Thermostabilities of Virion Activities of Newcastle Disease Virus: Evidence that the Temperature-Sensitive Mutants in Complementation Groups B, BC, and C Have Altered HN Proteins

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Four virion activities of Newcastle disease virus (hemagglutinating, neuraminidase, hemolytic, and infectious activities) were examined before and after heat stress in low-salt buffer and physiological salt buffer (phosphate-buffered saline). The hemagglutinating and neuraminidase activities of the Australia-Victoria wild-type (AV-WT) strain were thermostable at both salt concentrations tested, whereas the thermostabilities of the hemolytic and infectious activities were salt dependent (thermostable in phosphate-buffered saline but not in low-salt buffer). Virions of RNA⁺ temperature-sensitive (ts) mutants of AV-WT were tested for the stabilities of the four activities. Some mutants in groups B, BC, and C were as stable as AV-WT in all functions, but others were much less stable in all functions. The unstable mutants in groups B, BC, and C affirmed the assignment of the ts lesions of these mutants to the hemagglutinin/neuraminidase (HN) protein gene because HN function(s) are required for all four activities. The instability of these ts mutants was not related to their decreased virion HN protein content and was not due to physical loss of the HN protein from the virions. Three of four ts⁺ plaque-forming revertants of the least stable mutant, BC2, coreverted for stability, confirming that the unstable phenotype is indeed the result of the mutation responsible for the ts phenotype. Group D mutants were approximately as stable as AV-WT in hemagglutinating, neuraminidase, and hemolytic activities; this is consistent with this group representing a lesion in a gene other than the HN protein gene. However, the infectivities of two of the three group D mutants were less stable than the infectivity of AV-WT in low-salt buffer.

Like virions of several other paramyxoviruses, virions of Newcastle disease virus (NDV) have the ability to perform a variety of functions in vitro, including the following: hemagglutination (aggregation of erythrocytes), neuraminidase activity (enzymatic removal of neuraminic acid from carbohydrate-containing molecules), and hemolysis (lysis of erythrocytes by fusion with the cell membrane). Each of these functions has its counterpart in infection of cells (6, 7, 12, 32), and each has been studied as a model of steps of infection. Paramyxovirus hemagglutination and neuraminidase activity are functions solely of the hemagglutinin/neuraminidase (HN) glycoprotein (14, 17, 31, 37). The other virion glycoprotein, glycoprotein F, is required for fusion functions. This glycoprotein is synthesized as a precursor, F₀, which must be proteolytically cleaved to the disulfide-linked F₁₊₂ form in order to be active. Both HN and F₁₊₂ glycoproteins are required for hemolysis and

infectivity (14, 19-21). A third protein of the viral membrane is the non-glycosylated M protein, whose function in these activities is not clear. A possible involvement of M protein in hemolysis has been discussed elsewhere (23).

Six complementing groups of temperature-sensitive (ts) mutants were previously isolated from the Australia-Victoria wild-type (AV-WT) strain of NDV (38). Two groups, groups A and E, are deficient in RNA synthesis at nonpermissive temperatures (RNA⁻) (25, 38) and probably represent mutations in the L and P genes, respectively (22). The other four groups, groups B, BC, C, and D, contain mutants that are altered in steps other than RNA synthesis (RNA⁺ at nonpermissive temperatures). Virions of group B, BC, and C mutants grown in eggs at the permissive temperature possess reduced amounts of HN protein and consequently show reduced levels of HN protein functions compared with AV-WT (23, 24). However, these

virions are not temperature sensitive in their functions (23). In addition, the HN proteins of three mutants in these groups are altered in their migration rates in sodium dodecyl sulfate-polyacrylamide gels (24; M. E. Peeples, R. L. Glickman, J. P. Gallagher, and M. A. Bratt, manuscript in preparation). This finding suggests that the group B, BC, and C mutants all have lesions in the gene coding for the HN protein. In contrast, virions of the group D mutants contain normal amounts of HN protein and exhibit normal levels of HN protein function; they are, however, deficient in hemolytic activities and specific infectivities. These group D mutants have a lesion in the gene coding for another protein, possibly F protein but more likely M protein (23, 24; Peeples and Bratt, manuscript in preparation).

We studied the susceptibility to heat stress of AV-WT and the RNA⁺ mutant virions on the assumption that a ts lesion might alter the functional stability of the mutant protein. We reasoned that by analyzing the stabilities of several virion functions, we might be able to confirm the gene assignments of the RNA⁺ mutants and possibly describe the functional domains in which the mutants are defective. Group B, BC, and C mutants displayed a wide spectrum of stabilities. However, for individual mutants there was a strong correlation among the stabilities of the infectious, hemagglutinating, neuraminidase, and hemolyzing activities. Our finding of mutants from each of these groups with unstable HN protein activities strengthens the assignment of the lesions in groups B, BC, and C to the HN protein gene but does not distinguish functional domains. Spontaneous ts⁺ plaque-forming revertants of the most unstable mutant, BC2, showed coreversion of the stabilities of the HN protein functions and the amount of HN protein in virions, further strengthening this gene assignment.

MATERIALS AND METHODS

Virus. The ts mutants were isolated by Tsipis and Bratt (38) from wild-type virus (AV-WT) that was previously cloned from the Australia-Victoria (1932) strain of NDV (5). All virus stocks were grown at 36°C in the allantoic sacs of 10-day-old embryonated hen eggs. After the death of the majority of the embryos (46 to 64 h), allantoic fluid was harvested, and virus was concentrated and purified by differential centrifugation which included velocity sedimentation on a 20%/65% step gradient and density sedimentation on a linear sucrose gradient, as previously described (8, 39). Stocks were stored in a solution containing 40% sucrose, 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8), 0.03 M NH₄Cl, and 0.0005 M EDTA at -70°C.

Cell cultures. Primary and secondary chicken embryo cells were maintained in standard medium (11) at 39.5°C in a 5% CO₂ atmosphere. Confluent secondary

cultures (24 to 48 h after plating) in 60-mm tissue culture dishes were used for plaque titrations, as described previously (5). Infected plates were incubated at the permissive temperature (37.5°C) for 2 days before plaque enumeration.

Thermal inactivation. Equivalent concentrations (0.6 mg of protein per ml) of each virus stock were added to either low-salt buffer (0.01 M HEPES, pH 8.0, 0.03 M NH₄Cl) or phosphate-buffered saline (PBS; 0.0081 M Na₂PO₄, 0.0015 M KH₂PO₄, pH 7.5, 0.14 M NaCl) unless otherwise stated. Both buffers also contained 7% sucrose as a result of the addition of virions which had been purified and stored in sucrose. Diluted virus (0.2 to 0.6 ml) was placed in glass test tubes, covered, and heated in a circulating water bath for 30 min unless otherwise specified. Biological activities of virions were measured before and after heating, and the fraction of activity remaining was determined by dividing the activity after heating by the activity before heating.

Hemagglutination. Virus was diluted in either low-salt buffer or PBS, treated with sodium metaperiodate to prevent subsequent elution, and assayed as previously described (8). Chicken erythrocytes were added to a final concentration of 1.5×10^6 cells per ml. Hemagglutination titers were determined by the fractional dilution method (13), using the TiterTek system (Flow Laboratories). The inverses of the endpoint dilutions were used as titers.

Neuraminidase. 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 37.5°C. The amount of *N*-acetylneuraminic acid released from fetuin was determined by the colorimetric method of Aminoff (2). Background adsorbance due to sucrose was subtracted, and the amount of *N*-acetylneuraminic acid released was determined from an *N*-acetylneuraminic acid standard curve.

Hemolysis. Hemolytic activity was assayed by using the procedures of Clavell and Bratt (8). Quantification was done by the dilution method of Bratt and Clavell (4), and the amounts of virus protein required to lyse a specific percentage of erythrocytes (rather than the amounts of lysis by given quantities of virus) were compared. In each case the amount of virus protein required to hemolyze 20% of the chicken erythrocytes was determined, and the inverses of these values were used for comparisons.

Protein determinations. The protein concentration of each virus stock was determined by the method of Lowry et al. (16), using bovine serum albumin as a standard. The relative concentrations of major virion proteins were determined by centrifuging virions in a Spinco SW41 rotor at 39,000 rpm for 90 min, electrophoresing 65-μg samples of viral protein on 10% sodium dodecyl sulfate-polyacrylamide gels (3), staining the gels with Coomassie brilliant blue, tracing the stained bands of the wet gels with an Ortek densitometer, and calculating the areas under the peaks with a Wang digitizer. The HN glycoprotein concentration in virions is expressed as the amount of HN protein divided by the sum of the amounts of major virion protein NP and P protein (which could not be separated easily from NP protein on the tracing). F₁ was also a minor component that migrated in the NP protein-P protein region of these gels.

Revertant isolation. Spontaneous revertants (rever-

FIG. 1. Temperature dependence of thermal inactivation of the hemagglutinating activity of AV-WT. AV-WT diluted in low-salt buffer was heated at 40°C (○), 45°C (□), 50°C (△), 50.5°C (▲), 51°C (■), 52°C (●), or 55°C (◇) for up to 60 min and then tested for remaining hemagglutinating activity (HA).

tants able to produce wild-type-like plaques at nonpermissive temperatures) of ts mutant BC2 were selected from individual (to reduce the possibility of selecting multiple copies of the same revertant) permissive temperature (37.5°C) plaques of a BC2 virus stock. Plaque contents were collected into Hanks balanced salt solution supplemented with 2% calf serum and were used to inoculate an embryonated hen egg or secondary chicken embryo cells in culture. The allantoic fluid or cell culture fluid was titrated at both permissive (37.5°C) and nonpermissive (41.8°C) temperatures. After 2 days at 41.8°C, large plaques were picked immediately (BC2R1 and BC2R2) or circled and incubated for a third day at 37.5°C and picked (BC2R3 and BC2R4). Plaque contents were then re-cloned two more times by plaque formation at 41.8°C before injection into hen eggs for amplification. These egg stocks were used as seed stocks to infect other eggs (approximately 10^3 PFU/egg) to grow the stocks used. The spontaneous reversion rate was between 10^{-3} and 5×10^{-4} .

RESULTS

Stability of the hemagglutinating activity of AV-WT. AV-WT virions were incubated for up to 60 min at different temperatures between 40 and 55°C to allow determination of the highest temperature at which hemagglutinating activity was stable. The hemagglutinating activity of AV-WT virions was relatively stable (50% of activity remaining after 60 min) in low-salt buffer at temperatures up to 50.5°C, but stability de-

creased rapidly at higher temperatures (Fig. 1). Therefore, 50.5°C was used for all of the experiments described below.

Stabilities of the hemagglutinating activities of the ts mutants. Virions of RNA⁺ mutants were diluted in low-salt buffer and heated at 50.5°C to determine the stabilities of their hemagglutinating activities. The inactivation curves of representative mutants are shown in Fig. 2. The inactivation constants of all of the RNA⁺ mutants were calculated from such curves (Table 1). All mutants in group D, as well as all mutants in RNA⁻ mutant groups A and E (data not shown), were approximately as stable as AV-WT. In contrast, the stabilities of the hemagglutinating activities varied among mutants in groups B, BC, and C, ranging from mutants like B1, B7, and C2, which were nearly as stable as AV-WT, to mutants BC2 and B6, which were very unstable. Since virions of group B, BC, and C mutants have lower amounts of HN protein than virions of AV-WT and the group D mutants (and RNA⁻ group A and E mutants) (23, 24), decreased stability might be a reflection of less HN protein per se. However, as shown in Table 1, there was clearly no correlation between HN protein content and stability. For example, mutants B1, B5, and B7, which had the least HN protein, were approximately as stable as AV-WT, whereas BC2 and B6, which had more HN protein, were very unstable.

Decreased stability of hemagglutinating activity could result either from the physical loss of

FIG. 2. Thermal inactivation of representative ts mutants. The procedure used was the same as that described in the legend to Fig. 1, except that only 50.5°C was used. Symbols: ●, AV-WT; ○, C1; ■, BC1; □, B6; ▲, BC2. HA, Hemagglutinating activity.

TABLE 1. Comparison of inactivation constants for hemagglutinating activity with HN protein content in virions

Virus	$K_{HA} (\times 10^{-3})^a$	HN/(P + NP) ($\times 10^{-2}$) ^b
AV-WT	9.4	19
B1	18	0.78
B2	28	6.5
B4	120	3.8
B5	7.6 ^c	1.3
B6	160	6.1
B7	16	2.5
BC1	85	5.4
BC2	880	5.3
BC3	34	2.8
C1	34	5.1
C2	15	8.8
D1	15	24
D2	15	24
D3	7.9	18

^a $K_{HA} = (-\ln HA/HA_0)/t$, where HA/HA_0 is the fraction of hemagglutinating activity remaining and t is the time (in minutes). Values were determined from plots like those shown in Fig. 2.

^b HN protein concentration expressed as the amount of HN protein divided by the sum of the amounts of major virion proteins NP and P. From reference 23.

^c The greater thermolability of B5 reported previously (24) was not observed in recent experiments in which stocks grown from the original B5 seed stock were used.

the HN protein from virions or from a conformational change in the HN protein. To compare the amounts of HN protein associated with untreated and heat-treated virions, the two most unstable mutants, BC2 and B6, were studied. Table 2 shows that similar amounts of HN protein were associated with virions before and after heating. Therefore, the decreased stabilities of the BC2 and B6 hemagglutinating activities were not due to the loss of the HN protein from virions, as has been reported for the G protein in unstable vesicular stomatitis virus ts045 virions (9). Therefore, instability was more likely due to a conformational change in the HN protein.

Stability of neuraminidase activity. A conformational change affecting the hemagglutinating ability of HN protein might also affect the neuraminidase activity of that bifunctional protein. The stability of the neuraminidase activity of each mutant was determined. In Fig. 3A the neuraminidase activity remaining after heating is plotted against the hemagglutinating activity remaining after heating. Since all of the mutants apparently fell near a straight line, the stability of the neuraminidase activity of each mutant

correlated with the stability of the hemagglutinating activity of that mutant. This line has a slope very near 1.0 (Fig. 3A, dashed line), indicating equivalent loss of both functions. Using two strains of NDV, Pierce and Haywood (27) previously demonstrated equivalent losses of hemagglutinating and neuraminidase activities upon heating.

Stability of hemolytic activity. Heating virions in low-salt buffers at either pH 7.5 or pH 8.0 destroyed the hemolytic activity of AV-WT but had little effect on the hemagglutinating activity (Table 3). When the same heating experiment was performed with PBS, the hemolytic activity of AV-WT was stable. The salt concentration, rather than the specific salt or buffer used, was clearly the stabilizing factor, because hemolytic activity was unstable in low-salt (0.04 M) buffers but stable in similar buffers with higher salt concentrations (0.15 M).

Having determined conditions under which the hemolytic activity of AV-WT was stable, we determined the stabilities of the mutants. The stability of hemolytic activity is plotted against the stability of hemagglutinating activity for each mutant in Fig. 3B. (The stabilities of the hemagglutinating and neuraminidase activities of AV-WT [Table 3] and the mutants [data not shown] were similar in PBS and low-salt buffer.) The stabilities of the hemolytic activities of the group D mutants were similar to the stability of AV-WT hemolytic activity. In contrast, mutants in groups B, BC, and C demonstrated reduced stabilities of hemolytic activities that were comparable to the stabilities of the hemagglutinating activities of the mutants, since these mutants fell near the solid line drawn through AV-WT (Fig. 3B). However, the slope of this line is greater than 1.0 (Fig. 3B, dashed line), indicating that the stabilities of the hemolytic activities of all of these viruses were greater than the stabilities of the hemagglutinating activities. This difference might reflect a more stringent binding requirement for hemagglutination (binding two erythrocytes) than for hemolysis (binding a single erythrocyte).

TABLE 2. HN protein remaining associated with virions after heating^a

Virus	HN/(P + NP) ($\times 10^{-2}$) ^b	
	Zero time	30 min
AV-WT	34	39
B6	14	10
BC2	16	14

^a Before and after heating at 50.5°C for 30 min in low-salt buffer, virions were purified on step gradients, pelleted, disrupted, and electrophoresed, and the amounts of HN protein were determined.

^b See Table 1, footnote b.

FIG. 3. Comparison of stabilities of hemagglutinating activities (HA) with stabilities of neuraminidase (A), hemolytic (B), and infectious (C) activities. Virions were diluted in low-salt buffer for determinations of hemagglutinating and neuraminidase activities and in PBS for determinations of hemolytic and infectious activities. Solid lines are drawn between AV-WT and the origins. The dashed lines represent the theoretical equivalent losses of both functions.

Stability of infectivity. The stabilities of AV-WT and mutant infectivities were determined in both low-salt buffer and PBS. Table 4 shows that AV-WT infectivity, like hemolytic activity, was relatively unstable in low-salt buffer but stable in

TABLE 3. Stabilities of hemagglutinating, neuraminidase, and hemolytic activities of AV-WT in different buffers

Buffer	Remaining activities (%) ^a		
	Hem-agglutinating	Neuraminidase	Hemolytic
0.01 M HEPES (pH 8.0) + 0.03 M NH ₄ Cl	54	73	10
0.01 M phosphate buffer (pH 7.5) + 0.03 M NaCl	71	ND ^b	10
0.01 M HEPES (pH 8.0) + 0.14 M NH ₄ Cl	46	ND	100
PBS (0.01 M phosphate buffer [pH 7.5] + 0.14 M NaCl)	59	77	100

^a Activity after heating for 30 min at 50.5°C, divided by activity before heating, $\times 100$.

^b ND, Not determined.

PBS. The infectivities of some mutants in groups B, BC, and C were as stable as the infectivity of AV-WT under both salt conditions, whereas the infectivities of other mutants were less stable than AV-WT infectivity under both conditions. However, several of the mutants were relatively more sensitive to heating in low-salt buffer than in PBS, as shown in the ratios of remaining infectivity. Of the three mutants with the lowest

TABLE 4. Effect of salt concentration on stabilities of infectivity

Virus	Infectivity remaining (%) ^a		Low salt/PBS ($\times 10^{-2}$) ^b
	Low-salt buffer	PBS	
AV-WT	3.6	51	7.0
D1	0.64	58	1.1
D2	1.1	52	2.1
D3	2.0	26	7.7
B1	4.4	42	10
B2	3.2	40	8.0
B4	0.69	5.3	13
B5	3.1	33	9.4
B6	0.22	0.86	25
B7	4.0	45	8.9
BC1	0.58	16	3.6
BC2	0.03	0.20	15
BC3	0.24	52	0.46
C1	0.72	20	3.6
C2	6.7	46	14

^a Infectivity after heating for 30 min at 50.5°C, divided by infectivity before heating, $\times 100$.

^b Ratio of percentage of infectivity remaining after heating in low-salt buffer to percentage of infectivity remaining after heating in PBS.

TABLE 5. Stabilities and HN protein contents of revertants of BC2

Virus	K_{HA} ($\times 10^{-3}$) ^a	Neuraminidase remaining (%) ^b	Infectivity remaining (%) ^c		HN/(P + NP) ($\times 10^{-2}$) ^d
			Low-salt buffer	PBS	
AV-WT	7.6	96	3.6	51	23
BC2	540	0	0.03	0.2	2.5
BC2R1	26	87	1.1	75	29
BC2R2	740	0	0.004	1.0	10
BC2R3	7.6	99	0.15	48	30
BC2R4	7.6	ND ^e	0.12	56	26

^a Inactivation constant for hemagglutinating activity determined as described in Table 1, footnote *a*, in low-salt buffer.

^b Neuraminidase activity after heating at 50.5°C for 30 min divided by neuraminidase activity before heating, (low-salt buffer).

^c Infectivity after heating at 50.5°C for 30 min, divided by infectivity before heating, $\times 100$.

^d Amount of HN protein compared with the amounts of P and NP proteins in virions as described in the text.

^e ND, Not determined.

ratios, two were from the three group D mutants (D1 and D2) and one was from the 11 group B, C, and BC mutants (BC3). Since the hemagglutinating activities of D1 and D2 were stable, the instability of infectivity must have been due to another function required for infection.

The stability of the infectivity of each mutant in PBS is plotted against the stability of hemagglutinating activity in Fig. 3C. In general, the stabilities of infectivity correlated with the stabilities of hemagglutinating activity, although not as well as neuraminidase (Fig. 3A) and hemolytic (Fig. 3B) activities did. The slope of the line is less than 1.0 (Fig. 3C, dashed line), suggesting that infectivity was more sensitive to heating than hemagglutinating activity. Since infectivity requires virion functions other than attachment, such as penetration and primary transcription, the difference between the slopes may represent the stabilities of these other functions.

Revertants of BC2. The hemagglutinating activity of mutant BC2 was the most unstable of all the mutants. Revertants of BC2 were isolated and characterized to determine whether stability coreverted with the ability to plaque at the nonpermissive temperature. As shown in Table 5, three of the four revertants (BC2R1, BC2R3, and BC2R4) regained much of the stabilities of their hemagglutinating and infectious activities in PBS, confirming the association of the unstable phenotype with the *ts* phenotype. However, in low-salt buffer, the infectivities of these revertants, although increased compared with the mutant, were not as stable as the infectivity of AV-WT. In addition, BC2R1, which was the most stable of the revertants, produced plaques larger than those of AV-WT at the nonpermissive temperature. Thus, it appears that these

three *ts*⁺ revertants are pseudorevertants (not exact reversions to the wild-type gene sequence). The fourth *ts*⁺ revertant, BC2R2, remained as unstable in hemagglutinating and infectious activities under both salt conditions as BC2. This revertant produced smaller plaques at the nonpermissive temperature than the plaques produced by AV-WT. All of these findings indicate that BC2R2 is also a pseudorevertant.

Table 5 also shows that virions of each of the revertants contained greater amounts of HN protein than mutant BC2 and in most cases had reacquired wild-type levels, confirming the association of the reduced HN protein phenotype with the *ts* phenotype.

DISCUSSION

Thermostability studies of NDV have been used for comparisons of naturally occurring NDV strains (10, 15, 26, 27, 40). Wide variations in the stabilities of infectivity, hemagglutinating, and hemolytic activities have been found. In this study, we examined the changes in stability among *ts* mutants of a single strain of NDV. The variety of stabilities among these mutants suggests that these mutants represent many different mutational changes.

The finding that the hemagglutinating and neuraminidase activities of several of the group B, BC, and C mutants are unstable, coupled with the evidence that for paramyxoviruses only the HN protein is required for these functions (14, 17, 31, 37), provides further evidence that the lesions in these mutants are in the gene coding for the HN protein. The group D mutants and members of each of the RNA⁻ groups (groups A and E) have AV-WT-like HN protein stability, indicating that HN protein instability is

not a general characteristic of ts mutants of AV-WT, but rather is limited to members of the group B, BC, and C mutants. We have previously suggested the association of the group B, BC, and C mutants with lesions in the gene coding for the HN protein since virions of these mutants contain less HN protein and HN protein activity, and one mutant in each of these groups possesses an HN protein whose migration rate on sodium dodecyl sulfate-polyacrylamide gels (24; Peeples et al., manuscript in preparation) differs from that of AV-WT HN protein. It seems likely that the same amino acid change which resulted in the decreased stability of HN protein functions also resulted in lowered virion HN protein content. This conclusion is supported by our finding that for three of the four presumably single-step revertants of BC2, both stability and wild-type virion HN protein content coreverted with the ability to plaque at the nonpermissive temperature.

The group B and C mutants were originally described as separate groups because of low-level complementation. The BC group mutants were unable to complement either group B or C mutants although, like group B and C mutants, they did complement group A, D, and E mutants (38). If all members of groups B, BC, and C represent mutations in the gene coding for the HN protein, then the low-level complementation between group B and C mutants must be intracistronic, one of the possibilities suggested by Tsipis and Bratt (38). The fact that HN protein exists as a dimer in virions (18, 33, 35) suggests a mechanism for such intracistronic complementation. If an HN protein from a group B mutant were paired with an HN protein from a group C mutant and each was defective in a different domain, a functional dimer might result. The group BC mutations might be either in an area which cannot be complemented intracistronically or in an area affecting both domains represented by the group B and C mutations.

Whether the functional areas of the HN glycoprotein are located at the same or different sites on the molecule is unclear. Since influenza virus performs similar attachment and elution functions, with the hemagglutinating activity on the HA glycoprotein molecule and the neuraminidase activity on another molecule, the NA glycoprotein (7, 11), it seems possible that there might be two physically separate sites. Actually, several lines of evidence suggest that for paramyxoviruses these two functions occupy close but separable sites on the HN protein molecule. (i) Smith and Hightower (34, 36) have been able to separate genetically the two functions; a revertant of their clone of our ts mutant C1 has hemagglutinating activity but lacks neuraminidase activity. (ii) Portner (28, 29) has selected

Sendai virus variants by using monoclonal antibodies to the HN protein; some of these variants have changed their reactivity to other anti-HN monoclonal antibodies in either hemagglutination inhibition or neuraminidase inhibition tests but not both. (iii) Virions of one ts mutant of Sendai virus are temperature sensitive in hemagglutinating activity but not neuraminidase activity (29, 30). (iv) Halide ions (17) and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (29) inhibit the neuraminidases of simian virus 5 and Sendai virus, respectively, without affecting hemagglutinating activities. Therefore, it seems possible that the hemagglutinating and neuraminidase activities may reside at different sites on the HN protein molecule.

On the other hand, since virus adsorbs to erythrocytes via a cell receptor which includes sialic acid and can elute by cleaving the sialic acid from that receptor, it seems reasonable that there would be a single functional site on the HN protein molecule responsible for both hemagglutinating and neuraminidase activities. Our data seem to support this idea since all of the group B, C, and BC mutants, as well as the revertants of BC2, displayed similar stabilities for all functions. Actually, this pattern of similar stabilities in all HN protein functions was also found for members of one complementation group of Sendai virus ts mutants presumably with lesions in the gene for the HN protein (1) and also for the hemagglutinating and neuraminidase activities of two strains of NDV (27).

Although all of these thermostability studies support the single-site model of HN protein functions, an alternative interpretation must be considered. The thermosensitive area of the HN protein molecule might not be in functional areas at all, but rather in structural areas of the molecule. The integrity of this structural area may be required for all HN protein functions. In fact, we have already presented evidence that the group B, BC, and C mutants package less HN protein into their virions and that these virions have proportionally lower HN protein activities at either the permissive or nonpermissive temperature (23), probably indicating that the HN protein alterations involve areas other than functional areas of the HN protein molecule.

In any case, our results confirm the conclusions reached previously from viral fractionation (14, 21) and antibody inhibition studies (17, 37) that the HN protein is required not only in hemagglutinating and neuraminidase activities but also in hemolytic and infectious activities.

The stabilities of AV-WT hemolyzing and infectious activities are salt dependent. Both of these activities are relatively stable in PBS and 10 to 20 times less stable at low salt concentra-

tions. This salt dependence is not found with the hemagglutinating activity of either AV-WT virions or the ts mutants. A virion component other than HN protein (or possibly another region of HN protein) required for hemolysis is unstable under low-salt conditions. The fact that the stabilities of both hemolytic and infectious activities are salt dependent is consistent with the requirement of a hemolysis-like activity in infectivity, probably for virion entry or uncoating in cells. On the other hand, the salt-dependent stability of infectivity might also reflect the sensitivity of another virion function(s) required for infectivity, such as the virion polymerase.

We previously found that the group D mutant virions like AV-WT-like hemagglutinating and neuraminidase activities but lower specific infectious and hemolytic activities, suggesting mutations in a gene for a protein other than HN protein, which is required for hemolytic and infectious activities (23, 24). Consistent with this was our finding that the stabilities of the hemagglutinating and neuraminidase functions of these group D mutants were similar to the stabilities of AV-WT. However, despite the reduced hemolytic activity of the group D mutants, the remaining activity is about as stable as that of AV-WT. In contrast, members of one complementation group of Sendai virus mutants, although stable in hemagglutinating and neuraminidase activities, are all unstable in their hemolyzing activities (1).

The finding that the infectivities of two of the group D mutants are even more sensitive to heating than AV-WT infectivity under low-salt conditions may indicate a change in a virion function (other than HN protein functions) required for infectivity, possibly membrane-fusing activity. We cannot directly examine the stabilities of the fusing activity of AV-WT virions since AV-WT is not able to cause fusion from without (5). In addition, we cannot quantify the sensitivity of hemolytic activity to heating in low-salt buffer since even AV-WT loses its hemolytic activity under these conditions. We are currently looking for conditions under which AV-WT hemolytic activity is stable to heating in low-salt buffer so that the stabilities of AV-WT and group D mutant infectious and hemolyzing activities can be compared. A correlation between decreased stabilities of infectious and hemolytic activities in D1 and D2 would establish a functional marker for the lesion in these mutants.

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LITERATURE CITED

1. Adachi, A., T. Kanda, and H. Shibuta. 1980. Isolation and characterization of temperature-sensitive mutants of Sendai virus. *Microbiol. Immunol.* 24:1053-1068.
2. Aminoff, D. 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* 81:384-391.
3. Blattler, D. P., F. Garner, K. Van Slyke, and A. Bradley. 1972. Quantitative electrophoresis in polyacrylamide gels. *J. Chromatogr.* 64:147-155.
4. Bratt, M. A., and L. A. Clavell. 1971. Hemolytic interaction of Newcastle disease virus and chicken erythrocytes. I. Quantitative comparison procedure. *Appl. Microbiol.* 23:454-460.
5. Bratt, M. A., and W. R. Gallaheer. 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus. *Proc. Natl. Acad. Sci. U.S.A.* 64:536-543.
6. Bratt, M. A., and L. E. Hightower. 1977. Genetics and paragenetic phenomena of paramyxoviruses, p. 457-533. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 9. Plenum Publishing Corp., New York.
7. Choppin, P. W., and R. W. Compans. 1975. Reproduction of paramyxoviruses, p. 95-178. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 4. Plenum Publishing Corp., New York.
8. Clavell, L. A., and M. A. Bratt. 1972. Hemolytic interaction of Newcastle disease virus and chicken erythrocytes. II. Determining factors. *Appl. Microbiol.* 23:461-470.
9. Deutsch, V., and A. Berkalo. 1971. Analyse d'un mutant thermolabile du virus de la stomatite vésiculaire (VSV). *Ann. Inst. Pasteur (Paris)* 121:101-106.
10. Hanson, R. P., and J. Spalatin. 1978. Thermostability of the hemagglutinin of Newcastle disease virus as a strain marker in epizootologic studies. *Avian Dis.* 22:659-665.
11. Hightower, L., and M. A. Bratt. 1974. Protein synthesis in Newcastle disease virus-infected chicken embryo cells. *J. Virol.* 13:788-800.
12. Homma, M., and M. Ohuchi. 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural differences of Sendai viruses grown in eggs and tissue culture cells. *J. Virol.* 12:1457-1465.
13. Horsfall, F. L., Jr., and I. Tamm. 1953. Fractional dilution procedure for precise titration of hemagglutination viruses and hemagglutination-inhibiting antibodies. *J. Immunol.* 70:253-259.
14. Hsu, M. C., A. Scheid, and P. W. Choppin. 1979. Reconstitution of membranes with individual paramyxovirus glycoproteins and phospholipid in cholate solution. *Virology* 95:476-491.
15. Lomniczi, B. 1975. Thermostability of Newcastle disease virus strains of different virulence. *Arch. Virol.* 47:249-255.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
17. Merz, D. C., A. Scheid, and P. W. Choppin. 1981. Immunological studies of the functions of paramyxovirus glycoproteins. *Virology* 109:94-105.
18. Moore, N. F., and D. C. Burke. 1974. Characterization of the structural proteins of different strains of Newcastle disease virus. *J. Gen. Virol.* 25:275-289.
19. Nagai, Y., and H.-D. Klenk. 1977. Activation of precursors to both glycoproteins of Newcastle disease virus by proteolytic cleavage. *Virology* 77:125-134.
20. Nagai, Y., H.-D. Klenk, and R. Rott. 1976. Proteolytic

- cleavage of the viral glycoproteins and its significance for virulence of Newcastle disease virus. *Virology* 72:494-508.
21. Ozawa, M., A. Asano, and Y. Okada. 1979. Biological activities of glycoproteins of HVJ (Sendai virus) studied by reconstitution of hybrid envelope and by concanavalin A-mediated binding: a new function of HANA protein and structural requirement for F protein in hemolysis. *Virology* 99:197-202.
 22. Peeples, M. E., and M. A. Bratt. 1982. UV irradiation analysis of complementation between, and replication of, RNA-negative temperature-sensitive mutants of Newcastle disease virus. *J. Virol.* 41:965-973.
 23. Peeples, M. E., and M. A. Bratt. 1982. Virion functions of RNA⁺ temperature-sensitive mutants of Newcastle disease virus. *J. Virol.* 42:440-446.
 24. Peeples, M. E., J. P. Gallagher, and M. A. Bratt. 1981. Permissive temperature analysis of RNA⁺ temperature-sensitive mutants of Newcastle disease virus, p. 567-572. In D. H. L. Bishop and R. W. Compans (ed.), *The replication of negative strand viruses*. Elsevier/North-Holland Publishing Co., New York.
 25. Peeples, M. E., L. L. Rasenas, and M. A. Bratt. 1982. RNA synthesis by Newcastle disease virus temperature-sensitive mutants in two RNA-negative complementation groups. *J. Virol.* 42:996-1006.
 26. Picken, J. C., Jr. 1964. Thermostability of Newcastle disease virus, p. 167-188. In R. P. Hanson (ed.), *Newcastle disease virus, an evolving pathogen*. The University of Wisconsin Press, Madison.
 27. Pierce, J. S., and A. M. Haywood. 1973. Thermal inactivation of Newcastle disease virus. I. Coupled inactivation rates of hemagglutinating and neuraminidase activities. *J. Virol.* 11:168-176.
 28. Portner, A. 1981. Evidence for two different sites on the HN glycoprotein involved in neuraminidase and hemagglutinating activities, p. 465-470. In D. H. L. Bishop and R. W. Compans (ed.), *The replication of negative strand viruses*. Elsevier/North-Holland Publishing Co., New York.
 29. Portner, A. 1981. The HN glycoprotein of Sendai virus: analysis of site(s) involved in hemagglutinating and neuraminidase activities. *Virology* 115:375-384.
 30. Portner, A., P. A. Marx, and D. W. Kingsbury. 1974. Isolation and characterization of Sendai virus temperature-sensitive mutants. *J. Virol.* 13:298-304.
 31. Scheid, A., and P. W. Choppin. 1973. Isolation and purification of the envelope proteins of Newcastle disease virus. *J. Virol.* 11:263-271.
 32. Scheid, A., and P. W. Choppin. 1974. Isolation of paramyxovirus glycoproteins and identification of their biological properties, p. 177-201. In R. D. Barry and B. W. J. Mahy (ed.), *Negative strand viruses*. Academic Press, Inc., London.
 33. Scheid, A., M. C. Graves, S. M. Silver, and P. W. Choppin. 1978. Studies on the structure and functions of paramyxovirus glycoproteins, p. 181-193. In B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press, Inc., New York.
 34. Smith, G. W., and L. E. Hightower. 1980. Uncoupling of the hemagglutinating and neuraminidase activities of Newcastle disease virus (NDV), p. 623-632. In B. N. Fields, R. Jaenisch, and C. F. Fox (ed.), *Animal virus genetics*. Academic Press, Inc., New York.
 35. Smith, G. W., and L. E. Hightower. 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. *J. Virol.* 37:256-267.
 36. Smith, G. W., and L. E. Hightower. 1982. Revertant analysis of a temperature-sensitive mutant of Newcastle disease virus with defective glycoproteins: implication of the fusion glycoprotein in cell killing and isolation of a neuraminidase-deficient hemagglutinating virus. *J. Virol.* 42:659-668.
 37. Tozawa, H., M. Watanabe, and N. Ishida. 1973. Structural components of Sendai virus. Serological and physicochemical characterization of hemagglutinin subunit associated with neuraminidase activity. *Virology* 55:242-253.
 38. Tsipis, J. E., and M. A. Bratt. 1976. Isolation and preliminary characterization of temperature-sensitive mutants of Newcastle disease virus. *J. Virol.* 18:848-855.
 39. Weiss, S. R., and M. A. Bratt. 1974. Polyadenylate sequences on Newcastle disease virus mRNA synthesized in vivo and in vitro. *J. Virol.* 13:1220-1230.
 40. Westbury, H. A. 1979. Newcastle disease virus—some properties of Australian strains. *Avian Dis.* 23:564-570.