDCK) cells:

Influenza A/WSN/33(H0)-Hong Kong/8/68(N2) virus (WSN-HK virus) was obtained after mixed infection with WSN (H0N1) virus and UV-irradiated HK (H3N2) virus (16, 23) and selection of plaques on MDCK cells in the presence of antiserum to H3N1.

Influenza A/Hong Kong/8/68(H3)-WSN/33(N1) virus (HK-WSN virus) was isolated after mixed infection of MDCK cells with HK (H3N2) and UV-irradiated WSN (H0N1) viruses and selection in the presence of antiserum to H0N2.

Influenza A/FM/1/47(H1)-WSN/33(N1) virus (FM1-WSN virus) was obtained after mixed infection with RV 10 (H0N1) virus (described above) and with FM1 (H1N1) virus and selection of plaques in MDBK cells in the presence of antiserum to H0N2.

All recombinants were purified by plaque-to-plaque passage before preparation of allantoic fluid seed viruses.

RNA analysis. Labeling of virus-specific RNA with ^{32}P (as orthophosphoric acid in sterile water; New England Nuclear, Boston, Mass.) was done in MDCK cells according to previously published techniques (16). Purification of virus and isolation of RNA followed standard techniques (15, 16, 23). Separation of RNAs on 2.6% urea-polyacrylamide gels was done by methods described by Floyd et al. (4) and subsequently modified by us (15, 16, 20, 23).

Protein analysis. Labeling of virus-specific proteins was done in MDCK cells, using 40 to 50 μCi of [^{35}S]methionine per ml (Amersham Searle Corp., Arlington Heights, Ill.), according to previously published procedures (21). Cells were infected with virus at 1 to 10 PFU, and radioactive label was present throughout the incubation of 14 h. Virus was purified as described under RNA analysis, and proteins were separated on a 13% polyacrylamide slab gel using the Tris-glycine system (6, 9, 21). After drying of the gel and exposure to Kodak (no-screen) X-ray film, the appropriate lanes containing the sample were cut into 1-mm slices, and fractions were counted in a Packard liquid scintillation counter for quantitative measurement.

Cell cultures and plaque assay. MDBK and MDCK cells were grown on plastic dishes according to previously described methods (15, 16, 20, 23). Replication of viruses under liquid medium and plaque determinations in MDBK cells were performed in the absence of serum or trypsin in the overlay, except where indicated in the text. Plaque assay in MDCK cells was performed in accordance with previously described methods (26), with the following modifications: after a 1-h adsorption period the dishes were washed with phosphate-buffered saline and incubated for 1 to 2 h in the presence of liquid maintenance medium. The monolayers were washed again and inoculated with 0.2 ml of high-titered, receptor-destroying enzyme (RDE)-treated antiserum to the infecting virus. After 0.5 h, the dishes were washed twice with phosphate-buffered saline and covered with agar overlay containing 2 μg of trypsin per ml (TRL50S, Worthington Biochemicals Corp., Freehold, N.J.).

Trypsin and RDE treatment of virus. After infection of MDBK cells, medium was removed, pooled, centrifuged to remove cellular debris, and incubated for 30 min after the addition of 2 U of *Vibrio cholerae* neuraminidase (RDE) (Behringwerke, Marburg, West Germany), 10 μg of trypsin per ml, or trypsin and neuraminidase. The tubes then were chilled and diluted rapidly in cold phosphate-buffered saline containing 1% bovine albumin for determinations of infectivity. Hemagglutination (HA) titrations on these fluids were performed in glass tubes, using 1.0% sodium citrate as the diluent and 0.5% erythrocytes suspended in sodium citrate.

HA titrations, determination of egg infectivity (50% egg infective dose), and serological identification of recombinant viruses all were performed in accordance with previously used techniques (1, 14).

RESULTS

Genotyping of recombinant viruses. Figure 1 demonstrates the RNA patterns of HK virus (lane 3), WSN virus (lane 2), and the recombinant, WSN-HK (H0N2) virus (lane 1), described in Materials and Methods. It should be noted that HK and WSN viruses had been mapped previously (12, 13, 16, 19, 20). In addition, the surface proteins of all recombinant viruses had been identified by serological methods. Comparison of the migration patterns of the three viruses on several polyacrylamide gels demonstrates that the WSN-HK recombinant is isogenic with the WSN virus parent except for the neuraminidase gene, which is derived from HK virus.

Figure 2A demonstrates the RNA patterns of WSN virus (lane 1), HK-WSN (H3N1) virus (lane 2), and HK virus (lane 3). In this instance it is clear that the recombinant virus in lane 2 derives seven of its genes from the HK virus parent, deriving only the sixth RNA segment (neuraminidase gene) from WSN virus. Figure 2B is a longer exposure of the top portion of the same gel, included to provide better definition of the HK virus origin of the three slowest-moving RNAs in the recombinant.

Comparison of the RNA patterns in Fig. 3 demonstrates that the recombinant, FM1-WSN virus (lane 1), derives all of its genes from FM1 virus (lane 2) except for the sixth (neuraminidase) gene, which is derived from WSN virus (lane 3).

Plaque formation in MDBK cells. Table 1 summarizes data regarding the capacity of allantoic fluid seeds of these viruses to produce

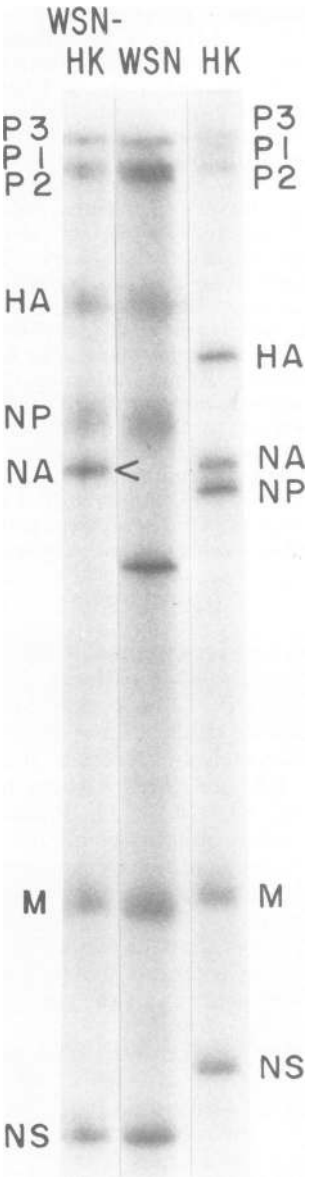

FIG. 1. *Analysis of ^{32}P-labeled RNAs of WSN-HK virus (lane 1), WSN virus (lane 2), and HK virus (lane 3). Migration is from top to bottom on a 2.6% urea-polyacrylamide gel (23 cm long) containing 0.15% N,N'-methylenebisacrylamide. The gene product of each of the RNA segments of WSN-HK and HK viruses is indicated. The arrow indicates the HK virus origin of the neuraminidase gene of WSN-HK virus.*

plaques in MDBK cells. It is evident that the parental HK and FM1 viruses were incapable of producing plaques even in the presence of normal serum. Similarly, the WSN-HK virus recombinant, which derives all of its genes from WSN virus except for neuraminidase, was unable to produce plaques. In contrast, the two recombinant viruses (HK-WSN and FM1-WSN), which derive only neuraminidase from WSN virus, were capable of producing plaques in MDBK cells. In the absence of serum, many FM1-WSN virus plaques were visible only microscopically, whereas in the presence of 0.125% serum both the number and size of plaques increased appreciably. With HK-WSN virus,

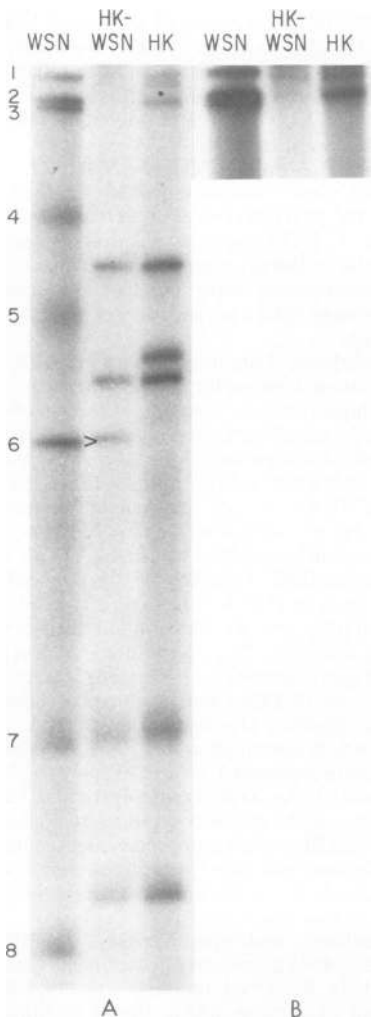

FIG. 2. *(A) Analysis of ^{32}P-labeled RNAs of WSN virus (lane 1), HK virus (lane 3), and the recombinant HK-WSN virus (lane 2). Conditions are the same as those in Fig. 1. RNAs of WSN virus are numbered 1 through 8. Arrow indicates the WSN virus origin of the neuraminidase gene (RNA 6) of HK-WSN virus. (B) Longer exposure of the top portion of the same autoradiogram as in (A) to demonstrate the HK virus origin of the three slowest-moving RNAs of the recombinant virus.*

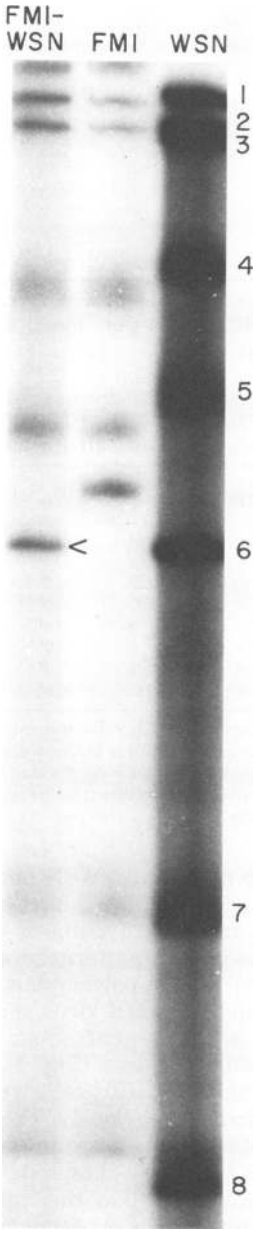

FIG. 3. *Analysis of ^{32}P-labeled RNAs of FM1-WSN virus (lane 1) and those of its two parents, FM1 virus (lane 2) and WSN virus (lane 3). Conditions are the same as in Fig. 1. RNAs of WSN virus are numbered 1 through 8. Arrow indicates that the sixth RNA (neuraminidase gene) of the recombinant (lane 1) is derived from WSN virus (lane 3).*

plaques were readily visualized even in the absence of serum, and addition of low concentrations of serum to the overlay medium did not have any effect.

Although RNA patterns and plaquing data have been presented for single representatives of the three recombinants, identical results were obtained with other viruses genotypically identical to each of the three recombinants. In addition, it should be noted that the same recombinational mixture, which contained more than 10^4 PFU of recombinants per ml containing WSN hemagglutinin and HK neuraminidase when assayed on MDCK cells, failed to produce any plaques of the same serotype on MDBK cells. This further reduces the likelihood that the particular WSN-HK viruses used were not representative.

These results demonstrate that all of the viruses containing WSN neuraminidase (WSN, HK-WSN, FM1-WSN) produce plaques on MDBK cells, whereas viruses lacking WSN neuraminidase (WSN-HK, HK, FM1) are not capable of producing plaques in these cells.

Effect of trypsin on viruses grown in MDBK cells. The mechanism by which WSN virus neuraminidase promotes productive infection and plaque formation in MDBK cells was investigated by studying the effects of neuraminidase (RDE) and trypsin on infectivity of MDBK cell-grown virus. Confluent monolayers were infected with FM1 virus at a multiplicity of approximately 10 50% egg infective doses/cell. Two hours after incubation under liquid overlay, the cells were washed and incubated for 30 min with anti-FM1 antisera. Sixteen hours after infection, virus yields either were left untreated or were treated with neuraminidase, trypsin, or trypsin and neuraminidase, and HA titers, egg infectivity, and PFU of the supernatant fluids were measured. (It should be emphasized that PFU determinations in MDCK cells involved the use of antiserum 3 h after infection to remove virus still present on

TABLE 1. *Plaque formation of parent and recombinant viruses on MDBK cells*

Virus	No serum		0.125% Normal rabbit serum	
	PFU/ml	Size (mm)	PFU/ml	Size (mm)
WSN	5.8×10^8	4.0–4.5	ND[a]	ND
HK	$<10^2$		$<10^2$	
WSN-HK	$<10^2$		$<10^2$	
HK-WSN	9×10^6	3.0–3.5	9.7×10^6	3.0–3.5
FM1	$<10^2$		$<10^2$	
FM1-WSN	3×10^{6b}	Pinpoint-microscopic	4.2×10^7	2.5–3.5

[a] ND, Not determined.

[b] Approximate count, determined by scanning of dishes under the microscope, where plaques could be identified, as confirmed by recovery of infectious virus from circled areas on the dish.

the cell membrane before agar overlay containing trypsin was added.)

The results, summarized in Table 2, demonstrate that trypsin treatment of MDBK cell-grown FM1 virus results in approximately a 1,000-fold increase in infectivity for eggs and a 10,000-fold increase of infectivity in MDCK cells. These results suggest that the failure of FM1 virus to produce plaques in MDBK cells is related to the production of a high proportion of noninfectious virus particles containing uncleaved hemagglutinin.

This hypothesis was tested further by a similar experiment comparing the effects of trypsin treatment on WSN, FM1, and FM1-WSN viruses grown in MDBK cells. The experimental design of this experiment was identical to that just described, except that a shorter period of incubation (11.5 h) was used. The effects of trypsin treatment of virus yields obtained from MDBK cells infected with each of these viruses are summarized in Table 3. (The effects of treatment with neuraminidase are not included because no discernible effects were observed.) It is evident that trypsin increases the infectivity of MDBK cell-grown FM1 virus by a factor of 4 $\times 10^3$. In contrast, only marginal effects of trypsin are observed on infectivity of FM1-WSN virus (which is distinguished from FM1 virus only by its neuraminidase). Similarly, trypsin causes only slight increases in infectivity of WSN virus grown in MDBK cells.

Similar results were obtained after trypsin treatment of HK, WSN-HK, HK-WSN, and WSN viruses in vitro (Table 4). In this experiment a longer period of incubation (20 h) was used to obtain as much virus from HK and HK-WSN virus-infected cells as possible. Once again, trypsin treatment produced much greater increases in infectivity with virus strains that do not contain WSN neuraminidase.

Cleavage of hemagglutinin of viruses with WSN or HK neuraminidase. The preceding observations suggested that in MDBK cells, viruses that do not contain WSN neuraminidase produce particles containing uncleaved hemagglutinin. To test this hypothesis directly, we examined the protein patterns produced on sodium dodecyl sulfate-polyacrylamide gels by WSN virus and WSN-HK virus, a recombinant that derives all of its genes from WSN virus, except for neuraminidase (Fig. 1). The conditions of infection of MDBK cells were the same as those described previously, except that 2% non-inactivated fetal calf serum (previously dialyzed for 10 h against phosphate-buffered saline) was incorporated in the liquid overlay. Serum was used in this experiment because its presence enhances the proportion of cleaved hemagglutinin of WSN virus in MDBK cells (3, 7). The presence of serum did not alter the infectivity titer of either virus yield (data not shown).

Figure 4 demonstrates the protein patterns of WSN and WSN-HK viruses. It is evident that with WSN virus most of the hemagglutinin is present in the cleaved form, HA1 and HA2. In contrast, the hemagglutinin of WSN-HK virus is present only in the uncleaved form, as a diffuse band migrating slightly more rapidly than the P proteins.

TABLE 2. *Effect of trypsin and neuraminidase on infectivity of FM1 virus grown in MDBK cells* [a]

Treatment	HA	EID_{50}/ml	PFU/ml[b]
None	128	$10^{4.9}$	2×10^8
RDE (neuraminidase, 2 U/ml)	256	$10^{4.9}$	5×10^8
Trypsin (10 μg/ml)	256	$10^{8.4}$	2.5×10^7
Trypsin and RDE	256	$10^{7.9}$	3.8×10^7

[a] Samples obtained 16 h after infection. EID_{50}, 50% egg infective dose.

[b] PFU in MDCK cells; 2 μg of trypsin per ml was added 3 h after infection after removal of residual virus by antiserum.

TABLE 3. *Effect of trypsin on infectivity of FM1, FM1-WSN, and WSN viruses grown in MDBK cells* [a]

Virus	Trypsin treatment	HA	PFU/ml (MDCK cells)
FM1	–	32	1.3×10^3
FM1	+	64	5.2×10^6
FM1-WSN	–	16	1.3×10^6
FM1-WSN	+	16	3.9×10^6
WSN	–	128	1.1×10^6
WSN	+	128	3.0×10^6

[a] Conditions of treatment with trypsin and assay of PFU on MDCK cells were identical to those described for Table 2.

TABLE 4. *Effects of in vitro treatment with trypsin on infectivity of HK, WSN-HK, HK-WSN, and WSN viruses grown in MDBK cells* [a]

Virus	Trypsin	HA	EID_{50}/ml	PFU/ml (MDCK cells)
HK	–	<4	10^1	<10
HK	+	<4	$10^{4.0}$	1.4×10^2
WSN-HK	–	128	$10^{3.7}$	2.2×10^3
WSN-HK	+	128	$10^{6.7}$	3.3×10^6
HK-WSN	–	8	$10^{4.7}$	1.9×10^4
HK-WSN	+	8	$10^{4.7}$	3.2×10^4
WSN	–	256	$10^{6.0}$	1.1×10^6
WSN	+	256	$10^{6.7}$	1.5×10^6

[a] PFU titration in MDCK cells and conditions of treatment with trypsin were similar to those described in Table 2, except that pooled samples were divided 20 h after infection for treatment or nontreatment with trypsin. EID_{50}, 50% egg infective dose.

DISCUSSION

In previous communications we speculated that newly developed techniques that permitted precise definition of the genetic composition of recombinant influenza A viruses would facilitate identification of the genes involved in strain-related differences in biological activity in different host cell systems (16, 23). We proposed that comparison of viruses, identical with respect to the derivation of all genes except one, would provide a basis for determining the relationship of that gene product to differences in "virulence."

These techniques have been used in the present experiments to demonstrate that neuraminidase determines the capacity of WSN virus, and of recombinants derived from WSN, to produce plaques in MDBK cells. Thus HK-WSN and FM1-WSN viruses that derive only the neuraminidase gene of WSN virus do produce plaques, whereas WSN-HK virus, which is identical to WSN except for its neuraminidase, does not (Table 1).

Observations of the effects of in vitro treatment with trypsin may explain the mechanism by which neuraminidase influences the production of infectious particles and plaque formation in MDBK cells. The infectivity of viruses that lack WSN neuraminidase was increased approximately 1,000-fold in permissive cell systems by trypsin treatment, whereas only slight increases in infectivity (less than 10-fold) were observed after trypsin treatment of viruses containing WSN neuraminidase (Tables 2 through 4). Moreover, recent experiments have provided evidence that FM1 virus produces small plaques in MDBK cells under 0.5 μg of trypsin per ml.

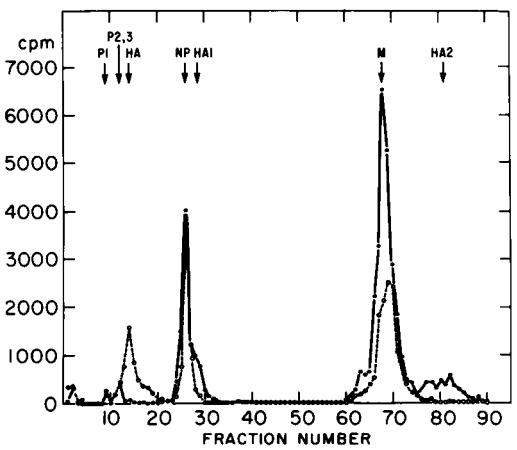

FIG. 4. *Protein analysis of WSN (●) and WSN-HK (○) viruses after infection of MDBK cells in the presence of 2% fetal calf serum. Virus labeled with [^{35}S]-methionine was disrupted with sodium dodecyl sulfate, and proteins were analyzed in adjacent lanes on a 13% polyacrylamide slab gel. After making 1-mm slices, fractions were counted in a liquid scintillation counter.*

These observations are consistent with the hypothesis that WSN neuraminidase removes sialic acid from a site(s) adjacent to the cleavage site on hemagglutinin, thus facilitating the proteolytic cleavage of HA to HA1 and HA2, an event that other investigators have shown is necessary for infectivity (5, 7). Other viral neuraminidases that are fully functional in other host cell systems apparently are not as effective in performing the same function in MDBK cells, possibly because of differences in host-directed glycosylation.

Direct evidence in support of this hypothesis was derived by analysis of the proteins of MDBK cell-grown WSN and WSN-HK viruses on sodium dodecyl sulfate-polyacrylamide gels. Under conditions in which the hemagglutinin of WSN virus is present as HA1 and HA2, all of the hemagglutinin of WSN-HK virus is uncleaved, and no cleaved HA1 and HA2 are detectable. WSN-HK virus, which derives all of its genes from WSN virus except for neuraminidase, produces noninfectious particles with uncleaved hemagglutinin in MDBK cells (Fig. 4).

In proposing this hypothesis we do not intend to imply that all strain-related differences in replication in MDBK cells are attributable to differences in neuraminidase. Differences in single-cycle yield of different viruses all containing WSN neuraminidase (Tables 3 and 4) must reflect differences in any or all of the other gene products. In addition, the plaque-enhancing effect of serum with FM1-WSN virus probably is related to the FM1 hemagglutinin and the previously observed effects of plasmin on cleavage of hemagglutinin (3, 7).

Previous studies demonstrated the importance of functional neuraminidase in repeated cycles of infection. Inhibition of neuraminidase by potent chemical inhibitors was shown to prevent plaque formation (10, 11, 17, 22) and, at 39.5°C, virus yields obtained after infection with temperature-sensitive neuraminidase mutants were shown to be aggregated because of residual sialic acid on virus particles (18). The present experiments suggest that in addition to preventing autoaggregation, neuraminidase may be required in some cell systems to facilitate cleavage of hemagglutinin. The observation that some neuraminidases do not fulfill this function equally well in all host cell systems opens the way to speculation that virulence of influenza viruses in particular host

cells may in part be related to appropriate combination of hemagglutinin and neuraminidase.

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ADDENDUM IN PROOF

Analyses of virus proteins obtained after mixed infection of MDBK cells with both WSN and WSN-HK viruses demonstrated the presence of only cleaved hemagglutinin (HA2 + HA2). These results further reduce the likelihood that the uncleaved hemagglutinin observed with WSN-HK virus alone is due to steric obstruction of the cleavage site by HK neuraminidase.

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