Mutation in the Matrix Protein of Newcastle Disease Virus Can Result in Decreased Fusion Glycoprotein Incorporation into Particles and Decreased Infectivity

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Virus particles produced in eggs by the group D *ts* mutants of Newcastle disease virus at permissive temperature display low infectious and hemolytic activities (M. E. Peeples and M. A. Bratt, *J. Virol.* 42:440-446, 1982). These lower activities correlate with a decreased incorporation of F₁₊₂ (fusion glycoprotein) into virus particles, compared with that for wild type. The incorporation of F₁₊₂ into virus particles of the group D mutants is also lower than that for wild type when grown in chicken embryo cells in culture at either permissive or nonpermissive temperature. The infectivity of virions from these mutants correlates with the amounts of F₁₊₂ in the virus particles, below a certain concentration, indicating that the quantity of F₁₊₂ in virus particles is a determining factor in the infectivity of those particles. In addition, one of these mutants, D1, produces an M (matrix protein) which migrates at a faster rate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three of four revertants of D1 have coreverted to wild-type M electrophoretic mobility, associating M with the *ts* lesion and the other observed phenotypes. In each of these revertants, as well as in three revertants each from D2 and D3, there has been coreversion from the low specific infectious and hemolytic activities to greater, and often wild-type, activities. There is also a coreversion for F₁₊₂ incorporation into virions. All of the revertants incorporate F₁₊₂ into virions more efficiently than their mutant parents. The coreversions associate those phenotypes with the *ts* lesion and, in the case of D1, with the M lesion as well.

Newcastle disease virus (NDV), an avian paramyxovirus, has a single-stranded genome encoding six major proteins found both in infected cells and virions. The NP (nucleocapsid protein), P (phosphoprotein), and L (large protein) are located in the nucleocapsid with the genomic RNA and are involved in RNA synthesis. The two glycoproteins, F (fusion) and HN (hemagglutinin-neuraminidase), as well as a non-glycosylated M (matrix protein), are associated with the plasma membrane of the infected cell and the membrane of the virion. HN serves as the attachment glycoprotein and also possesses neuraminidase activity. F is synthesized as a precursor, F₀, which is cleaved to yield the disulfide-linked F₁₊₂. As it is for Sendai virus (3, 13) and other members of the family Paramyxoviridae (15), the cleavage of F is required for NDV to function in hemolysis, cell-to-cell fusion, and an early event in infection (14, 23, 25).

M presumably lies between the lipid membrane and the nucleocapsid. Its function(s) is not well understood, but it has been implicated (i) as a possible control element in viral RNA synthesis for another paramyxovirus, Sendai virus (19), and the rhabdovirus vesicular stomatitis virus (VSV) (5, 7, 30, 37), (ii) in recognition of altered cell membrane by viral nucleocapsids during maturation of VSV (21), (iii) in interactions with actin (10), (iv) in interactions with both the nucleocapsid and glycoprotein of Sendai virus (39), and (v) possibly in the organization of membrane glycoproteins (38).

Of the six complementation groups of *ts* mutants originally isolated from the wild-type Australia-Victoria strain (AV-WT) of NDV (35), five have been assigned to the genes for three of the proteins: the RNA⁻ group A has been assigned

to the L gene (17, 26), the RNA⁻ group E has been assigned to the P gene (26), and three RNA⁺ groups (B, C, and BC) have all been assigned to the HN gene (27-29; M. E. Peeples, R. Glickman, J. P. Gallagher, and M. A. Bratt, manuscript in preparation). The remaining RNA⁺ group, D, which complements the B, C, and BC group mutants (as well as the RNA⁻ mutants), has no detectable problem with its HN and therefore probably represents the gene for either M or F.

Virions of the group D mutants are less infectious and less hemolytic than AV-WT (the wild-type virus from which the mutants were derived [27]) and all the other RNA⁺ mutants. In addition, cells infected with the group D mutants, although able to fuse from within at permissive temperature, are unable to do so at nonpermissive temperature (35). These properties of the group D mutants as well as other properties described herein and elsewhere would be consistent with their possessing lesions in the gene for F. However, the finding that mutant D1 produces an M of altered electrophoretic migration (28) raises the possibility that the originally selected *ts* phenotype is a result of an alteration in the gene for M.

In this report, cells infected by the group D mutants and virus particles released from them have been examined. Slower production of virus particles possessing reduced specific infectivity has been correlated with the altered plaque-forming ability of these viruses. Also, lower specific infectivity of virus grown in cell culture at nonpermissive temperature and of virus grown in eggs at permissive temperature is correlated with lower F₁₊₂ content of these virus particles.

The question of whether the altered migration of M is caused by the same lesion that causes the *ts* phenotypes or, alternatively, whether it reflects a second, unrelated lesion

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has been addressed by the isolation and characterization of revertants. All 10 revertants regained or approached wild-type levels of biological activities. In addition, three of four *ts*⁺ revertants of mutant D1 produced an M which coreverted to the AV-WT migration rate. Thus, it seems likely that the gene for M contains the *ts* lesion represented in the group D mutants and that this is responsible for the defective F₁₊₂-related phenotypes.

MATERIALS AND METHODS

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 tissue culture dishes were used for plaque titrations (4) and for radioactive labeling of infected cell proteins or virus.

Virus. The *ts* mutants were isolated from the Australia-Victoria (1932) strain of NDV (4) by Tsipis and Bratt (35). AV-WT and *ts* mutant virus stocks were grown in the allantoic sac of 10-day-old embryonated hen eggs at 37°C. After the death of the majority of embryos (46 to 64 h), allantoic fluid was harvested, and virus was concentrated and purified by differential centrifugation, including density banding in linear sucrose gradients as described previously (6, 36). Stocks were stored at -70°C and titrated by plaquing at permissive (37.5°C) or nonpermissive temperature (41.8°C).

Radioactive labeling of cells. Cell cultures were infected at a multiplicity of infection of 10 and incubated with standard medium. Six hours after the start of infection, the cultures were washed with Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) and labeled for 1 h with 20 µCi of [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml as described by Hightower et al. (12). Cultures were washed with HBSS, lysed in gel sample buffer (0.125 M Tris-hydrochloride [pH 6.8], 20% glycerol, 6% sodium dodecyl sulfate [SDS], 0.0001% bromophenol blue), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Radioactive labeling of virions from cell culture. Cell cultures in 100-mm dishes were infected at a multiplicity of infection of 10 at room temperature, washed with HBSS, and incubated with 10 ml of standard medium at either 37.5 or 41.8°C. At 4 h postinfection, the cultures were washed with HBSS and incubated with 3 ml of labeling medium containing 100 µCi of [³⁵S]methionine (Amersham) per ml. At 8 h after infection, the labeling medium was replaced with fresh labeling medium. At 12 h after infection, the medium was removed and pooled with the 8-h yield. Virus was purified by centrifugation, including a low-speed spin and a step gradient as described previously (36). The interface was collected from the bottom of the tube with a peristaltic pump and sampled for infectivity and trichloroacetic acid-precipitable radioactivity. The interface fraction was then diluted in HBSS and pelleted by centrifugation at 39,000 rpm in a Spinco SW41 rotor. The pellet was suspended in sample buffer for SDS-PAGE.

Radioactive labeling of virions from eggs. Approximately 1,000 PFU of virus in 0.1 ml of HBSS was injected into the allantoic sac of 10-day-old embryonated hen eggs. One day later, 0.3 mCi of [³⁵S]methionine in 0.1 ml of HBSS was also injected into the allantoic sac. The eggs were refrigerated, and the allantoic fluid was harvested 48 h after infection; the virus was purified as described above for virions from cell culture.

SDS-PAGE. Radioactively labeled infected cell or virion

proteins were displayed by SDS-PAGE and detected by autoradiography. Samples disrupted in the gel sample buffer described above, but lacking 2-mercaptoethanol (non-reduced), were electrophoresed in slab gels by the method of Laemmli (16). Samples disrupted in sample buffer which also included 10% 2-mercaptoethanol (reduced) were electrophoresed in slab gels by a variation of the Laemmli method (1). The gels were dried and exposed to Kodak X-Omat AR film. The autoradiograms were scanned with an Ortek densitometer, and the areas under the peaks were integrated with a Wang digitizer.

Revertant isolation. To minimize the possibility of multiple isolations of the same revertant, mutant stocks were plaqued at low density at permissive temperature, and single clones were then selected by removing an agar plug over the plaque, dispersing the plug in 0.5 ml of HBSS, and injecting 0.1 ml into one embryonated hen egg. After the death of the embryos, allantoic fluid was removed, and infectivity titers were determined at both permissive and nonpermissive temperatures by the plaque assay. Plaques formed at 41.8°C were selected and cloned several times at nonpermissive temperature, and a single revertant from each original permissive-temperature clone was chosen for study.

Hemolytic activity. Egg-grown virus stocks were assayed for hemolytic activity 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 required to hemolyze 20% of the chicken erythrocytes was determined, and the inverse of this value was used for comparison (27).

Antiserum. Phosphate-buffered saline (0.5 ml) containing 200 µg of AV-WT was UV irradiated for 15 min at a distance of 78 cm from a Sylvania G15T8 germicidal lamp to destroy its infectivity (average of 50 hits), emulsified with 0.5 ml of complete Freund adjuvant, and injected subcutaneously and intramuscularly into a rabbit. Two weeks later, the injections were repeated. Ten days after the second injection, blood was removed by cardiac puncture, and the serum was isolated, heat inactivated (30 min at 56°C), and stored at -20°C.

RESULTS

Growth and plaquing ability. At permissive temperature, *ts* mutant D2 produced plaques similar in size to those of AV-WT, and *ts* mutants D1 and D3 produced somewhat smaller plaques (Fig. 1). Figure 2A shows that in single cycles of infection at permissive temperature, mutant D2 produced infectious virus with kinetics similar to those of AV-WT; the kinetics of D1 and D3 infectious virus production appear to be somewhat delayed, and slightly lower (usually two- to three-fold) maximum yields were reached. Delayed production of infectious virus and lower maximum yields could, in the multiple cycles of replication required to produce plaques, result in smaller plaque size.

At nonpermissive temperature, AV-WT produces plaques (albeit somewhat smaller ones than those at permissive temperature) (data not shown), and the group D mutants produce no plaques (10³ to 10⁴ fewer than at permissive temperature) (35). The kinetics of infectious virus production at nonpermissive temperature by AV-WT were similar to those at permissive temperature, but the maximal amount of infectious virus was approximately six-fold lower (Fig. 2). This lower yield could account for the smaller size of AV-WT plaques at this temperature. For the group D mutants at nonpermissive temperature, the kinetics of infectious virus production were considerably slower, and the maximal

amount of virus produced was reduced ca. 60-fold. With so little infectivity being produced by infected cells, in retrospect it seems reasonable that these mutants do not produce plaques at nonpermissive temperature.

Virus particle production. To determine whether the lower yield of infectious virus, especially at nonpermissive temperature, might be due to the production of fewer virus particles, or possibly to the production of equivalent amounts of virus particles with lower specific infectivity, cell cultures were infected, incubated at either permissive or nonpermissive temperature, and metabolically labeled with [35 S]methionine. Table 1 shows the quantification of virus particles, and the infectivities associated with them, which have accumulated in the fluids of these cultures by 12 h. The mutant-infected cells released 50 to 69% as much label into virus particles as did AV-WT-infected cells at permissive temperature and 38 to 100% as much at nonpermissive temperature. We assume that the amount of label incorporated into virus particles provides a reasonable estimate of the amount of virus particles. Therefore, although the lower infectivity yields produced by mutant-infected cells at permissive temperature (Fig. 2 and Table 1) might be due to a somewhat lower production of virus particles, the dramatically lower yields produced at nonpermissive temperature cannot be due solely to decreased virus particle production.

Table 1 also shows the relative infectivities and specific infectivities of the virus particles released from mutant-infected cells. At permissive temperature, similar amounts of infectivity were produced in mutant D1-, D2-, and AV-

WT-infected cells. Mutant D3-infected cells produced a somewhat lower infectivity. In contrast to permissive temperature, infectivity released from mutant-infected cells at nonpermissive temperature was only 4 to 24% that observed in AV-WT infection. More importantly, the specific infectivity of the virus particles produced by these mutants at nonpermissive temperature was only ca. 20% that of AV-WT. (The fact that the infectivity produced by mutant-infected cells at nonpermissive temperature is not as low in these experiments as in the experiment presented in Fig. 2 may be related to the fact that infectivity remaining due to the inoculum was neutralized by antiserum in Fig. 2 but not in the experiments presented in Table 1.) Thus, it seems likely that the combination of slightly lower virus particle production and lower specific infectivity accounts for the lower yields of infectious virus from the mutant-infected cells at nonpermissive temperature.

Proteins of cell culture-grown virus particles. To determine whether a gross difference in the amount of a particular virion protein incorporated into virus particles might account for the reduced plaquing abilities and specific infectivities of virus produced by mutant-infected cells at nonpermissive temperature, the protein compositions of radioactively labeled virus particles were examined. Figure 3 shows these proteins displayed by SDS-PAGE under reducing conditions, and Table 2 shows their quantification from these gels. The amounts of both HN and M in mutant virus particles were quite similar to those of AV-WT, regardless of the temperature of production, with the exception of the HN

FIG. 1. Relative plaque sizes at permissive temperature. Chicken embryo cells in 60-mm culture dishes were infected with AV-WT (A), D1 (B), D2 (C), or D3 (D) and overlaid with agar in a plaque assay. Dishes were incubated at 37.5°C for 3 days and photographed with indirect lighting.

FIG. 2. Viral growth curves. Two 35-mm dishes of cells were infected with AV-WT (●), D1 (▲), D2 (■), or D3 (▼) at a multiplicity of infection of 5 at room temperature for 45 min, washed with HBSS, and incubated with 1 ml of standard medium for 30 min at 37.5°C to allow penetration to occur. The medium was replaced with 0.5 ml of a 1:50 dilution of rabbit anti-AV-WT serum to neutralize the remaining inoculum. One plate infected with each virus was incubated at 37.5°C, and the other was incubated at 41.8°C for 20 min. The diluted serum was removed, the cells were washed with HBSS, and 2 ml of standard medium was added to each dish, which was again incubated at either 37.5 or 41.8°C. At 4, 6, 8, 10, and 12 h after infection, 10- μ l samples were withdrawn from each dish, diluted into 1 ml of HBSS with 2% calf serum, and frozen at -20°C. Viral infectivity was titrated at 37.5°C. The virus produced at 37.5°C is shown in panel A, and that produced at 41.8°C is shown in panel B.

content of group D mutants, which is greater than that of AV-WT at nonpermissive temperature. Mutant B2, included as a control in Table 2, is defective in its HN gene (27, 28) and is known to package less HN into these cell culture-grown virus particles, as previously reported for egg-grown virus (27). In addition, with the exception of M of D1, the relative migration rates of all of the viral proteins in mutant virus particles are similar to those of AV-WT produced under similar conditions. As seen previously (28), M of D1 migrates more rapidly (corresponding to an apparent molecular mass difference of ca. 1,000 fewer daltons) than M of AV-WT, D2, or D3.

It is interesting that the HN packaged into both AV-WT and mutant virus particles at permissive temperature migrates more rapidly than that packaged at nonpermissive temperatures. These differences appear to reflect differences in carbohydrate composition (Peeples et al., in preparation).

The amount of F₁₊₂ in virus particles cannot be deter-

mined from these gels because under reducing conditions, the larger species, F₁, migrates with and is obscured by NP, and the smaller species, F₂, is not retained in the gel. To separate F₁₊₂ from NP, virus particle proteins were electrophoresed under nonreducing conditions (Fig. 4) in which F₁₊₂ migrates as a doublet at a rate similar to F₀. (In these gels, NP migrates as a triplet, P migrates both as a monomer in the NP region and a trimer near the top of the gel, HN migrates as a dimer, also near the top of the gel [22, 31], and M migrates near its normal position as a smear rather than as a discrete species.) Clear-cut differences in the amounts of F₁₊₂ can be seen among these virus particle preparations, particularly when they have been quantified as in Table 2.

Compared with AV-WT particles (or those of mutant B2, which possesses a mutation in the HN gene), those particles produced by the group D mutants at permissive temperature have less F₁₊₂ (49 to 67% of the levels for AV-WT) (Table 2). The amount of F₁₊₂ in the group D mutant virus particles

TABLE 1. Virus particles and infectivities released from infected cells^a

Virus	Physical properties			Infectivity			Specific infectivity		
	A	B	C	A	B	C	A	B	C
AV-WT	1.0	0.37 $\pm$ 0.15	1.0	1.0	0.22 $\pm$ 0.13	1.0	1.0	0.43 $\pm$ 0.11	1.0
D1	0.50 $\pm$ 0.16	0.15 $\pm$ 0.02	0.38 $\pm$ 0.03	0.63 $\pm$ 0.27	0.010 $\pm$ 0.003	0.044 $\pm$ 0.018	1.20 $\pm$ 0.22	0.071 $\pm$ 0.027	0.20 $\pm$ 0.010
D2	0.69 $\pm$ 0.10	0.39 $\pm$ 0.07	1.00 $\pm$ 0.24	0.90 $\pm$ 0.23	0.060 $\pm$ 0.025	0.24 $\pm$ 0.012	1.20 $\pm$ 0.18	0.12 $\pm$ 0.05	0.21 $\pm$ 0.06
D3	0.51 $\pm$ 0.12	0.24 $\pm$ 0.07	0.64 $\pm$ 0.19	0.36 $\pm$ 0.11	0.032 $\pm$ 0.018	0.15 $\pm$ 0.08	0.65 $\pm$ 0.18	0.086 $\pm$ 0.035	0.17 $\pm$ 0.09

^a Trichloroacetic acid-precipitable radioactivity, infectivity, and infectivity-to-radioactivity ratio of virus (produced in chicken embryo cells at each temperature) relative to AV-WT grown at 37.5 or 41.8°C in the same experiment. Values are the average of three to four experiments $\pm$ standard deviation. (A) 37.5°C virus/37.5°C AV-WT; (B) 41.8°C virus/37.5°C AV-WT; (C) 41.8°C virus/41.8°C AV-WT.

FIG. 3. Reduced SDS-PAGE of [35 S]methionine-labeled virions grown in chicken embryo cells at 37.5°C (p) or 41.8°C (n).

correlates with their plaque size at permissive temperature: D2 incorporates the most F_{1+2} of the three mutants and makes the largest plaques, and D1 and D3 incorporate less F_{1+2} and make smaller plaques. Therefore, both virus particle production and the F_{1+2} content of these particles correlate with permissive-temperature plaque size.

In nonpermissive-temperature infection, AV-WT incorporates less F_{1+2} into its virus particles than at permissive temperature; this also correlates with a somewhat smaller plaque size. The group D mutants incorporate nearly undetectable amounts of F_{1+2} into virus particles at nonpermissive temperature, correlating with their inability to produce plaques.

The dramatically lower amounts of F_{1+2} in virus particles of group D mutants produced at nonpermissive temperature also correlate with the lower specific infectivities of these virus particles. However, the effect of lower amounts of F_{1+2} in permissive-temperature-grown virus particles, compared with those in AV-WT infection, is not reflected in lower specific infectivity. This finding with cell culture-grown virus is particularly puzzling because permissive-temperature-grown virus of the group D mutants produced in eggs has a lower specific infectivity than AV-WT (27). The quantity of F_{1+2} in egg-grown virus particles had not previously been determined, due to the low efficiency of staining of F_{1+2} with Coomassie blue.

Proteins of egg-grown virus particles. To resolve this specific infectivity discrepancy, which is dependent on the source of the virus, AV-WT and the group D mutants were produced in both eggs and tissue culture at permissive temperature in the presence of [35 S]methionine, and the

relative amounts of M, HN, and F_{1+2} were compared in Table 3. For each virus and each source, the virus particles contained similar amounts of M. In contrast, egg-grown AV-WT virus contained less of both viral glycoproteins, HN and F_{1+2} . Egg-grown virus of the three group D mutants and mutant B2 as well contained amounts of HN similar (although considerably less than cell culture-grown virus) to the lower amount in egg-grown AV-WT. Egg-grown viruses of both AV-WT and mutant B2 contained relatively less F_{1+2} than their cell culture-grown counterparts. However, egg-grown group D mutants showed greatly reduced amounts of F_{1+2} . Thus, the lower specific infectivity (and hemolytic activity) of egg-grown group D mutant virus (27) correlates with the 20%-or-less incorporation of F_{1+2} compared with that of AV-WT (Table 3).

Coreversion of infectious and hemolytic activities with plaquing at nonpermissive temperature. To determine whether the phenotypes and genotypes described above for these mutants are related to their *ts* lesions, spontaneously occurring revertants were isolated. A total of 10 revertants were isolated from the three group D mutants on the basis of their ability to form plaques at nonpermissive temperature (ts^+). This is indicated by their relative efficiencies of plating (Table 4). All 10 have also regained the specific infectious activity of AV-WT and at least part of their specific hemolytic activity (Table 5). Coreversion of these two functions with ts^+ plaquing indicates that the lower infectious and hemolytic activities of egg-grown virus are related and that both phenotypes are related to the *ts* lesions.

Coreversion of M migration with ts^+ plaquing. The revertants isolated from *ts* mutant D1 were of particular interest since coreversion of M migration from a position corresponding to 40,000 daltons, like that of the mutant, to one corresponding to 41,000 daltons, like that of AV-WT, would suggest a connection between M and the phenotypes described above. In extracts from infected cells (data not shown) and in virus particles released from them (Fig. 5), it is clear that M protein reverted to AV-WT-like migration in three of four D1 revertants. Only M of D1R3 retains a migration rate comparable to that of its mutant parent. Of these four revertants, all produce large plaques at nonpermissive temperature except for D1R3 which, unlike its mutant parent, produces small plaques (Table 4). D1R3, therefore, appears to have reverted only partially in its ability to produce AV-WT-like plaques at nonpermissive temperature; this may be related to its apparently unchanged M.

Coreversion of incorporation of F_{1+2} with ts^+ plaquing. None of the group D mutants is altered in its ability to incorporate HN or M into virions (33, 34). It is apparent from

TABLE 2. Amounts of M, HN, and F_{1+2} in chicken embryo cell-grown virus^a

Virus	M			HN			F_{1+2}		
	A	B	C	A	B	C	A	B	C
AV-WT	1.0	0.73 ± 0.05	1.0	1.0	0.57 ± 0.04	1.0	1.0	0.36 ± 0.09	1.0
D1	0.79 ± 0.10	0.86 ± 0.17	1.2 ± 0.23	0.91 ± 0.09	0.98 ± 0.24	1.7 ± 0.3	0.49 ± 0.20	0.021 ± 0.036	0.062 ± 0.094
D2	0.90 ± 0.30	0.87 ± 0.15	1.2 ± 0.15	0.94 ± 0.02	0.93 ± 0.21	1.6 ± 0.3	0.67 ± 0.15	0.050 ± 0.018	0.094 ± 0.071
D3	0.81 ± 0.08	0.76 ± 0.05	1.0 ± 0.10	0.91 ± 0.09	0.81 ± 0.10	1.4 ± 0.1	0.51 ± 0.11	0.037 ± 0.025	0.095 ± 0.079
B2	0.78	0.51	0.72	0.14	0.11	0.18	1.1	0.34	0.94

^a The amounts of M, HN, F_{1+2} , and NP+P in each preparation were determined by scanning autoradiograms (2) of SDS-PAGE like those presented in Fig. 3 and 4. The ratio of M, HN, or F_{1+2} to NP was determined for each condition and is presented here relative to that of AV-WT in the same experiment. Values are the average of three to four experiments ± standard deviation except for B2, which represents a single experiment. M was quantified from both reduced and nonreduced SDS-PAGE, HN was quantified from reduced SDS-PAGE, and F_{1+2} was quantified from nonreduced SDS-PAGE. (A) 37.5°C virus/37.5°C AV-WT; (B) 41.8°C virus/37.5°C AV-WT; (C) 41.8°C virus/41.8°C AV-WT.

FIG. 4. Nonreduced SDS-PAGE of [^{35}S]methionine-labeled virions grown in chicken embryo cells at 37.5°C (p) or 41.8°C (n).

Fig. 5 that revertants of D1 incorporate amounts of HN and M similar to their mutant parent D1 and to AV-WT at both permissive and nonpermissive temperatures, as do all revertants of D2 and D3 (data not shown). The ability of the revertants to incorporate the disulfide-linked F_{1+2} was determined from SDS-PAGE under nonreducing conditions (Fig. 6). The amounts of F_{1+2} in the revertant virus particles are quantified in Table 6. All of the revertants incorporate F_{1+2} more efficiently than their mutant parents: three revertants incorporate F_{1+2} in a pattern similar to AV-WT (D1R4, D1R5, and D2R3), but the other seven have become temperature insensitive. Unlike AV-WT, which incorporates less F_{1+2} into virus particles at nonpermissive temperature, these seven revertants incorporate F_{1+2} equally well at both permissive and nonpermissive temperatures.

The specific infectivities of virus produced in cell culture at both temperatures are also presented in Table 6. As described previously, the specific infectivities in group D mutants grown in cell culture at permissive temperature are similar to AV-WT. Revertant virus grown at permissive temperature is also similar to AV-WT. However, the specific infectivities of the revertants grown at nonpermissive temperature have increased, between 2-fold (D1R5) and 20-fold (D3R2), over those of their mutant parents; there is, therefore, a rough correlation between the ability to incorporate F_{1+2} at nonpermissive temperature and its infectivity. The fact that all of the revertants do not regain AV-WT-like specific infectivity when grown at nonpermissive temperature despite AV-WT-like (or better) incorporation of F_{1+2} suggests that they are not true revertants (pseudorevertants) and that a problem other than incorporation of F_{1+2} still exists for them.

DISCUSSION

We have examined plaque-forming ability, ability to produce physical and infectious particles, and the biochemical makeup of virus particles produced under three conditions by *ts* group D mutant-infected cells. Analysis of 10 revertants of these mutants indicates that the *ts* defect affects the incorporation of F_{1+2} into virus particles. In addition, analysis of the revertants of mutant D1 indicates that its lesion is in M, implying a transmembranal interaction of M and F_{1+2} at some point(s) during viral maturation.

We have found at least two factors which play a role in determining the differences in plaque-forming ability and plaque size of the group D mutants and AV-WT: physical particle production and infectivity of those particles. Virus particle infectivity is related to the incorporation of F_{1+2} as will be discussed below. At permissive temperature, mutants D1 and D3 produced infectious virus more slowly and with a lower final yield than AV-WT (and mutant D2) due to lower physical particle production; this correlates with a smaller plaque size. One possible explanation for the slower production of viruses might be that they enter the cell more slowly; these mutants, in fact, have lower hemolytic activities (16), supposedly an *in vitro* correlate of penetration into a cell.

At nonpermissive temperature, each of the three mutants produced infectious virus more slowly than at permissive temperature and with 60-fold lower final yields than AV-WT. This slower production of infectious virus by the mutants correlates with their inability to form plaques at the higher temperature.

Although the group D mutant-infected cells produced less infectivity, they produced virus particles with at least 40% the efficiency of AV-WT at both permissive and nonpermissive temperatures. At permissive temperature, these mutant virus particles contained less F_{1+2} than AV-WT (49% to 67%); at nonpermissive temperature, these virus particles contained very little F_{1+2} (less than 10% as much as in AV-WT). It thus appears that little, if any, F_{1+2} is required for the assembly of virus particles. By studying another *ts* mutant of AV-WT, Smith and Hightower (34) reached an apparently contrary conclusion, namely, that F_{1+2} is required for the assembly of virus particles. They found that their stock of mutant C1 had not only the HN lesion described by Peebles and Bratt (27) but also an additional lesion, presumably in the gene for F; however, no physical evidence for an altered F was presented. One explanation is that although a full complement of F_{1+2} is not required for virus particle assembly, a small amount is required. Another explanation is that a critical mass of glycoprotein molecules is required for particle formation and may be supplied by either HN or F_{1+2} . A lesion in both glycoproteins could

TABLE 3. Amounts of M, HN, and F_{1+2} in virus grown in chicken embryo cells and eggs at permissive temperature^a

Virus	M			HN			F_{1+2}		
	A	B	C	A	B	C	A	B	C
AV-WT	1.0	1.2	1.0	1.0	0.19	1.0	1.0	0.39	1.0
D1	1.1	0.82	0.67	0.97	0.18	0.94	0.50	0.080	0.20
D2	1.1	1.1	0.93	0.81	0.23	1.2	0.86	0.058	0.15
D3	1.1	0.62	0.50	0.81	0.14	0.74	0.58	0.061	0.15
B2	0.91	1.3	1.0	0.22	0.12	0.62	1.5	0.50	1.3

^a The ratios of M, HN, and F_{1+2} to NP+P in each virus preparation were determined as described in footnote a of Table 2 and are presented here relative to those of AV-WT. (A) cell-grown virus/cell-grown AV-WT; (B) egg-grown virus/cell-grown AV-WT; (C) egg-grown virus/egg-grown AV-WT.

diminish the quantity of both to the point that maturation was impossible.

It is interesting that at nonpermissive temperature, at which almost no F_{1+2} is incorporated into group D mutant virus particles, more HN is incorporated, compared with AV-WT. Possibly, the drastically lower F_{1+2} content of these virus particle membranes allows more room for or necessitates the incorporation of HN.

Mutations, temperature of replication, and source of virus have all been examined in this report; each can affect both the amount of F_{1+2} in the virus particles produced and the infectivity of these particles. Under conditions in which F_{1+2} has been incorporated at low efficiency (20% or less than that of AV-WT for cell-grown virus from nonpermissive temperature or for egg-grown virus from permissive temperature), the virus particles have a lower specific infectivity. It appears that a critical number of F_{1+2} molecules may be required for infectivity. However, under conditions in which F is incorporated more efficiently, the correlation does not hold; virus particles of group D mutants are as infectious as those of AV-WT when produced at permissive temperature in cell culture, despite the fact that they contain only 49 to 86% (Tables 2 and 3) as much F_{1+2} . There are at least two possible explanations. (i) Cell-grown virus produced at permissive temperature may have an overabundance of F_{1+2} , such that the 50% packaging of F_{1+2} is still enough to render particles as infectious as those of AV-WT; when fewer than a critical or threshold number of F_{1+2} molecules are packaged, the virus particles are less infectious. (ii) Comparable infectious subpopulations of virus with similar protein content may exist in AV-WT and the group D mutant virus particles, but if noninfectious subpopulations with much lower F_{1+2} content existed within the mutant stocks, they would lower the average amount of F_{1+2} in virus particles. It is also possible that the lower infectivity of egg-grown group D mutant viruses might simply reflect possession of reduced amounts of F_{1+2} in a background of reduced HN content.

TABLE 4. Plaque efficiency at permissive and nonpermissive temperatures

Virus	PFU/ml $\times 10^9$ at:		EOP (41.8°C/ 37.5°C) ^a
	37.5°C	41.8°C	
AV-WT	20	27	1.4
D1	7.0 (s) ^b	0.0011	1.6×10^{-4}
D1R1	16	16	1.0
D1R3	34	22 (s)	0.65
D1R4	26	25	0.96
D1R5	28	30	1.1
D2	16	0.0037	2.3×10^{-4}
D2R1	40	56	1.4
D2R2	41	47	1.1
D2R3	52	54	1.0
D3	5.8 (s)	0.0097 (s)	1.7×10^{-3}
D3R1	15	22	1.5
D3R2	50	50	1.0
D3R3	40	44	1.1

^a EOP, Efficiency of plaquing. Values represent the efficiency of one stock of each virus in forming plaques at 41.8°C relative to 37.5°C.

^b (s), Production of plaques smaller than those produced by AV-WT.

TABLE 5. Specific infectious and hemolytic activities of egg-grown virus

Virus	PFU/ μ g of protein ^a $\times 10^6$	1/20% hemolysis ^b $\times 10^{-2}$
AV-WT	4.5	32
D1	1.0	7.1
D1R1	4.8	37
D1R3	4.5	26
D1R4	5.9	18
D1R5	4.8	15
D2	0.91	7.0
D2R1	6.2	13
D2R2	6.2	17
D2R3	6.5	10
D3	0.97	7.8
D3R1	4.0	20
D3R2	5.3	20
D3R3	5.0	20

^a PFU (at 37.5°C) in a purified stock divided by the protein concentration of that stock.

^b Hemolytic activity of each virus expressed as the inverse of the amount of virus (micrograms of protein) required to hemolyze 20% of the chicken erythrocytes.

Elsewhere (27, 28), we have shown that the B, C, and BC mutants produce virus particles with as little as 5% of the normal complement of the other glycoprotein, HN. Interestingly, the virus particles of these mutants, which all possess proportionally reduced HN functions, are just as infectious as those of AV-WT. Thus, it appears that reduced HN content per se does not result in reduced specific infectivity but that the reduced amount of F_{1+2} does.

The reduced incorporation of F_{1+2} into group D mutant virus particles is not due to the inability of infected cells to synthesize the precursor, F_0 (unpublished data). However, the intracellular F species might be unstable (8), unable to

FIG. 5. Radioactively labeled virus proteins of AV-WT, D1, and four revertants of D1 reduced and displayed by SDS-PAGE and detected by autoradiography. Virus was grown in cell culture in the presence of [³⁵S]methionine at either permissive (p) or nonpermissive (n) temperature.

FIG. 6. Radioactively labeled virus proteins of AV-WT, D1, and four revertants of D1 displayed by SDS-PAGE in the absence of a reducing agent and detected by autoradiography. Virus is the same as that shown in Fig. 5. p, Permissive temperature; n, nonpermissive temperature.

migrate to the cell surface, or unable to organize into budding virus particles. These possibilities are presently being examined.

By characterizing revertants, we have confirmed the association of the group D mutant phenotypes with each other and with the *ts* lesions of these mutants. Along with the ability to plaque at nonpermissive temperature, all 10 revertants coreverted to a large extent to greater hemolytic and infectious activity of egg-grown virions (27) and regained the ability to efficiently incorporate F_{1+2} into virions.

Coreversion of M migration in three of four revertants of mutant D1 with ts^+ plaquing confirms the assignment of the *ts* lesion of D1 to its M. In addition, it confirms the association between M and the F-related phenotypes: lower hemolytic and infectious activities of egg-grown virus and decreased incorporation of F_{1+2} , especially at nonpermissive temperature. The lower infectious and hemolytic activities correlate with and are probably caused by the reduced incorporation of F_{1+2} into virus particles. This reduction indicates an interaction between M and F_{1+2} before or during virus formation. A physical relationship between F_{1+2} and M has not been found in cross-linking studies of NDV and Sendai virus (18), but similar studies with VSV have found M and G to be in close proximity (9).

Of the 10 revertants isolated, at least 7 are pseudorevertants; they contain second compensating mutations in the originally mutated codon, in another site within the gene, or in another gene. These seven revertants are much more efficient in incorporating F_{1+2} into virus particles at the nonpermissive temperature. In addition, these seven revertants have been tested for the heat stability of their infectivity and have been found to be significantly different from AV-WT or their mutant parents (unpublished data).

The isolation of pseudorevertants seems to be a common event, for they are also a large fraction of the revertants of HN mutants of groups B, C, and BC (unpublished data). The isolation of numerous pseudorevertants provides an important function: it indicates that these are indeed revertants and not just chance wild-type virus contaminants.

The most interesting of these pseudorevertants are D1R1 and D1R3. M of D1R1 has reverted to wild-type migration although that of D1R3 has not, but both are much more efficient in F_{1+2} incorporation into virions at nonpermissive

temperature. These revertants, therefore, appear to represent two solutions to the same problem. D1R1 has incorporated a second mutation in M which has reverted its migration. D1R3 has incorporated a second mutation in either M or F. Both solutions for the D1 lesion also increase the efficiency of F incorporation into virions at nonpermissive temperature. Determination of the exact location of each reversion may confirm and extend our interpretation of M interaction with F_{1+2} .

An alternative possibility which we cannot rule out is presented by our evidence for interaction between M and F and by the fact that as yet we have no designated complementation group representing lesions in F. It is possible that mutants with lesions in either M or F could be assigned to the same complementation group. In other words, D1 might have a lesion in M, and D2 and D3 might have their lesions in F. This possibility seems unlikely since the mutants are similar in most of the phenotypes described and their revertants also show similar patterns.

M is thought to be specifically recognized by the nucleocapsid in maturation. Fractionation studies of Sendai virus and simian virus 5 particles have found M to be associated with nucleocapsid structures (21, 32, 39). McSharry et al. (20) found that phenotypically mixed particles containing all of the VSV proteins also contained the two simian virus 5 glycoproteins but no other simian virus proteins. These data suggested a specific NP-M interaction but no specific M-glycoprotein interaction. In *in vitro* mixing experiments using components of detergent lysed virus, Yoshida et al. (39) showed that Sendai virus glycoproteins would associate with nucleocapsid structures only in the presence of M, indicating that M may act by recognizing nucleocapsids as well as viral glycoproteins. Our data are consistent with a specific M-F interaction but do not rule out interactions between M and other glycoproteins.

TABLE 6. F_{1+2} content and specific infectivity of virus grown in cell culture

Virus	Ratio of F_{1+2} ^a to NP+P at:		Specific infectivity ^b at:	
	37.5°C	41.8°C	37.5°C	41.8°C
AV-WT	1.0	0.34	1.0	0.43
D1	0.41	0.021	1.2	0.071
D1R1	1.2	1.6	0.61	0.46
D1R3	1.6	1.6	1.4	0.32
D1R4	0.69	0.23	1.0	0.15
D1R5	1.1	0.28	0.74	0.13
D2	0.69	0.050	1.2	0.12
D2R1	1.3	1.4	0.85	0.30
D2R2	1.1	1.3	0.56	0.37
D2R3	1.1	0.52	1.5	1.1
D3	0.62	0.037	0.65	0.086
D3R1	0.83	1.2	0.80	0.41
D3R2	1.3	1.3	1.2	1.3
D3R3	1.4	1.1	1.2	1.2

^a The amounts of F_{1+2} and NP+P in each virus preparation were determined by scanning autoradiograms of nonreduced SDS-PAGE like those presented in Fig. 4. The ratio of F_{1+2} to NP+P was determined and is presented here relative to the F_{1+2} /NP+P ratio of AV-WT grown at 37.5°C.

^b The infectivity-to-radioactivity ratio of each partially purified preparation is presented here relative to AV-WT grown at 37.5°C in the same experiment.

M of Sendai virus has been implicated in virus budding by analysis of a *ts* mutant (38). A recent study indicates that M interacts with actin (10). If M is involved in budding, that function is relatively intact in the group D mutant-infected cells, which produce between 40 and 100% as many infectious virus particles as those cells infected by AV-WT at both permissive and nonpermissive temperatures.

M of VSV has been shown to be involved in condensing the nucleocapsid into a tight cytoskeleton (24). M in NDV might maintain the nucleocapsid in a spherical shape (32), but this shape does not appear as specific as that found in VSV. M has also been shown to be involved in transcription control of VSV (5, 7, 30, 37). Possible alterations in such functions in the group D mutants have not yet been explored.

It is clear that the M alteration of mutant D1 is related to its *ts* lesion since these properties coreverted in three of four revertants. However, it is still conceivable that a lesion in another viral protein, perhaps F, might somehow affect the migration of M. Such an interaction might be required for a post-translational modification of M, a modification that would decrease the migrational rate of M from that of D1 M (40,000) to that of AV-WT M (41,000). Unless this interaction and modification occur within 5 min of synthesis, however, this possibility seems unlikely since in pulse-chase studies of AV-WT-infected cells, M is present in a single, discrete species from a 5-min pulse through a 5-h chase (unpublished data). In addition, cells coinfecting with D1 and AV-WT produce both forms of M, indicating that the presence of the wild-type "modifier" does not change the migration rate of D1 M back to that of AV-WT (unpublished data).

Finally, we must point out that we have not absolutely ruled out the relatively unlikely possibility that the M alteration represents one *ts* lesion, that the F-related *ts* phenotypes represent another *ts* lesion, and that both *ts* lesions have reverted in three of the four revertants. This explanation seems unlikely since the reversion frequency for D1 (10^{-5} to 10^{-6}) is similar to the reversion frequency for D2 and D3 (10^{-4} to 10^{-5}). Assuming that the D2 and D3 reversions represent single-step mutations, two *ts* reversions should be found at a frequency of $(10^{-4})^2$: 10^{-8} or less. Such a low frequency is beyond the sensitivity of our screening procedures. Definitive proof must await sequencing and comparison of the F and M genes or proteins of AV-WT, mutants, and revertants.

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