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Virion Functions of RNA$^+$ Temperature-Sensitive Mutants of Newcastle Disease Virus

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Virions from Newcastle disease virus mutants in four temperature-sensitive RNA$^+$ groups were grown in embryonated hen eggs at the permissive temperature, purified, and then analyzed for biological properties at both the permissive and nonpermissive temperatures. At the permissive temperature, virions of mutants in groups B, C, and BC (11 mutants) were all lower in specific (per milligram of protein) hemagglutination, neuraminidase, and hemolysis activities compared with the wild type. These deficiencies were related to decreased amounts of hemagglutinin-neuraminidase glycoprotein in the virions. Activities of these mutant virions at both the permissive and nonpermissive temperatures were similar, indicating that hemagglutinin-neuraminidase synthesized at the permissive temperature was not temperature sensitive in function. The three group D mutants displayed a different pattern. At the permissive temperature, they had wild-type hemagglutination and neuraminidase activities but were deficient compared with the wild type in hemolysis. Again, functions were similar at both temperatures. Most of the B, C, and BC mutants had specific infectivities similar to that of the wild type despite lower hemagglutination, neuraminidase, and hemolysis functions. However, the D mutants were all less infectious. This evidence is consistent with a shared hemagglutinin-neuraminidase defect in the B, C, and BC mutants and a defect in either the F glycoprotein or the M protein in the D mutants.

Virions of Newcastle disease virus (NDV), like those of other paramyxoviruses, consist of a single strand of viral RNA, six to seven virus-specific polypeptides, and membrane lipids from their most recent host cells. Associated with the envelope of the virion are a variety of functions which can be measured in vitro: hemagglutination (aggregation of erythrocytes), neuraminidase (enzymatic removal of neuraminic acid from carbohydrate-containing molecules), and hemolysis (destroying erythrocytes). Another envelope function, measurable on tissue culture cells in the absence of RNA and protein synthesis, is fusion from without (FFWO). Hemagglutination and neuraminidase are both functions of the hemagglutinin-neuraminidase (HN) glycoprotein (9, 22). Hemolysis (and probably FFWO) is a function of the F glycoprotein but also requires HN (9, 16). Hemadsorption, neuraminidase, and fusion from within (FFWI) are also properties associated with the surfaces of infected cells once viral proteins have been synthesized.

The F glycoprotein is synthesized as an inactive precursor, F_0; depending on the strain of NDV and the host cell, either F_0 may be cleaved during maturation to its active form of disulfide-linked F_{1+2} (15), or the virus may elsewhere encounter an appropriate protease for activation (7, 22). For most strains of NDV, no detectable cleavage is required for HN to function, but such cleavage is required for at least two strains (14). A third membrane-associated protein, M, contains no carbohydrate and appears to be associated with the inner surface of the membrane. There is no assayable function of M, but it may perform an organizing role in virion maturation and possibly virion structure as well.

Six complementation groups of temperature-sensitive mutants have been derived from the Australia-Victoria strain of NDV (25). Two of these groups, A and E, are deficient in RNA synthesis at the nonpermissive temperature (RNA$^-$), while the four other groups, B, BC, C, and D, are deficient in something other than RNA synthesis (RNA$^+$). Assignment of the B, BC, C, and D defects to particular proteins has not yet been done.

To study the in vitro functions of these temperature-sensitive mutants, we purified and concentrated virus stocks grown at the permissive temperature in hen eggs. We determined the specific neuraminidase, hemagglutinating, and hemolyzing activities relative to protein concen-

tration for each mutant at both the permissive and nonpermissive temperatures. In addition, we determined the specific infectivity and relative amount of HN in the virions. The B (six members), C (two members), and BC (three members) group mutants were similar in that they were deficient compared with the wild type (AV-WT) in neuraminidase, hemagglutinating, and hemolyzing activities assayed at the permissive temperature; all of the deficiencies can be related to a decreased HN protein content in their virions. In general, the decreased HN content did not affect the specific infectivity of these mutants. At the permissive temperature, the D group mutants (three members) were deficient only in hemolyzing activity and specific infectivity compared with AV-WT. Neuraminidase, hemagglutinating, and hemolyzing activities of most mutants, irrespective of complementation group, were similar when assayed at the permissive and nonpermissive temperatures, suggesting that the temperature-sensitive defects in these mutants are manifest mainly in the infected cell during virion maturation rather than in activity in completed virions. It appears that the B, C, and BC mutants share a defect in the HN protein, whereas the D mutants are defective in another virion protein, perhaps F or M.

MATERIALS AND METHODS

Cell cultures. Primary and secondary chicken embryo cells were maintained in standard medium (6) at 39.5°C in a 5% CO_2 atmosphere. Confluent secondary cultures (24 to 48 h after plating) in 60-mm tissue culture dishes were used for plaque titrations.

Virus. The temperature-sensitive mutants were isolated by Tsipis and Bratt (25) from wild-type virus AV-WT previously cloned from the Australia Victoria (1932) strain of NDV (4). Virus stocks were grown in the allantoic sac of 10-day-old embryonated hen eggs at 36°C. Allantoic fluid was harvested after the death of the majority of embryos (46 to 64 h), and virus was concentrated and purified by differential centrifugation, including density banding in linear sucrose gradients as described previously (5, 26). Stocks were stored at −70°C.

Plaque assays. Infectivity was titrated as described previously (4). Plates were incubated at the permissive temperature, 37.5°C.

Hemagglutination. Equivalent volumes of virus stocks were treated with sodium *meta*-periodate and assayed as previously described (5). The hemagglutination titers were determined by the fractional dilution method (8), using the Titertek system (Flow Laboratories, Inc.). Chicken erythrocytes were present at a final concentration of 1.5×10^6 cells per ml. The inverse of the endpoint was the hemagglutinating titer. Specific hemagglutination was the titer divided by the protein concentration.

Neuraminidase. Fetuin was used as the substrate for the neuraminidase activity of the virus stocks. Samples of virus were incubated with 0.25 mg of fetuin in 0.5 ml of 0.4 M sodium acetate (pH 7.0) for 1 h at either 37.5 or 41.8°C. The *N*-acetylneuraminic acid released was determined colorimetrically by the method of Aminoff (1). Background adsorbance due to sucrose in the virus stocks was subtracted, and the amount of *N*-acetylneuraminic acid released was determined from an *N*-acetylneuraminic acid standard curve. Specific neuraminidase activity is expressed as nanomoles of *N*-acetylneuraminic acid released in 60 min/μg of viral protein.

Hemolysin. The hemolytic activities of virus stocks were assayed by the dilution method of Bratt and Clavell (3) in which amounts of virus required to lyse a specific percentage of erythrocytes are compared. The amount of virus protein required to hemolyze 20% of the chicken erythrocytes was determined, and the inverse of this value was then used for comparison.

Protein determinations. The protein concentration of each virus stock was determined by the method of Lowry et al. (11), with bovine serum albumin used as a standard. The concentrations of major virion proteins in each stock were determined by pelleting virions in a Spinco SW41 rotor at 39,000 rpm for 90 min, electrophoresing 20 μg of viral protein on 10% sodium dodecyl sulfate-polyacrylamide gels (2), staining the gels with Coomassie brilliant blue, tracing the stained bands of the wet gel with an Ortek densitometer, and calculating the areas under the peaks with a Wang digitizer.

RESULTS

Hemagglutination and neuraminidase. The hemagglutinating and neuraminidase functions of the B, C, BC, and D mutants and AV-WT virions grown at the permissive temperature were assayed at both the permissive and nonpermissive temperatures. The specific activities are shown in Fig. 1. Most of the mutants, as well as AV-WT, displayed similar activities at 37.5 and 41.8°C. However, the specific neuraminidase and hemagglutinating activities of the group B, C, and BC mutants was significantly lower than those of AV-WT and the D group mutants.

Polypeptides of mutant virions. To determine whether the lower neuraminidase and hemagglutinating activities of the B, C, and BC mutants were associated with lower amounts of the HN polypeptide, virions were electrophoresed and stained. The amount of HN was quantified and expressed (Table 1) in relation to the amount of P protein plus NP protein (P+NP) in each virion preparation. All members of the B, C, and BC groups had less HN (5 to 50% AV-WT) in their virions, whereas group D mutants were similar to AV-WT. The correlation between neuraminidase and hemagglutinating activity and the amount of HN in each mutant is shown in Fig. 2. In general, the neuraminidase activities of all of the mutants correlated well with their HN contents (Fig. 2A). Since neuraminidase is an enzymatic function, it seems reasonable that a decreased concentration of enzyme should result in a similar decrease in activity. Hemagglutinat-

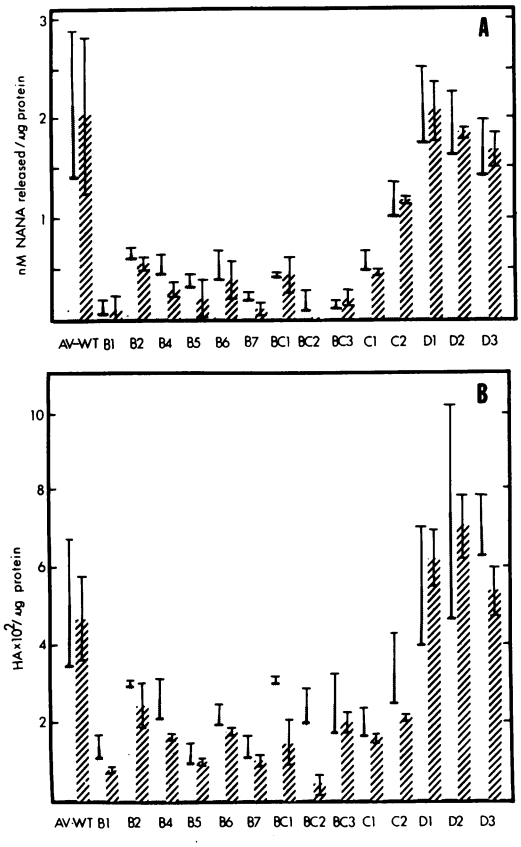

FIG. 1. Neuraminidase (A) and hemagglutinating (B) activities of temperature-sensitive mutants assayed at the permissive (shaded bars) and the nonpermissive (cross-hatched bars) temperature. NANA, *N*-Acetylneuraminic acid. The vertical lines indicate standard deviations of two to four determinations.

ing activities correlated fairly well with HN content, but the B, C, and BC mutants all had hemagglutinating activities slightly higher than that expected from HN content. Since hemagglutination measures the attachment of a virion to two erythrocytes, perhaps by as few as two HN molecules, the total number of HN molecules available on the virion may be relatively unimportant, making hemagglutination a complex function. In any case, it appears that the decreased neuraminidase and hemagglutinating activities can adequately be explained by lower amounts of the HN polypeptide rather than by decreased functioning of the HN polypeptides.

The amount of M protein relative to P+NP was similar for all mutants and AV-WT (Table 1). The amount of F polypeptide in these virion preparations could not, however, be determined. Under reducing conditions, F_1 migrates with and is obscured by P+NP, whereas F_2, the cleavage fragment, is not detected. Under non-

TABLE 1. Relative amount of virion HN and M proteins[a]

Virus	Amt ($\times 10^{-2}$ of: HN/ P + NP	M/ P + NP
AV-WT	17	70
B1	0.78	69
B2	6.5	75
B4	3.8	71
B5	1.3	76
B6	6.1	78
B7	2.5	88
BC1	5.4	75
BC2	5.3	72
BC3	2.8	87
C1	5.1	70
C2	8.8	77
D1	17	83
D2	24	98
D3	18	78

[a] Virions were pelleted, disrupted, and electrophoresed. Stained bands of viral proteins were quantitated and compared.

reducing conditions, the disulfide-linked F_1+F_2 migrates as a diffuse larger band separate from P+NP, but unfortunately this band stains inefficiently with Coomassie brilliant blue (approximately three times less efficiently than NP or M [unpublished data]) and therefore was not quantifiable.

Hemolysis. The hemolytic activities of the temperature-sensitive mutants and AV-WT were tested (Fig. 3). At the permissive temperature, all of the mutants had lower specific hemolytic activities than that of AV-WT. The lower hemolytic activities of the B, C, and BC group mutants probably reflect the decreased HN content of the virions (Fig. 4A). The lower hemolytic activities of the D group mutants cannot be

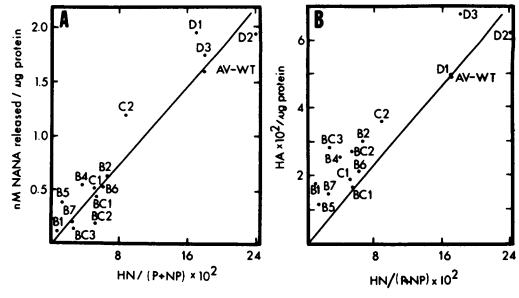

FIG. 2. Relationship between the amount of HN polypeptide and neuraminidase (A) or hemagglutinating (B) activities. HN/P+NP ratios (Table 1) are plotted against permissive-temperature activities of mutants (Fig. 1). NANA, *N*-Acetylneuraminic acid. The line is drawn between the origin and AV-WT.

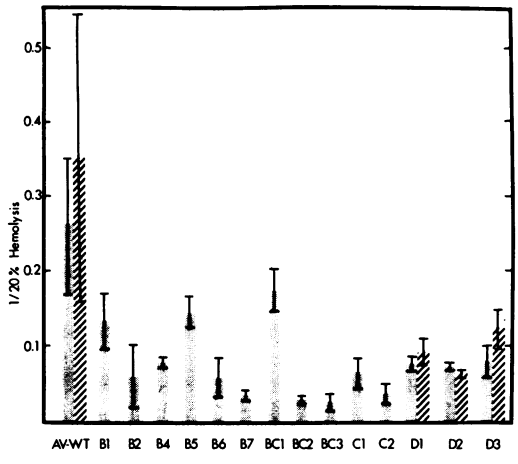

FIG. 3. Hemolytic activity. All of the mutants were assayed at the permissive temperature (shaded bars). AV-WT and the D group mutants were also assayed at the nonpermissive temperature (cross-hatched bars). Each mutant was assayed at least three times; the vertical lines represent the standard deviations.

explained by an HN defect and must be related instead to a defect in another virion component.

Assays for hemolytic activities were also done at the nonpermissive temperature for the group D mutants and AV-WT. The hemolytic activity of each of the D mutants and AV-WT was similar whether assayed at the permissive or the nonpermissive temperature (Fig. 3). Since the defects in B, C, and BC mutants appear to lie in the maturation of the HN polypeptide into virions, they were tested only once at the nonpermissive temperature. They, too, were found to have activities similar at the permissive and nonpermissive temperatures (data not shown).

Infectivity. The specific infectivity (permissive-temperature titer relative to protein concentration) of each stock was calculated (Table 2). Most of the B, C, and BC mutants had specific infectivities similar to that of AV-WT. B7 is a possible exception. On the other hand, the D group mutants were uniformly less infectious. Several stocks of each D group mutant have been tested and consistently show low specific infectivities.

A comparison of the specific infectivity and the amount of HN in each stock is shown in Fig. 4B. There appears to be no correlation between the amount of HN and infectivity. Virions of mutants in groups B, C, and BC, which contain as little as 5% of the amount of HN as do those of AV-WT, are just as infectious. In contrast, the group D mutants, all of which have amounts of HN similar to that of AV-WT, are much less infectious, indicating that their defects probably lie elsewhere.

For comparison, specific infectivity was plotted against hemagglutinating activity (Fig. 5A) and hemolytic activity (Fig. 5B). For most of the B, C, and BC mutants, these activities do not correlate with infectivity. Hemagglutinating activities of the group D mutants do not correlate with infectivity, but their hemolytic activities do. Therefore, the B, C, and BC mutant defects in hemolytic activity appear to be unrelated to infectivity, whereas the D group mutant defects in hemolysis are related to infectivity.

The plating efficiency (ability to plaque at the nonpermissive temperature compared with the permissive temperature) of each of these virus stocks was similar (Table 2) to those previously described (25).

Variations among stocks of the same virus. Even though the same cloned seed stock was used to grow each subsequent stock of a particular virus, variations among stocks were found. For this reason, several stocks of AV-WT were compared (Table 3). Although a scattering of values was found for each virion function, such variation between stocks is insufficient to account for the defects observed with the mutants; thus, their observed defects seem to truly reflect their mutations rather than to be merely stock variations.

RNA$^-$ mutants with defects presumably in their genes for the P (E group) or L (A group) proteins (17) would not be expected to show variation in either HN content or the activities attributed to the surface of the virions. This prediction was borne out by the data shown in Table 3. The neuraminidase, hemagglutinating, and hemolytic activities and the amount of HN polypeptide were all similar to those of the AV-WT stocks. However, the specific infectivities of A1 and E1 were somewhat lower than that of

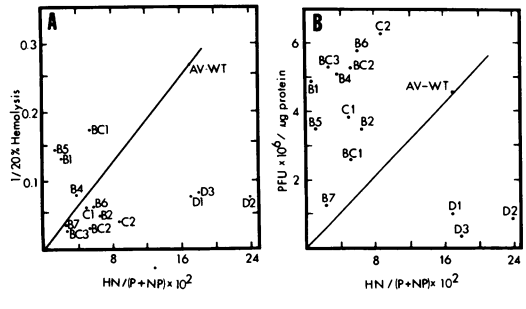

FIG. 4. Relationship between relative amount of HN polypeptide and hemolytic activity (A) or infectivity (B). HN/P+NP ratios (Table 1) were plotted against hemolytic activity at the permissive temperature (Fig. 3) and infectivity (Table 2) values. The line is drawn between the origin and AV-WT.

TABLE 2. Efficiency of plating and specific infectivities

Virus	PFU ($\times 10^6$) per μg of protein	Plating efficiency[a]
AV-WT	4.6	5.0×10^{-1}
B1	4.9	1.4×10^{-2} (s)
B2	3.5	1.1×10^{-1} (s)
B4	5.1	1.5×10^{-1} (s)
B5	3.5	3.3×10^{-1} (s)
B6	5.8	1.7×10^{-1} (s)
B7	1.5	8.6×10^{-2} (s)
BC1	3.1	8.4×10^{-2} (s)
BC2	5.3	2.2×10^{-3}
BC3	5.3	7.6×10^{-5} (s)
C1	3.8	9.8×10^{-2}
C2	6.3	1.3×10^{-1}
D1	1.0	2.2×10^{-4}
D2	0.86	5.9×10^{-3}
D3	0.33	4.5×10^{-3}

[a] Plating efficiency is defined as the ratio of plaques formed at 41.8°C to those formed at 37.5°C. (s), Small plaque phenotype (41.8°C).

AV-WT, presumably owing to a defect in another component of infectivity, probably an RNA-synthesizing function (25; M. E. Peeples, L. L. Rasenas, and M. A. Bratt, J. Virol., in press).

DISCUSSION

The preliminary characterization of these temperature-sensitive mutants of NDV described four RNA^+ groups: B, BC, C, and D (25). It was suggested that the low complementation between the B and C group mutants might reflect intracistronic complementation between mutants in the same gene. The BC group of mutants was unable to complement either the group B or C mutants. The results presented here suggest that the B, C, and BC mutants should be assigned to the HN gene. All members of the B, C, and BC groups have a lower amount (5 to 50%) of HN polypeptide per virion and lower hemagglutinating, neuraminidase, and hemolyzing activities than does AV-WT. Smith and Hightower (23) previously reported that mutant C1 had less HN in its virions as well as lower HN functions as compared with the parent AV-WT. The sole RNA^+ temperature-sensitive mutant of Sendai virus also had less HN in its virions (20). It is not clear whether the decreased amount of virion HN is directly related to these temperature-sensitive defects or is indirectly related to any type of change in the HN protein. The lower amount of HN in C1 virions may be due to the instability of intracellular HN (G. W. Smith and L. E. Hightower, personal communication).

In addition to decreased amounts of HN in virions, it was previously reported that, in infected chick embryo cells, one member from group B was temperature sensitive for hemadsorption, another was temperature sensitive for FFWI, and one member from group C had poor FFWI function at both temperatures (25), all of which factors might be related to an HN defect.

It would, of course, be possible that the defects in all of the B, C, and BC mutants which appear to cause less efficient packaging of the HN polypeptide into virions actually lie in a second protein which interacts with HN during virion formation. This possibility seems unlikely for two reasons: (i) the hemagglutinins of several, but not all, of these mutants are thermolabile (18; M. E. Peeples, R. L. Glickman, and M. A. Bratt, submitted for publication) and, since only the HN is required for paramyxovirus hemagglutination (9, 13, 21, 24), the thermolability of the mutant hemagglutinin should indicate an alteration in the HN protein itself; and (ii) the electrophoretic migration rate of the HN of temperature-sensitive mutants B2, C1, and BC3 is altered (18; Peeples et al., in preparation), also indicating a difference in the HN molecule.

The lower HN content in the B, C, and BC virions correlates with lower virion neuraminidase, hemagglutinating, and hemolyzing activities at the permissive temperature. Thus, the functional defects in these permissive-temperature-grown virions can be directly explained by the lower amount of HN in the virion. As yet, there is no evidence for defects in a functional domain of the HN molecule. It may be that defects in the functional regions of the HN polypeptide are lethal. Smith and Hightower (23) isolated a mutant derived from C1 which had no neuraminidase activity, but mutations in the hemagglutinin function have not been reported.

Interestingly, the lower hemagglutinating and

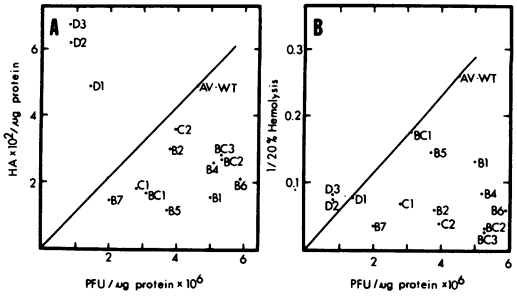

FIG. 5. Relationship between infectivity and hemagglutinating (A) or hemolytic (B) activities. Infectivity (Table 2) was plotted against permissive-temperature hemagglutinating (Fig. 1) or hemolyzing activities (Fig. 3). The line is drawn between the origin and AV-WT.

TABLE 3. Comparison of AV-WT and temperature-sensitive RNA⁻ mutant stocks

Virus	PFU ($\times 10^6$) per μg of viral protein	NANA[a] (nM) released per h per μg of viral protein	Hemagglutination titer per μg of viral protein	Hemolysin[b]	HN $\times 10^{-2}$/ P + NP[c]
AV-WT[d]	3.8 ± 0.80	3.0 ± 0.64	1,195 ± 262	0.35[e]	34 ± 3.9
A1	1.9	3.2	1,012	0.29	45
A4	3.3	3.6	748	0.22	38
A7	3.9	2.8	853	ND[f]	42
E1	1.3	3.2	839	ND	39

[a] NANA, *N*-Acetylneuraminic acid.
[b] Inverse of viral protein concentration required to hemolyze 20% of the chicken erythrocytes.
[c] Relative density of stained HN to P + NP bands from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of virions.
[d] Mean ± standard deviation for four to six different stocks of AV-WT.
[e] Average of two stocks.
[f] ND, Not determined.

hemolyzing activities of the B, C, and BC mutants are not reflected in their specific infectivities; the majority of these mutants are equally as infectious as AV-WT. Apparently, only a limited number of HN polypeptides per virion are required to allow the necessary attachment to occur; a full complement of HN is not required. The hemagglutinating activities of these mutants, a receptor function of the virion, are lower than that of AV-WT, possibly because the adsorption of one virion to two erythrocytes is more demanding than the ability of a virion to adsorb to a single cell to initiate infection. Experiments are presently under way to determine the relative elution rates of these mutants. Less neuraminidase might result in more efficient adsorption, counteracting the defect in the quantity of receptors.

If the virion membrane must fuse with the cell membrane as the next step in infection, it seems paradoxical that the B, C, and BC mutants with lower hemolytic activities are as infectious as AV-WT. Since hemolytic activity is thought to be a reflection of the fusing function of the virus, this activity is thought to be necessary for infection. It might be that the lower hemolytic activity determined at a single point, as described in this report, reflects a lower rate rather than the endpoint of hemolytic activity. However, rate studies with several mutants have not borne this out (unpublished data). The lower hemolytic activities of these mutants seem to reflect the lower amount of HN protein. It has not been determined whether the decreased receptor function (9), neuraminidase function (10), or another function of HN required for hemolysis (16) was responsible.

The D group mutants have been shown to be defective in their specific infectivity and hemolyzing activities. Since the levels of their hemolytic activities correlate with their levels of infectivity, the lower hemolytic function of the D mutants might be responsible for the lower infectivity. The location of the defect in the D group does not appear to be the HN polypeptide, but whether it is in the F or M polypeptide is unclear. These mutants are all temperature sensitive for FFWI in infected cells (25; unpublished data). FFWI, like FFWO, is thought to be mediated through the same mechanism as is hemolysis. Both hemolysis and FFWI require the F polypeptide in its cleaved form (7, 12, 15, 22). Preliminary unpublished results indicate that the F protein is present in D mutant virions from infected chick embryo cells in a cleaved form, but the defect might lie in another part of the molecule. However, virions from cultured cells may differ from egg-grown virions. The M polypeptide of mutant D1 has an altered electrophoretic mobility, but whether that change is related to its defect is uncertain (18). The M polypeptide might be involved in both hemolysis and infectivity as an organizer.

Presumably, the lower amount of HN in B, C, and BC mutants are in each case caused by the same amino acid substitution responsible for temperature sensitivity. The defects of the group D mutants are not as clear but result in lower hemolysin and infectivity. All of these mutants were isolated for their temperature sensitivity, yet we have found little evidence for temperature-sensitive function in their virions. This is unlike the temperature-sensitive RNA+ mutant of Sendai virus whose hemagglutinin function was temperature sensitive in addition to being present in lower amounts in virions (19). Probably the temperature-sensitive defects affect a stage in the virus life cycle before or during virion assembly. Perhaps the defects reflected in the virions grown at the permissive temperature are merely exacerbated at the nonpermissive temperature. Presently we are growing radioac-

tively labeled virions at both temperatures and analyzing cell surface functions in mutant infected cells to approach these questions.

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