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

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Biological and molecular properties of a temperature-sensitive mutant (C1) of Newcastle disease virus and its revertants were analyzed. C1 exhibited three temperature-sensitive alterations (plaque formation, virion assembly, and cytopathogenicity) and several defects which were also present at the permissive temperature. C1 virions contained low amounts of hemagglutinin-neuraminidase glycopolypeptides and consequently were deficient in hemagglutinating and neuraminidase activities. These virions also contained defective fusion glycoproteins which rendered them poorly hemolytic and slow to penetrate cultured chicken embryo cells. The biological activities of the membrane glycoproteins were recovered sequentially in a series of plaque-forming revertants. The coreversion of hemolysis, membrane-penetrating activities, and cytopathogenicity in the first-step revertant (S1) suggested that fusion glycoproteins were major contributors to cellular destruction. This revertant also provided evidence of a role for fusion glycoproteins in virion assembly. From S1 we isolated a large-plaque-forming revertant (L1) that assembled wild-type amounts of biologically active hemagglutinin-neuraminidase glycoproteins into virions. Although it was normal for hemagglutination, L1 had less than 3% of the neuraminidase activity of the wild type, demonstrating that these two activities can be uncoupled genetically. The neuraminidase deficiency of L1 did not impair its virulence in ovo or its reproduction in cultured cells.

The envelope of the avian paramyxovirus Newcastle disease virus (NDV) contains two types of biologically active glycoproteins which form physically (36) and antigenically (4, 39) distinct spikes on the surfaces of virions (reviewed in references 11 and 14). One population of spikes is composed of hemagglutinin-neuraminidase glycoproteins (HN) which attach virions to cellular receptors, an activity that is conveniently assayed by agglutination of erythrocytes. HN are also responsible for neuraminidase activity (36), which releases virions from neuraminic acid-containing receptors and which may have a role in membrane fusion (21). A second population of spikes contains the disulfide-linked fusion glycopolypeptides F_1 and F_2 ($F_{1,2}$), which are derived by proteolytic cleavage from F_0 precursor molecules (37). These spikes confer membrane fusion and hemolytic activities on virions and are required for efficient penetration of host cells (29). Cells that accumulate HN and $F_{1,2}$ in plasma membranes during infection acquire hemadsorbing and neuraminidase activities (reviewed in reference 11) and may also undergo fusion from within (FFWI) (7).

We have studied the biological activities of the glycoproteins of virulent NDV strain AV (Australia-Victoria, 1932) with temperature-sensitive (*ts*) mutants originally isolated by Tsipis and Bratt (reviewed in references 8 and 44). Some of these mutants have been partially characterized (32, 44). Each independently isolated mutant was temperature sensitive for plaque formation at a nonpermissive temperature of 41.8°C. The mutants were classified as either RNA⁺ or RNA⁻ based on their ability to induce virus-specific RNA synthesis in infected cells at 41.8°C. Four complementation groups (B, C, BC, and D) of RNA⁺ mutants and two complementation groups (A and E) of RNA⁻ mutants have been described.

A representative mutant from complementation group C was chosen for revertant analysis. *ts* mutant C1 exhibited a variety of phenotypic alterations which were probably due to mutations in both of the envelope glycoprotein genes. The biological activities of fusion glycoproteins (F) and HN were sequentially recovered in a series of plaque-forming revertants. The coreversion of the altered properties of the mutant

provided insights into the roles that each glycoprotein plays during infection.

(A preliminary account of this work was presented at the ICN-University of California at Los Angeles Symposium on Animal Virus Genetics held in Keystone, Colo., in 1980 [41].)

MATERIALS AND METHODS

Viruses and cell culture. The RNA⁺ mutant C1, derived from strain AV by nitrosoguanidine mutagenesis (44), was obtained from M. A. Bratt (University of Massachusetts Medical School, Worcester). For establishing working stocks, viruses were cloned in chicken embryo (CE) cells, amplified in 10-day-old embryonated chicken eggs (SPAFAS), and purified as previously described (13, 18).

Secondary cultures of CE cells were prepared as previously described (10, 13). Unless stated otherwise, the cultures were infected at input multiplicities of 3 PFU per cell.

Assays of hemagglutination, neuraminidase activity, and hemolysis. Egg-grown virions were further purified by equilibrium density ultracentrifugation as previously described (42). Protein determinations were done by the method of Lowry et al. (25).

Viral agglutination of chicken erythrocytes at 4°C was quantified by the pattern method. End-point dilutions were read at 4 h and did not change for at least 20 h at 4°C. Hemagglutinating units were expressed as the reciprocal of the highest dilution capable of agglutinating the erythrocytes.

Neuraminidase assays were performed by the thio-barbituric acid method with fetuin or neuramin lactose as the substrate (3). Released *N*-acetylneuraminic acid (NANA) was measured spectrophotometrically and quantified with NANA standard curves. For identifying viral plaques containing neuraminidase activity, the chromogenic substrate 2-(3'-methoxyphenyl)-*N*-acetyl- α -neuraminic acid (Research Resources Branch, National Institute of Allergy and Infectious Diseases) and a diazonium salt of 4-amino-2,5-dimethoxy-4'-nitroazobenzene (Koch-Light Laboratories, Ltd.) were used as previously described (31).

Hemolytic activities of the purified virions were determined by the quantitative comparison procedure described by Bratt and Clavell (6).

Hemadsorption and neuraminidase assays of infected-cell surfaces. Infected or mock-infected CE cells were incubated for 10 h at 37.5 or 42.5°C. For hemadsorption assays, we used techniques similar to those described by Carver and Marcus (9). Washed plates received 2 ml of 2% chicken erythrocytes in phosphate-buffered saline and were incubated for 15 min at 4°C. The plates were washed several times with phosphate-buffered saline. CE cells and attached erythrocytes were then lysed in 3 ml of 0.03 M NH₄OH, and the hemoglobin concentration was determined spectrophotometrically at 540 nm (12). The amount of hemadsorption to the mock-infected cells was subtracted from the levels determined for the infected cells.

For neuraminidase assays, plates were washed twice with 0.4 M sodium acetate buffer (pH 5.2). Next, 0.5 ml of fetuin (12.5 mg/ml in the same buffer) was added to each plate, and incubation was continued for

1 h at 37.5°C on a rocker platform. Released NANA was assayed as described above for virions.

Viral penetration of host cells. A total of 100 to 200 PFU were added to CE cell cultures and allowed to adsorb for 1 h at 4°C. Control experiments showed that the adsorption of the wild type (wt) and mutants was complete by this time. The cultures were then shifted to 37.5°C. After various incubation periods, replicate cultures received partially purified anti-NDV immunoglobulin in concentrations which inactivated 95% of the infectivity of the inoculum during a 10-min incubation period at 37.5°C. After 10 min of antibody treatment, the medium was removed from the cultures, plaque assay medium was added, and incubation was continued for 3 days at 37.5°C. The percentages of PFU which survived antibody inactivation were calculated by comparisons with cultures that received no antiserum.

Radioisotopic labeling of virions. At 2 h postinfection, the cultures were washed twice with wash medium (minimal essential medium containing 5% [0.75 mg/liter] of the normal concentration of methionine and 5% dialyzed calf serum) and then incubated for 15 h at 37.5°C in the same medium supplemented with 25 μ Ci of [³⁵S]methionine (1,100 Ci/mmol; New England Nuclear Corp.) per ml. The medium was collected from the cultures, and cellular debris was removed by differential centrifugation. The virions were centrifuged for 1 h at 83,000 $\times$ *g* through a pad of 20% (wt/vol) sucrose in phosphate-buffered saline and were then collected in gel sample buffer for storage at -20°C.

SDS-PAGE. The radioisotopically labeled virions and infected cells were dissolved in gel sample buffer (42) and heated to 100°C for 3 min. Virion proteins were separated on 11.5% polyacrylamide slab gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Laemmli (23). Electrophoresis was performed at a constant current of 20 mA for 16 h. For analyses of cell extracts, 6% gels were subjected to electrophoresis at 20 mA for 7.5 h. The gels were acid fixed and prepared for fluorography as described by Laskey and Mills (24). Dried gels were exposed to Kodak XR-5 X-ray film for 1 to 5 days at -70°C. The fluorograms were scanned with a densitometer.

Preparation of antisera. Rabbits received weekly intravenous injections of UV-inactivated strain AV which had been purified by equilibrium density ultracentrifugation. After 5 weeks of injections, the rabbits were bled, and the immunoglobulin G fraction was recovered from the serum by ammonium sulfate precipitation (15). This fraction contained antibodies to all of the major viral proteins (unpublished data). The hemagglutination inhibition titer (320 U/ml) was determined as described by Maeno et al. (26).

RESULTS

The phenotype that was originally used to isolate C1 was the capacity to form plaques at 37.5°C but not at 41.8°C (44). In our experiments, a temperature of 42.5°C was required to block plaque formation. For isolating revertants, CE cells were infected at limiting dilutions with a cloned stock of C1, overlaid with plaque assay

medium, and incubated at 42.5°C for 3 days. Putative revertant plaques were picked, and the resulting inocula were injected into embryonated chicken eggs which were incubated at 37.5°C to produce amplified virus stocks. The isolates were plaque purified between egg passages. A stable revertant (S1) which formed tiny plaques at 42.5°C was identified in the first round of isolation (Fig. 1). A spontaneously arising second-step revertant (L1) which formed large plaques at 42.5°C was obtained from a cloned stock of S1. L1 was subsequently found to have a major deficiency in neuraminidase activity, allowing the isolation of a third type of revertant (N1) which had partially regained this activity. This type of revertant was identified by the addition of the chromogenic neuraminidase substrate 2-(3'-methoxyphenyl)-*N*-acetyl- α -neuraminic acid and a diazonium salt to agar overlays after plaque assays at 37.5°C (31). The formation of dark-red plaques indicated the presence of neuraminidase activity.

***ts* phenotypes of C1.** The relative plaque sizes of wt, C1, and each of the C1 revertants on CE cells at permissive and nonpermissive temperatures are compared in Table 1. C1 formed smaller plaques than wt at 37.5°C and did not form plaques at 42.5°C. Full plaque-forming capacity at 42.5°C was restored in two steps. At the nonpermissive temperature the first-step revertant S1 formed tiny plaques, whereas the second-step revertant L1 formed plaques that were the same size as wt plaques. The wt and the two large-plaque-forming revertants generated slightly larger plaques at the nonpermissive temperature than at the permissive temperature.

The single-cycle yields of infectious virus in CE cells at both temperatures are shown in Table 1. The permissive-temperature yields correlated well with the plaque sizes at 37.5°C. Although all viruses showed reduced yields of infectious virus at 42.5°C, the relative ratios of

the yields at 42.5°C to the yields at 37.5°C indicated that C1 was particularly temperature sensitive for infectious-particle production. Based on the yield ratios, we concluded that this *ts* phenotype was repaired in S1. Both L1 and the neuraminidase revertant N1 gave higher yields of infectious virus than did wt at 42.5°C.

C1 was also temperature sensitive for virion assembly. An electron microscopic examination of infected cells revealed physical particles budding from plasma membranes at 37.5°C but not at 42.5°C (data not shown).

Cytopathic effects (CPE) in cultured CE cells were determined by light microscopy for wt, C1, and the revertants. With the exception of C1, each of the viruses listed in Table 1 caused CPE in cell cultures at both incubation temperatures. C1 was temperature sensitive for this phenotype. C1 caused disruption of cells at 37.5°C but not at 42.5°C, even after 20 h of incubation. In contrast, CPE were detected in wt-infected cultures at 10 h of incubation.

Defects of C1 repaired in the first-step revertant S1. We examined the biological activities associated with the F of purified wt, C1, and revertant virions produced in ovo at 37.5°C. The relative hemolytic activities of the viruses are summarized in Table 2. C1 had less than 2% of wt hemolytic activity at the permissive temperature. At this temperature S1 virions demonstrated a 20-fold increase in hemolytic activity over C1 virions, whereas the large-plaque-forming revertants showed additional twofold increases in activity.

F are needed for efficient penetration of host cells by NDV (30). Therefore, the relative rates of penetration of CE cells, measured as the escape of wt, C1, and S1 from antibody neutralization, provided another indicator of F activity. Wt virions rapidly escaped neutralization when the infected cultures were shifted from 4 to 37.5°C (Fig. 2). After a 20-min incubation peri-

FIG. 1. Procedure for the isolation of plaque-forming revertants from *ts* mutant C1. The relative sizes of plaques formed by each virus on CE cells incubated at permissive (upper row) and nonpermissive (lower row) temperatures are shown. C1 was isolated after nitrosoguanidine (NTG) mutagenesis of wt AV (44). Details of the isolation of revertants are given in the text. Spontaneously occurring small (S1)- and large (L1 and N1)-plaque-forming revertants were amplified in embryonated chicken eggs (○) and plaque purified between egg passages. N1, which had partially regained viral neuraminidase activity, was identified in a plaque assay of L1 by the addition of the chromogenic neuraminidase substrate 2-(3'-methoxyphenyl)-*N*-acetyl- α -neuraminic acid (MPN) to the agar overlay (31). Plaques containing the active enzyme turned dark red (●).

TABLE 1. Phenotypic characteristics

Virus	Relative plaque size at:		11-h yield ^a (PFU per cell) at:		CPE ^b at:		MDT ^c
	37.5°C	42.5°C	37.5°C	42.5°C	37.5°C	42.5°C	
wt	○	○	7.2	1.0 (0.14)	+++	+++	41
C1	○	○	0.61	0.01 (0.02)	++	—	75
S1	○	○	0.64	0.12 (0.19)	++	++	71
L1	○ ^d	○	8.9	7.5 (0.84)	++	+++	39
N1	○ ^d	○	6.3	4.9 (0.78)	++	+++	38

^a Viruses (multiplicity of infection, 3) were adsorbed to cells for 1 h at 4°C. The cells were washed five times and then incubated for 11 h at the indicated temperatures. Virus released into the medium was titrated by plaque assay at 37.5°C. These values supersede earlier results (41). Numbers in parentheses represent the yield at 42.5°C divided by the yield at 37.5°C.

^b CE cell cultures were infected (multiplicity of infection, 5 PFU per cell) and incubated for 20 h at 37.5 or 42.5°C. Cells were fixed with 95% methanol, stained with Giemsa stain, and examined by light microscopy for CPE. CPE included loss of cells from the plate, presence of vacuoles, disruption of parallel array, and formation of thin, refractile cells.

^c Embryonated eggs were injected with 10³ PFU and candled periodically during the course of incubation at 37.5°C. The time in hours postinfection when one-half of the embryos were dead was the mean embryo death time (MDT). An average of 36 eggs were used for each determination.

^d L1 and N1 produced cloudy plaques at 37.5°C. Vital staining revealed viable cells within the plaques.

od, approximately 50% of the adsorbed virus was resistant to antibody neutralization, and virtually all of the infectious virus penetrated the cells within 1 h. In contrast, C1 escaped neutralization much more slowly. Only 50% of the adsorbed virus had penetrated the cells by 2 h. The rate of C1 penetration of CE cells was slightly faster at 42.5°C than at 37.5°C, indicating that C1 virions produced at 37°C were not temperature sensitive for penetration (data not shown). S1 had an intermediate rate of penetration, which is consistent with its partial recovery

of hemolytic activity. Approximately 50% of the adsorbed S1 virions escaped neutralization by 1 h of incubation, half the time required for an equivalent amount of C1 to penetrate cells.

Strain AV induces FFWI in CE cells; however, neither C1 nor any of its revertants was capable of inducing FFWI at either 37.5 or 42.5°C. Therefore, this property could not be

TABLE 2. Biological activities of purified virions^a

Virus	Hemagglutination (HAU ^b per µg of protein)	Neuraminidase concn (nM) ^c	Hemolysis	
			µg of protein per ml ^d	% of wt activity
wt	1,024	7.4	3.5	100
C1	64	3.3	280	1.3
S1	64	3.2	13	27
L1	1,024	0.2	7.0	50
N1	1,024	2.6	6.0	58

^a Virions were grown in 10-day-old embryonated chicken eggs at 37.5°C and purified by equilibrium gradient ultracentrifugation.

^b HAU, Hemagglutinating units.

^c NANA released from fetuin per microgram of viral protein per hour at 37.5°C and pH 5.2. Values were reproducible to within ±0.1 nM. Similar results were obtained when neuraminidase served as the substrate (unpublished data).

^d Virus concentrations required to lyse 10% of a 4% (vol/vol) suspension of chicken erythrocytes in 1 h at 37.5°C. The procedure of Bratt and Clavell (6) was used for quantitative comparison of hemolytic activities.

FIG. 2. Rates of penetration of wt (●), C1 (○), and S1 (□) virions into CE cells. Virus was adsorbed to cultures at 4°C and then warmed to 37.5°C to initiate penetration. After various periods of incubation, anti-viral antibody was added to replicate cultures to neutralize virus which had not entered the cells. Surviving infectious viruses were scored by plaque assay after removal of antibody. The incubation times shown include the 10-min antibody treatments.

used to assess the possible temperature-sensitive nature of altered F.

To find molecular changes in F, we analyzed by SDS-PAGE the assembly into virions of proteins synthesized by wt-, C1-, and S1-infected cells (Fig. 3). The relative amounts of radioactive $F_{1,2}$ in virions were quantified by liquid scintillation counting of dissolved gel slices. Duplicate infected cultures were incubated for 7 h at 37.5°C and were then divided for subsequent incubations at either 37.5 or 42.5°C. In wt infections, all of the major types of viral structural proteins synthesized during a short radioisotopic labeling period at 42.5°C were represented in virions released from the cells during the subsequent chase period. However, the relative amount of $F_{1,2}$ incorporated into virions at 42.5°C was only about 25% of that incorporated into virions at 37.5°C (data not shown), indicating that the accumulation of wt $F_{1,2}$ in virions was temperature sensitive. The pattern of proteins incorporated into virions by C1-infected cells at 42.5°C was aberrant. Few or no newly synthesized envelope proteins (HN dimer, $F_{1,2}$, and M) were assembled. The radioactive nucleocapsid proteins, NP and P, that were detected in released virions presumably were associated with unlabeled envelope components synthesized before the shift to 42.5°C. S1 showed a partial recovery of the wt pattern, which is consistent with its regained ability to produce infectious virus at 42.5°C (Table 1). Newly syn-

FIG. 3. Densitometer scans of fluorograms of [^{35}S]methionine-labeled polypeptides assembled into virions released from wt-, C1-, and S1-infected cells. Infected cultures were incubated at 37.5°C for 7 h, shifted to 42.5°C, labeled for 30 min, and then incubated for 2 h in medium supplemented with a 10-fold excess of nonlabeled methionine (150 mg/liter). Virions were collected by ultracentrifugation and dissolved in gel sample buffer without 2-mercaptoethanol for analysis by SDS-PAGE under nonreducing conditions. The NP regions of the scans of the C1 and S1 polypeptide patterns were normalized to wt NP; the gel origins are shown on the left.

FIG. 4. Thermostability of infectivity. Virus stocks were diluted to 5×10^7 PFU per ml of phosphate-buffered saline and incubated at 52°C. Samples were removed at the indicated times and diluted 10-fold into ice-cold minimal essential medium. The amount of infectious virus in each sample was determined by plaque assay at 37.5°C. Symbols: ●, wt; ○, C1; □, S1; △, L1; ▲, N1.

thesized envelope components, as well as nucleocapsid proteins, were detected in released S1 virions. S1-infected cells assembled about one-third of the relative amount of HN into virions as did wt-infected cells. As described below, this result was expected. An unexpected result was that S1-infected cells incorporated three times more $F_{1,2}$ into virions relative to other proteins than did wt-infected cells. In control experiments, wt-, C1-, and S1-infected cells assembled similar relative amounts of $F_{1,2}$ into virions at 37.5°C.

Tsipis and Bratt showed that C1 infectivity is more rapidly inactivated by heat than is wt infectivity (44). We compared the thermostabilities of wt, C1, and revertant virions to determine whether this phenotype was repaired as a discrete step in any of the revertants. The results shown in Fig. 4 confirmed the earlier observations on the sensitivity of C1 virions to heat. Each of the revertants, however, was almost as stable as wt. Thus, normal thermostability of infectivity was regained in the first-step revertant S1. The repair of this phenotype correlated with the partial recovery of hemolytic and cell-penetrating activities in S1, suggesting that defective F may have been responsible for the thermolabile phenotype of the C1 parent.

Non-temperature-sensitive defects of C1 repaired in the second-step revertant L1. We also

examined the biological activities associated with the HN of purified wt, C1, and revertant virions produced in ovo at 37.5°C. The C1 parent and the small-plaque-former S1 were deficient in both hemagglutinating and neuraminidase activities, even at the permissive temperature (Table 2). In the transition from S1 to L1, hemagglutinating activity was restored to wt levels; however, additional neuraminidase activity was lost. L1 virions contained less than 3% of wt neuraminidase activity. N1, which was originally identified as a neuraminidase-positive plaque in the 2-(3'-methoxyphenyl)-*N*-acetyl- α -neuraminic acid assay, demonstrated a 10-fold increase in enzyme activity over its parent L1 while maintaining wt levels of hemagglutinating activity.

To determine whether any aspect of the synthesis and assembly of mutant HN into a biologically active form was temperature sensitive, we assayed the surfaces of infected cells incubated at 37.5 and 42.5°C for hemadsorbing and neuraminidase activities. The adsorption of chicken erythrocytes to cells at 10 h postinfection served as a measure of attachment function. Additional cell layers were fixed and incubated with fetuin, and the NANA released by cell-bound neuraminidase was assayed. Neither activity had significant temperature-sensitive characteristics in C1- or revertant-infected cells (Table 3).

To find alterations in HN which might account for the observed biological defects of C1 and the revertants, purified [³⁵S]methionine-labeled virions grown in cultured CE cells at 37.5°C were analyzed by SDS-PAGE under reducing conditions. Densitometer scans of virion polypeptides which were separated on gels are shown in Fig. 5. Compared with wt, the small-plaque-forming viruses C1 and S1 contained less HN in virions relative to other polypeptides. The amount of HN in these viruses was about 35% of that in wt, based on either quantification of areas under the peaks or determination of radioactivity in the gel. This quantity agreed well with the levels of

FIG. 5. Densitometer scans of fluorograms of [³⁵S]methionine-labeled virion polypeptides separated by SDS-PAGE under reducing conditions. Migration was from left to right. The dashed line marks the position of wt HN relative to the faster-migrating HN of the mutants. The portion of each scan containing 50- to 80-kilodalton polypeptides is shown.

neuraminidase activity in virions (Table 2). Virions of both large-plaque-forming revertants contained wt levels of HN. Although the relative quantities of HN in virions varied among C1 and the revertants, each had HN that exhibited greater electrophoretic mobilities on gels than did wt HN. Preliminary results obtained by Peeples and co-workers suggested that an alteration in the polypeptide, rather than in the carbohydrate moiety, was responsible for the altered electrophoretic mobility of mutant HN (32).

The reduced levels of HN present in C1 and S1 virions might have been due to lower-than-normal rates of synthesis, increased degradation of the polypeptides, or an assembly defect. To evaluate the nature of the defect, we pulse-labeled cells infected with wt, C1, or L1 with

TABLE 3. Biological activities of infected cells^a

Virus	Hemadsorption		Neuraminidase	
	37.5°C	42.5°C	37.5°C	42.5°C
wt	1.0	1.4	1.0	1.1
C1	0.15	0.14	0.11	0.07
S1	0.18	0.20	0.10	0.11
L1	0.80	1.0	0.01	0.01
N1	0.78	0.92	0.13	0.10

^a Values are expressed as fractions of the activities of wt-infected cells incubated at the permissive temperature. Results represent averages of several determinations. The contribution of adsorbed input virus, assayed at 1 h postinfection, was negligible.

radioactive methionine at 37.5°C. Cultures were incubated at the permissive temperature in medium containing excess nonlabeled methionine. The polypeptide compositions of cells collected after various chase periods were analyzed on polyacrylamide gels, and the amounts of radioactive HN in gel slices were plotted as a function of chase time (Fig. 6). The rates of synthesis of HN were approximately the same in wt-, C1-, and L1-infected cells, since the amounts of radioactively labeled HN in the cells immediately after pulse-labeling were similar for each virus (data not shown). During the chase, HN was lost from infected cells with first-order kinetics. The half-life of newly synthesized HN in C1-infected cells (1.8 h) was approximately one-third of that of HN in wt- and L1-infected cells (5.8 h). The magnitude of the loss of the HN of C1 from infected cells was too large to be due to the incorporation of HN into virions. Only about 1% of the viral proteins synthesized in NDV-infected cultures are assembled into virus particles (19), and soluble forms of HN comparable to the soluble G glycoprotein of vesicular stomatitis virus (22) have not been detected (unpublished data). Thus, the HN of C1 appeared to be metabolically unstable in infected cells. Similar results were obtained with cells incubated at the nonpermissive temperature (data not shown), which is consistent with the accumulation of low levels of hemadsorbing and neuraminidase activities in C1-infected cells at both temperatures (Table 3). The stabilization of HN correlated with the restoration of wt levels of the glycoproteins in L1-infected cells and virions.

An additional altered phenotype of C1 which coreverted with hemagglutinating activity in the large-plaque formers was virulence for chicken embryos. The three large-plaque formers (wt, L1, and N1) had shorter mean embryo death times than did the two small-plaque formers (C1 and S1) (Table 1). Similar relationships were found when infected eggs were incubated at 42.5°C, although death times were prolonged for each virus, suggesting that the conditions for infection were suboptimal.

DISCUSSION

Our results indicated that both the HN and F of C1 were partially defective and that both contributed to the variety of altered biological properties of this mutant. Based on indirect evidence, we concluded that the alteration responsible for temperature-sensitive plaque formation, CPE, and virus production by C1 was in the F. All of these *ts* phenotypes coreverted with F activities. However, in the absence of a direct measurement of temperature-sensitive fusion activity or a temperature-sensitive molecular

FIG. 6. Metabolic stabilities of HN. At 7.5 h postinfection, wt (●)-, C1 (○)-, and L1 (△)-infected CE cells were pulse-labeled for 20 min with [³⁵S]methionine (25 μ Ci per ml of medium) at 37.5°C. The cultures were then washed and incubated in medium containing excess nonradioactive methionine. After the chase periods indicated, cell extracts from replicate cultures were prepared and analyzed by SDS-PAGE. HN bands were located by fluorography and excised from the gel, and radioactivity in the dissolved slices was quantified in a liquid scintillation spectrometer. The host cell background was determined by normalization as described previously (18) and subtracted from the total counts to obtain the virus-specific radioactivity. The amount of radioactivity present in HN after each chase period was plotted as the percentage of HN present immediately after the pulse-labeling. Error bars represent standard deviations ($n = 3$).

property of F, this point has not been proved beyond doubt.

Several lines of evidence suggested that C1 had defective F. C1 virions had less than 2% of wt hemolytic activity. S1 showed a 20-fold improvement in hemolytic activity relative to C1, with no change in HN functions. The full extent of the recovery of fusion activity could not be assessed directly in S1 because, like C1, it carried an HN defect that affected attachment, a function required for hemolysis (reviewed in reference 20). Additional increases in the hemolytic activities of the large-plaque-forming revertants, which had regained wt levels of hemagglutination, were probably due to improved attachment of virions to erythrocytes. Overall, at least 50% of the wt levels of hemolytic activity was restored.

Results from cell penetration experiments corroborated the hemolysis data, providing additional evidence of an F defect in C1. The rate of escape of infectious virus from antibody neutralization, presumably by the fusion of viral envelopes with host plasma membranes, was mark-

edly reduced in C1 as compared with wt. S1 showed a substantial improvement in cell-penetrating activity. HN function(s) affects the rate of penetration in this type of experiment, even though initial attachment is carried out at 4°C (unpublished data); therefore, the measurement of penetrating activity by S1, which contains one-third of the wt number of HN molecules, is a minimum estimate of recovery.

C1-infected cells developed hemadsorbing and neuraminidase-containing plasma membranes but showed no budding virions at the nonpermissive temperature. Since S1-infected cells produced virions, it is likely that F are involved in the budding process directly or in an earlier step required for budding, such as the binding of M or nucleocapsids to modified plasma membranes. Both HN and F are transmembrane proteins, at least in Sendai virus (5), and thus have the potential to interact with internal components of virions. RNA⁺ *ts* mutants of Sendai virus which direct the assembly of virions containing F but few HN molecules have been described (1, 34). Taken together, these results suggest that F, but not HN, may be required for virion assembly.

Molecular evidence of a change in F in the transition from C1 to S1 came from analyses of the assembly of proteins into virions at the nonpermissive temperature. The F of S1 accumulated to much higher relative levels in virions than did the F of C1 or even wt. Although indirect effects of other repaired gene products on F assembly are possible, the simplest explanation for these data and for the recovery of hemolytic and cell-penetrating activities measured at the permissive temperature is that a reverting mutation in the F gene restored activity to the F of S1 relative to C1 and improved the assembly of F into virions at 42.5°C well beyond wt levels. The behavior of F during virion assembly in wt- and S1-infected cells provides a possible explanation for the lower wt yields of PFU at 42.5°C than at 37.5°C and for the higher-than-wt yields of L1 and N1 at 42.5°C (Table 1). A reduced accumulation of wt F in virions produced at 42.5°C could result in a decrease in the specific infectivity of virions or in the release of fewer particles. The full effect on the virus yield of the change in the incorporation of F into virions which arose at the S1 step would not be realized until full recovery of viral attachment function occurred in the large-plaque-forming revertants L1 and N1.

C1-infected cells did not exhibit CPE over the normal time course of lytic wt infections in CE cells at 42.5°C. S1 was cytopathic at the nonpermissive temperature, suggesting that F may be involved in cell killing. In the past, F have been linked to the destruction of cells and tissues

because of their role in the cell-to-cell spread of infection by viral penetration of cells or by FFWI (17, 28, 30). The mechanism by which F might cause cell destruction during a single cycle of infection is unknown; however, the mechanism does not appear to involve cell-to-cell fusion, since S1 was not capable of inducing FFWI at detectable levels.

Portner et al. (34) found that Sendai *ts* mutant 271 was less cytopathic than wt. Since mutant-infected cells accumulated reduced amounts of HN, these glycoproteins were implicated in cell killing. However, the mechanism of cell killing need not be the same for NDV and Sendai virus, which have very different time courses of infection and cell death, and F may not be the only paramyxoviral proteins involved in cell killing. It may be pertinent that the uncleaved F₀ precursor is the primary intracellular form of the F of Sendai virus (and of avirulent NDV strains), whereas intracellular cleavage occurs during virulent NDV infection (40). Activation by intracellular proteolytic cleavage might be a prerequisite for cell damage mediated by F.

Bratt and co-workers have reported that C1 has a thermolabile hemagglutinin (32). Our C1 mutant and its S1 revertant both had thermolabile hemagglutinins, despite a major recovery in the thermostability of infectivity of S1. Therefore, the defect repaired in the C1-to-S1 transition, presumably a defect in thermolabile F, was the major contributor to the thermolability of the infectivity of C1. A comparison of the properties of the independently prepared working stocks of C1 from our laboratory and from the laboratory of Bratt and co-workers showed similar HN defects but differences in F activities. Presumably these differences arose during the recloning and amplification of the stocks (unpublished data).

In addition to defects in F, the cell-to-cell spread of C1 infection was further restricted by a non-temperature-sensitive defect in the HN. C1 virions had low hemagglutinating and neuraminidase activities, and the surfaces of infected cells had low HN activities at both permissive and nonpermissive temperatures. Analyses of virion polypeptide compositions showed that C1 and S1 virions contained about one-third of the amount of HN found in wt virions. A corresponding reduction in neuraminidase activity in these mutants indicated that the amount, but not the specific activity, of this enzyme was significantly reduced in mutant virions. The HN synthesized in C1-infected cells were metabolically unstable. Half-life determinations of radioactively labeled HN showed that those of C1-infected cells turned over three times more rapidly than newly synthesized HN of wt-infected cells. Whether the threefold increase in the

degradation of HN in C1- and S1-infected cells was the cause or the effect of their accumulation to one-third of normal levels in virions and presumably on cell surfaces remains to be clarified. The metabolic instability of HN was repaired in the second-step revertant L1. Wt levels of the glycoproteins were assembled into L1 virions, and hemagglutinating activity was completely restored. L1-infected cells had wt levels of hemadsorbing activity at both permissive and nonpermissive temperatures.

We have found that many *ts* mutants of strain AV belonging to the other RNA⁺ complementation groups (B, BC, and D) also contain metabolically unstable HN (unpublished data). Our results, which show that most of the RNA⁺ *ts* mutants of NDV contain reduced levels of HN in virions, with corresponding reductions in hemagglutinating and neuraminidase activities, agree with those of Peeples et al. (32). Previous studies by Thiry (43) revealed that many of the NDV mutants generated by hydroxylamine and nitrous acid form small plaques on cultured cells and that several of these mutants have hemagglutinin deficiencies. Apparently, there is a relatively high probability of isolating mutants of NDV that are defective in HN when either conditional or nonconditional changes in plaque sizes are scored. Among RNA⁺ *ts* mutants of Sendai virus, the single mutant identified by Portner et al. (33) had an HN defect, and most of the 14 mutants isolated by Adachi et al. (1) had deficiencies in HN functions. Therefore, the relatively frequent isolation of HN mutants may not be unique to NDV.

The conformational change which must have occurred in the stabilization of the HN of L1 severely reduced neuraminidase activity. L1 virions and plasma membranes of cells infected by this revertant contained less than 3% of the wt levels of the enzyme. Despite the elimination of 97% of wt neuraminidase activity, L1 made large plaques and produced infectious virus in CE cells at both 37.5 and 42.5°C. Interestingly, L1 was also as virulent as wt for chicken embryos. It is possible that neuraminidase activity is not required for efficient reproduction of NDV in cultured cells or embryonated eggs. Alternatively, strain AV may have a large excess of neuraminidase, and the residual 3% in L1 may be adequate.

Several groups have demonstrated correlations between plaque size among NDV strains and virulence for either embryos or adult birds (16, 35, 38). Each of the revertants showed the appropriate increases in virulence in ovo as plaque sizes increased. Neuraminidase phenotypes, on the other hand, did not correlate with virulence; large differences in enzyme activities among the large-plaque formers had little effect

on mean embryo death times. Previous attempts to correlate neuraminidase content of virions and virulence yielded contradictory results (2, 27). In our studies, the recovery of hemagglutinating activity, not neuraminidase activity, in the large-plaque-forming revertants appeared to be the more important factor in determining virulence in ovo. Presumably, the increased attachment function allowed more efficient cell-to-cell spread of infection.

The genetic uncoupling of hemagglutinating and neuraminidase activities has provided additional opportunities for studying the functional interactions of HN with cells. The effects of the L1 neuraminidase deficiency on attachment and elution properties of NDV will be described elsewhere.

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