Noncytopathic Mutants of Newcastle Disease Virus Are Defective in Virus-Specific RNA Synthesis

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We have studied virus-specific RNA synthesis in cells infected by six noncytopathic (nc) mutants of the Australia-Victoria wild-type strain (AV-WT) of Newcastle disease virus (NDV) (19). The rates of NDV-specific RNA synthesis in mutant infection were three to sevenfold lower than those observed in wild-type infection. Velocity sedimentation of this NDV-specific RNA revealed that the lower rates of synthesis in mutant infection correlated with reduced accumulation of 18S and 35S mRNA. Electrophoresis in polyacrylamide-urea gels showed that accumulation of all of the 18S mRNA species was reduced and no new species could be detected. Primary transcription appeared unaltered in mutant infection. Cells infected with two naturally occurring avirulent strains of NDV also showed less accumulation of 18S mRNA. Electrophoresis of this RNA resulted in patterns which differed from those obtained with RNA from either AV-WT or nc mutant infection. Complementation for RNA accumulation between the nc mutants and RNA⁻ temperature-sensitive mutants of AV-WT (32) suggested a common defect in the nc mutants. Analysis of plaque-forming revertants of five of the nc mutants revealed that viral RNA synthetic capacity, cell killing, and plaque-forming ability correlated absolutely. These results suggest that viral RNA synthesis and cytopathogenicity may be causally related. In addition, several of the plaque-forming (and cell-killing) revertants were found to be unable to induce fusion from within in infected cell cultures. This result, coupled with the finding that several of the nc mutants are capable of wild-type levels of fusion from within, suggests that the ability to cause such fusion does not correlate with the ability to kill cells.

Newcastle disease virus (NDV) is a negative-strand RNA virus, represented in nature by many strains which vary extensively in their virulence for wild and domestic fowl. The virulent strains of NDV characteristically show considerable cytopathic effects in chicken embryo cell cultures, both in multiple cycles of growth (e.g., plaque formation) and in single cycles of growth (13). In contrast, avirulent strains of NDV cause little or no cytopathic effects in these cells (13). Virulent and avirulent strains exhibit several other consistent phenotypic differences. For example, avirulent strains tend to accumulate less virus-specific mRNA than do virulent strains (2, 18). Also, in certain cell types, avirulent strains accumulate a form of the F₀ polypeptide in virions and are thus unable to spread from cell to cell or grow in multiple cycles (20, 24). This last observation explains the observed inability of avirulent strains to cause plaques in chicken embryo cell cultures. However, none of these consistent differences have been shown to be directly relevant to cell killing, per se.

Several other possible determinants of cell

killing have been proposed for paramyxoviruses. Viral ability to cause cell fusion and the susceptibility of host cell membranes to fusion have been correlated with cell killing by measles virus and simian virus 5 (12, 15). The reduced cytopathogenicity of a temperature-sensitive mutant of Sendai virus led Portner et al. to postulate a role for the HN polypeptide in cell killing by that virus (26).

We have previously isolated six independent mutants of NDV which are less cytopathic than their virulent parent, but are nevertheless capable of infectious virus production (19, 20). These mutants were selected in the hope of identifying the viral phenotype(s) involved in cell killing and, ultimately, of determining the contribution of cell killing to virulence in vivo. Although these noncytopathic (nc) mutants are unable to form plaques in chicken embryo cell cultures within 2 days of incubation, they form hemadsorbing spots. The mutants are also less cytopathic than wild-type virus (AV-WT) during single cycles of infection but produce similar amounts of infectious virus. In addition, all of them exhibit extended mean embryo death

times, suggesting they may be reduced in their virulence for the adult animal (19, 20).

We have now characterized the nc mutants and revertants derived from them with respect to their capacity for RNA synthesis and ability to cause FFWI (3). All of the mutants share a defect in the accumulation of virus-specific mRNA, and thus resemble the naturally occurring avirulent strains of NDV (2, 18). This defect in RNA accumulation, and not ability to cause FFWI, correlates with the noncytopathic phenotype.

Terminology. The term "noncytopathic" is potentially misleading for several reasons. In common usage, "cytopathology" describes a variety of deviations from normal cellular morphology or behavior. However, the nc mutants are not necessarily unable to cause all such effects; as shown herein, several of the nc mutants still possess the ability to cause fusion from within (FFWI), a classical cytopathic effect of paramyxoviruses. Furthermore, we have characterized the nc mutants under specifically defined culture conditions and within a limited time frame. For example, the mutants fail to make plaques on chicken embryo cells within 2 to 3 days of incubation; with longer periods of incubation or at elevated temperature they may, eventually, form plaques (19). Also, while the mutants show a lack of cell killing within 12 to 24 h (as measured by light microscopy and loss of protein from cultures infected at a multiplicity [MOI] of 5), they will eventually kill cells. Thus, within defined limits, the nc mutants are significantly reduced in a variety of measurable effects on chicken embryo cells, and we have retained the general designation of "noncytopathic" to describe their behavior.

The concept of cell killing, or cell death, is also elusive, being operationally defined in a variety of ways (including that immediately above). Whatever the definition, cytopathology and cell death are not equivalent terms. Cell death is an end result; a cytopathic effect may or may not lead to that end. In fact, we provide evidence below that, in this system, FFWI does not correlate with and, thus, does not cause cell killing or plaque formation. (Furthermore, cell killing and plaque formation are not necessarily equivalent events, although, in this system, they do appear to correlate.)

Finally, in all of our previous studies (19, 20) and in all but the fusion studies described herein, we have employed experimental conditions which suppress FFWI by the wild-type virus—namely, pH 7.2 and an MOI of 5 (3). These conditions were specifically chosen (i) because the pH is relatively physiological and identical to that used for plaque formation; (ii) so that

cytopathic effects other than FFWI could be measured and, thus, (iii) allow us to assess the contribution of FFWI to the altered cell killing phenotypes of the nc mutants. In fact, even under conditions which encourage FFWI (pH 8.2, MOI of 1), the nc mutants appear to cause less cell killing and do not form plaques (data not shown). Thus, we have used the term "cytopathogenicity" to denote the ability of the virus to cause a variety of cytopathic effects, excluding FFWI.

(Portions of this study were presented at the Annual Meeting of the American Society for Microbiology, New Orleans, La., 1977; The Negative Strand Virus Conference, Cambridge, England, 1977 [20]; the Fourth International Conference for Virology, The Hague, The Netherlands, 1978; and at the ICN-UCLA Symposium on Animal Virus Genetics, Keystone, Colo., 1980 [M. A. Bratt and C. H. Madansky, *J. Supramol. Struct. Suppl.* 4, p. 281, 1980]. Portions of this study were contained in the Ph.D. thesis of C.H.M., Harvard University, Cambridge, Mass., 1979.)

MATERIALS AND METHODS

Cell culture. Details of the preparation of the secondary cultures of chicken embryo cells used in all experiments have been described (19).

Virus strains and mutants. The wild-type virus used in this study was the Australia Victoria (1932) strain of NDV (AV-WT). The selection of the six nc mutants from AV-WT and their preliminary characterization have been described (19).

All revertants appeared spontaneously at frequencies of ca. 10^{-2} . Plaque-forming revertants of mutants nc9 and nc12, as well as the spot-size revertant (nc4S1) of nc4 and its plaque-forming second-step revertant (nc4S1R1), were isolated from individual clones which had been amplified by growth in chicken embryo cell cultures for 12 to 24 h. Plaque-forming revertants of nc7 and nc17 were isolated from mutant stocks grown in embryonated hen eggs.

Two naturally occurring avirulent strains of NDV were employed for comparative purposes: NJ-LaSota and B1-Hitchner. Titers for the avirulent strains were obtained by using a plaque assay in the presence of $0.3 \mu\text{g}$ of *N*-acetyl trypsin (Sigma Chemical Co.; 24, 28) per ml. Representatives of RNA⁻ complementation groups A and E of the temperature-sensitive mutants derived previously from AV-WT by Tsipis and Bratt (32) were employed in the complementation studies. All stocks of virus were prepared and purified as described previously (14).

Chemicals and solutions. Actinomycin D was a gift of Merck Sharp & Dohme. Cycloheximide was obtained from Sigma. Proteinase K was obtained from EM Laboratories. Standard medium consisted of Eagle minimal essential medium supplemented with 2.5% calf serum, 2.5% tryptose phosphate broth, and 0.07% NaHCO_3 . RNA labeling medium consisted of standard medium supplemented with $5 \mu\text{g}$ of actinomycin D per ml. [$5,6\text{-}^3\text{H}$]uridine (4.1×10^4 mCi/mmol) was obtained

from New England Nuclear Corp. Solubilizing buffer consisted of 0.01 M Tris-hydrochloride (pH 8.5), 0.1 M NaCl, 0.001 M EDTA, 1% 2-mercaptoethanol, and 1% (wt/vol) sodium dodecyl sulfate (SDS).

RNA accumulation protocols. (i) Total RNA. Secondary cultures of chicken embryo cells seeded in 60-mm tissue culture plates were infected at an MOI of 5 for 30 min at 39.5°C, and 3 ml of standard medium was added to each culture. At 3.5 h postinfection, the medium was replaced with 2 ml of labeling medium per culture. At 4 h postinfection, the medium was replaced with 2 ml of labeling medium containing 25 μ Ci of [³H]uridine per ml. At various intervals, medium from duplicate cultures was removed by aspiration, the cultures were washed twice with ice-cold Hanks balanced salts solution (pH 7.4), and 2 ml of ice-cold 5% trichloroacetic acid per culture was added. After 2 h at 4°C, samples of the trichloroacetic acid were taken as a measure of the uptake of radioactive precursor (trichloroacetic acid-soluble counts per minute), and the remainder was then removed by aspiration. The cultures were washed with Hanks balanced salt solution until the pH was restored to 7.4. Solubilizing buffer (2 ml per culture) was added, and trichloroacetic acid-precipitable material was again precipitated with the addition of an equal volume of ice-cold 25% trichloroacetic acid. The precipitates were collected under vacuum on membrane filters (HAWP 25; Millipore Corp.). Dried filters were placed in toluene-containing Omnifluor (New England Nuclear) and counted in a Beckman scintillation counter.

(ii) Primary transcription. Cell cultures seeded in 100-mm dishes were pretreated with standard medium containing 50 μ g of cycloheximide and 20 μ g of actinomycin D per ml. After 45 min at 39.5°C, the cells were infected at an MOI of 100 at 39.5°C in 0.5 ml of the same medium containing 100 μ Ci of [³H]uridine per ml. After 30 min, an additional 3 ml of the [³H]uridine-containing medium was added per culture. Cultures were incubated at 39.5°C for various intervals and processed for liquid scintillation as described above for total accumulation.

(iii) Complementation for RNA accumulation. Cell cultures in 60-mm tissue plates were infected singly at an MOI of 10 or in the appropriate pairs at an MOI of 5 for each virus for 30 min at 39.5°C. Cultures were then processed as described above for total RNA accumulation except that the temperatures of incubation were either 37.5 or 41.8°C, respectively, the permissive and nonpermissive temperatures of the temperature-sensitive mutants (32).

RNA extraction. Cell cultures in 100-mm tissue culture plates, uninfected or infected at an MOI of 5, were labeled with 100 μ Ci of [³H]uridine per ml between 4 and 10 h postinfection and washed with ice-cold Hanks balanced salt solution, and the cells were solubilized in 0.01 M Tris (pH 8.5), 0.5% SDS and 1 mg of proteinase K per ml as described by Clinkscales et al. (7). The digests were then extracted with phenol-chloroform-isoamyl alcohol, and the RNA was precipitated twice with ethanol (7).

Velocity sedimentation. Velocity sedimentation of extracted RNA was performed as described by Collins and Bratt (8). Samples were sedimented through 15 to 30% linear sucrose-SDS gradients for 4

h at 180,000 $\times g$ in a Beckman SW41 rotor at 22°C. Fractions (0.3 ml) were collected and precipitated with cold trichloroacetic acid, and radioactivity was determined as described above.

Electrophoresis. Electrophoresis of extracted, [³H]uridine-labeled virus-specific RNA was done on polyacrylamide-6 M urea gels as described by Floyd et al. (11) and McGeoch et al. (22) except that samples were boiled for 3 min and quenched immediately in an ice bath before electrophoresis. This treatment was employed to denature any partially base-paired species of RNA in the samples (30).

Electrophoresis was performed at 115 V (constant voltage) for 12 h at 4°C. Autoradiography of the dried slab gels and densitometer analysis have been described (7).

FFWI. FFWI was assayed under conditions derived from those of Bratt and Gallaher (3). Duplicate non-confluent cultures, either uninfected or infected at an MOI of 1, were incubated at 39.5°C at pH 8.5 until 12 h postinfection. The culture plates were then fixed and stained with Giemsa. (It should be noted that these conditions differ from the MOI of 5 and pH of 7.2 used in the other experiments with the nc mutants, described herein and previously [19, 20], and which suppress FFWI by AV-WT [3]).

Quantification was done by ordering the cultures relative to one another with respect to their degree of FFWI on an arbitrary scale of 0 to 8. All cultures were examined blindly and ranked by at least two individuals.

MDT. Mean embryo death time (MDT) was determined as described previously (19).

RESULTS

Rate of RNA synthesis. Figure 1 illustrates the kinetics of accumulation of virus-specific RNA in chicken embryo cells between 4 and 10 h after infection with AV-WT, the nc mutants, and two avirulent strains. Five of the nc mutants and the avirulent strains accumulated RNA at 14 to 21% the rate for AV-WT. Mutant nc4 consistently exhibited the highest rate of any of the mutants (37%). (Mutant nc16, which was reported [20] to behave similarly to nc4 in this respect, now behaves like the other mutants.) The lower rate of RNA accumulation by the nc mutants and avirulent strains was not the result of lower levels of infection since 99% of the cells in cultures infected at an MOI of 5 used in these experiments were hemadsorption positive (19) at 10 h postinfection. Nor were the differences in rates of RNA accumulation the result of differences in uptake of [³H]uridine, since measurements of intracellular trichloroacetic acid-soluble radioactivity were equivalent in all cultures. An example of such a measurement is shown for an nc9-infected culture in Fig. 1A (inset). The lower rates of accumulation are also unlikely to have resulted from more rapid degradation of RNA since shorter labeling periods (30 min), which should reduce the effects of degradation,

FIG. 1. Kinetics of total virus-specific RNA accumulation. Actinomycin D-resistant trichloroacetic acid-precipitable counts per minute. (A) AV-WT (—●—); nc9 (---x---); nc7 (---●---); nc12 (—x—); B1-Hitchner (—□—). (B) AV-WT (—●—); nc17 (---○---); nc4 (—○—); nc16 (----△----); NJ-LaSota (—△—). (A) and (B) represent separate experiments. Uninfected background counts per minute (4×10^3 cpm) have been subtracted from all values. (inset) Kinetics of uridine uptake. Trichloroacetic acid-soluble counts per minute. Uninfected (---●---); AV-WT (---○---); nc9 (---x---).

did not increase the rates of accumulation of RNA by the mutants or the avirulent strains relative to AV-WT (not shown). Thus, the lower rates of RNA accumulation appear to result from lower rates of RNA synthesis.

Virions of NDV contain an RNA-dependent RNA polymerase which, upon entry of the virus into the host cell, transcribes messenger RNA using the genome as template (5, 10, 16). This "primary transcription" can proceed in the presence of inhibitors of protein synthesis which block the formation of 50S RNA molecules and, thus, the amplification of mRNA transcription. Since the rate of total virus-specific RNA synthesis appeared reduced in mutant infection, it was of interest to determine whether primary transcription was also reduced in mutant and avirulent strain infection. Figure 2 shows that AV-WT and all of the nc mutants accumulated essentially equivalent levels of RNA in the presence of cycloheximide. The avirulent strains NJ-LaSota and B1-Hitchner consistently accumulated significantly higher levels of RNA under these conditions.

Characterization of RNA. To determine whether specific size classes of RNA were reduced in mutant infection, we labeled infected cells between 4 and 10 h postinfection, and the RNA was extracted and subjected to velocity sedimentation. Figure 3A shows a typical pattern for AV-WT. It has been shown previously that the 18S peak represents exclusively single-stranded, polyadenylic acid-containing mRNA which codes for all of the known NDV polypeptides (7, 9) except the L polypeptide, encoded by a 35S mRNA (23, 30; B. Spanier Collins, Ph.D. thesis, Harvard University, Cambridge, Mass., 1975). The 35S region (fractions 12–16) also contains partially base-paired material, presumably replicative intermediates (30); and the 50S region contains single-stranded, genome-size RNA, as well as partially base-paired material, presumably transcriptive intermediates (30).

In contrast with the pattern for AV-WT, the pattern shown in Fig. 3B for mutant nc9 reveals a dramatic reduction in the ratio of 18S to 50S RNA; nc mutants 7, 12, 16, and 17 exhibited essentially the same pattern (not shown). Con-

sistent with the data in Fig. 1, mutant nc4 (Fig. 3C) accumulated somewhat more 18S (and less 50S RNA) than the other mutants, though the ratio still differed from that of the wild-type.

As previously reported (2, 18), the naturally occurring avirulent strains of NDV characteristically exhibit less accumulation of 18S RNA relative to 50S RNA than do virulent strains. Figure 3D shows the pattern obtained with the avirulent strain, NJ-LaSota. This pattern and that obtained with the avirulent strain B1-Hitchner (not shown) were clearly similar to the patterns obtained with the nc mutants.

To establish which species of 18S to 35S RNA were being measured in the experiments described above, we compared the virus-specific RNA extracted from cells infected with AV-WT, the nc mutants, and the naturally occurring avirulent strains by polyacrylamide-urea gel electrophoresis. Figure 4 shows an autoradiogram of such a gel. Although the total accumulation of radioactivity was reduced in mutant infection (Fig. 1), in each case the same 18S mRNA's were found in essentially the same proportions as in AV-WT infection. We have had difficulty in obtaining quantitative data on

FIG. 2. Kinetics of accumulation of primary transcripts. Cells were pretreated with cycloheximide and actinomycin D and labeled with [^3H]uridine, and the trichloroacetic acid-precipitable counts per minute were determined. AV-WT (—●—); nc9 (---x---); nc12 (—x—); nc7 (---●---); nc17 (---○---); nc4 (—○—); nc16 (---△---); NJ-LaSota (—△—); B1-Hitchner (—□—). Uninfected background counts per minute (10^3 cpm) have been subtracted from all values.

FIG. 3. Velocity sedimentation of virus-specific RNA. Cells were infected and labeled, and the RNA was extracted and subjected to velocity sedimentation through 15 to 30% sucrose gradients containing SDS. (A) AV-WT (—●—); uninfected (—○—). (B) nc9. (C) nc4. (D) NJ-LaSota. The 18 and 28S markers were located by absorbance at 254 nm. The unlabeled arrow indicates the location of 50S RNA.

the 35S mRNA but it, too, appeared proportionally reduced in mutant infection. The avirulent strains accumulated several species of 18S RNA which migrated differently from the 18S species of AV-WT. These results compare well with previous reports of differences in the migration rates of mRNA species of AV-WT and NJ-LaSota (8).

Characterization of plaque-forming revertants. To determine the relationship between the noncytopathic and low-RNA-accumulation phenotypes, we selected revertants from five of the six nc mutants which, like AV-WT, form plaques within 2 days of incubation. Figure 5 shows almost identical RNA patterns for AV-WT and nc12R1, a plaque-forming revertant of nc12. Similar results were obtained from plaque-forming revertants of other nc mutants. The coreversion of RNA synthetic capacity with plaque-forming ability strengthens the correlation between these two phenotypes. Further evidence for this is summarized in Table 1, where RNA accumulation by the nc mutants and their plaque-forming revertants have been compared. In every case reversion to plaque-forming ability was accompanied by increased

NDV-specific RNA synthesis. Furthermore, revertants forming the clearest, most-distinct plaques usually showed the greatest restoration of RNA synthetic capacity. Plaque-forming revertants producing less-distinct, fuzzy plaques had only partially reverted for RNA synthesis. We have also found that the plaque-forming revertants are more cytopathic in single-cycle experiments (data not shown).

Mutant nc4 has been previously reported to produce hemadsorption-positive spots smaller than those of most of the other nc mutants (19, 20). A size revertant (nc4S1) which still does not form plaques, but produces larger hemadsorption-positive spots than mutant nc4, showed no reversion for RNA synthesis. However, a second step plaque-forming revertant (nc4S1R1) derived from nc4S1, did show reversion for RNA synthesis. Thus, RNA synthetic ability and plaque-forming ability coreverted in every case, while ability to spread (indicated by spot size) appeared unrelated to RNA synthetic or plaque-forming ability.

Table 1 also summarizes the FFWI phenotypes of the nc mutants and their plaque-forming revertants. Mutants nc4, nc16, and nc17 were

FIG. 4. Polyacrylamide-urea gel electrophoresis of virus-specific mRNA. This autoradiogram compares the patterns produced by each of the nc mutants and several avirulent strains (NJ, NJ-LaSota; B1, B1-Hitchner). Two different preparations of RNA from AV-WT-infected cells are shown (AV). The autoradiogram is a composite of two exposure times to compensate for differences in labeling between samples.

capable of wild-type levels of FFWI. Thus, the ability to cause FFWI does not correlate with cytopathogenicity. Furthermore, the plaque-forming revertants of mutants nc9 and nc12 were unable to induce FFWI. Although it is unclear why mutants nc9 and nc12, themselves, are unable to cause FFWI, it is quite clear that the abilities to cause FFWI and to kill cells do not go hand in hand. Mutant nc7 was also unable to induce FFWI. Elsewhere we will show that this inability is related to a defect in the post-translational processing of the F_0 polypeptide (Madansky and Bratt, in preparation).

Finally, Table 1 shows that several of the plaque-forming revertants exhibited a shorter MDT than their noncytopathic parents. Thus, the lesions affecting cytopathogenicity in the nc mutants were also responsible for their extended MDTs, a result which strengthens the connection between cytopathogenicity and virulence.

Complementation with RNA⁻ temperature-sensitive mutants. To learn which gene(s) is altered in the nc mutants to give reduced levels of RNA synthesis, we devised complementation tests to measure the enhancement of RNA accumulation in cells coinfecting

with nc mutants and the RNA⁻ temperature-sensitive mutants previously isolated from the same parental strain (32). Those RNA⁻ mutants fall into two complementation groups, A and E (tsA; tsE). Table 2 shows that the tsA and the tsE mutants complemented each other for RNA accumulation. This finding is consistent with the complementation for virus production previously described by Tsipis and Bratt (32). Coinfection of the nc mutants and a representative of tsA mutants did not result in enhanced RNA accumulation, whereas coinfection with the tsE mutant consistently resulted in enhanced accu-

FIG. 5. Velocity sedimentation of NDV-specific RNA from cells infected with AV-WT (—□—), nc12 (—●—), and its revertant, nc12R1 (—○—).

TABLE 1. *Properties of the nc mutants and their revertants*

Virus	Virus-specific RNA ^a			Relative spot or plaque size ^b	FFWI ^c	MDT ^d
	1 ^e	2	3			
AV-WT	100	100	100	○	8, 8	42
nc9	20	—	25	●	1, 2	63
nc9 R1	75	—	62	○	1, 0	40
nc9 R2	43	—	65	○	1, 0	—
nc9 R3	68	—	—	○	0, 1	—
nc9 R4	—	—	34	○	0, 1	—
nc12	18	—	4	●	2, 2	63
nc12 R1	86	—	73	○	0, 1	40
nc12 R2	24	—	34	○	1, 0	—
nc12 R3	55	—	160	○	1, 0	—
nc12 R4	16.5	—	30	○	1, 0	—
nc17	15	—	—	●	7, 8	63
nc17 R1	86	—	—	○	7, 8	48
nc17 R2	75	—	—	○	7, 8	—
nc7	—	20	24	•	0, 1	74
nc7 R1	—	58	84	○	0, 1	63
nc7 R2	—	—	45	○	—	—
nc4	—	30	—	●	7, 8	74
nc4 S1	—	28	—	●	4, 3	63
nc4 S1R1	—	93	—	○	6, 7	44
nc16	—	16	—	•	8, 8	86
Uninfected	—	—	—		1, 0	∞

^a Values represent the actinomycin-D-resistant incorporation of [³H]uridine into trichloroacetic acid-insoluble material between 4 and 10 h postinfection. Values are expressed as the percentage of AV-WT (2).

^b Relative size of hemadsorbing spots (●) or plaques (○) after 2 days of incubation at 39.5°C. (○) Fuzzy plaques. (○) Clear plaques.

^c Cultures infected and fixed and stained (3) were ordered relative to one another with respect to their degree of FFWI on an arbitrary scale of 0 to 8. All cultures were examined blindly and ranked by at least two individuals. The data represent the results of two separate experiments.

^d MDT determined as described previously (19); average of several experiments.

^e Experiment number.

mulation. In other experiments, coinfection of the nc mutants and other members of tsA yielded similar results. In addition, coinfection of pairs of the nc mutants showed no enhancement of RNA accumulation. Table 2 also shows that RNA accumulation was not enhanced in coinfections between mutants in either of the temperature-sensitive complementation groups and the naturally occurring avirulent strains, NJ-LaSota and B1-Hitchner.

It should be noted that several of the mutants exhibited different levels of RNA accumulation at 41.8°C (Table 2) than at 39.5°C (Fig. 1). These effects of temperature on total RNA accumulation were reflected in the levels of accumulation of virus-specific 18S RNA, as revealed by velocity sedimentation analysis of RNA extracted from cells infected with the nc mutants at various temperatures (data not shown). Mutant nc7 consistently accumulated RNA at ca. 50% the levels of AV-WT (Table 2). Thus, the relatively low complementation index (1.6) shown for nc7 × tsE1 in Table 2 actually reflects significant complementation; even a twofold en-

hancement would have brought RNA accumulation by nc7 up to wild-type levels.

We have also found that tsA mutants characteristically inhibited AV-WT RNA accumulation by 50% in dual infection (Table 2; Madansky and Bratt, unpublished data). As yet, we have no explanation for this finding.

DISCUSSION

nc mutants are defective in virus-specific RNA synthesis. Our six nc mutants exhibited a common defect in virus-specific RNA synthesis. Their rates of total virus-specific RNA accumulation were reduced to 14 to 37% of the levels observed in wild-type infection. These reduced rates of accumulation did not appear to result from either reduced uptake of radioactive precursor, lowered levels of infection, or increased rates of degradation of RNA in mutant infection. Thus, the nc mutants appear to share a defect in RNA synthesis. Since primary transcription, measured as RNA accumulation in the presence of cycloheximide, did not appear to be altered, all types of virus-specific RNA synthesis

TABLE 2. *Complementation for RNA Accumulation*

Virus	Virus-specific RNA in single infection ^a		Virus-specific RNA in double infection with tsA3 mutant ^a at 41.8°C	Complementation index (x A3) ^b	Virus-Specific RNA in Double Infection with tsE1 ^a at 41.8°C	Complementation index (x E1) ^b
	37.5°C	41.8°C				
AV-WT	78	108	45	0.4	92	0.8
tsA3	100	8	— ^c	—	41	4.6
tsE1	106	6	41	4.6	—	—
nc9	—	17	13	0.7	52	2.3
nc12	—	20	11	0.5	88	3.4
nc7	—	48	30	0.6	86	1.6
nc17	—	15	8	0.4	95	4.5
nc4	—	10	0	∞	52	3.1
nc16	—	12	0.4	0.03	52	2.9
NJ-LaSota	—	12	13	0.65	18	1.0
B1-Hitchner	—	9	13	0.72	19	1.3

^a Counts per minute $\times 10^{-3}$ with uninfected background of 7.0×10^{-3} subtracted.

^b Complementation index = counts per minute in double infection/sum of counts per minute in single infections.

^c —, Not done.

are not equally reduced in mutant infection. This finding is consistent with the possibility that the nc mutants might be defective in some step in the amplification of viral mRNA transcription. However, we do not yet have any information concerning the levels of individual primary transcripts; a reduction in the level of a minor species such as the 35S mRNA could have little effect on the overall accumulation of primary transcripts, but a potentially dramatic effect on total viral RNA synthesis.

Velocity sedimentation of total viral RNA extracted from mutant-infected cells showed a significant decrease in the accumulation of 18S mRNA. Polyacrylamide-urea gel electrophoresis indicated that the accumulation of all the 18S mRNA's, and possibly the 35S mRNA (8, 23, 30, 33), was reduced. Furthermore, no new species were produced.

We did not observe a reduction in the level of RNA sedimenting in the 50S region comparable to that observed in the 18S region of gradients of total intracellular nc mutant RNA. However, from the data presented here, it is impossible to conclude that 50S replication is unaffected in mutant infection; a reduction in the single-stranded, genome-size RNAs (which comprise less than 30% of the RNA sedimenting in the 50S region [30]) would not necessarily be detectable by velocity sedimentation analysis.

Whatever the cause of the reduced RNA synthesis in mutant infection, it is interesting to note that in mutant infections, intracellular accumulation of the L polypeptide (presumably involved in transcription [10, 17, 31]) is reduced to levels 5 to 20% of those observed in wild-type infection, while that of the other NDV polypep-

tides is only reduced to 30 to 50% (20; Madansky and Bratt, in preparation).

Each of the nc mutants is able to complement for RNA accumulation with the tsE mutant, but not with the tsA mutants (or with each other). This uniformity is consistent with our finding that none of the nc mutants can complement with any other for plaque formation (Madansky and Bratt, unpublished data). Coupled with the uniformity of the RNA phenotypes, these results suggest that these six independently isolated nc mutants have lesions in the same gene. Positive complementation with the tsE mutant indicates that the lesion is not in the *E* gene. Whether the lesion is actually in the *A* gene is difficult to assess because of the behavior of the tsA mutants in coinfections with wild-type virus; mutants in this group inhibit the accumulation of virus-specific RNA by AV-WT.

Comparison with avirulent strains. Comparisons between avirulent and virulent strains of NDV have revealed several consistent phenotypic differences (reviewed by Madansky and Bratt [19]). Among these is the finding that avirulent strains accumulate far less 18S viral RNA than do virulent strains (2, 18). We have confirmed this finding and have shown that, as in the case of the nc mutants, this is reflected in lower rates of total virus-specific RNA accumulation. Polyacrylamide-urea gel electrophoresis of the 18S mRNA species of the two avirulent strains studied here, NJ-LaSota and B1-Hitchner, showed relative migration rates for several species which were different from those of AV-WT. Differences in migration rates of the mRNA's of AV-WT and the NJ-LaSota strain in aqueous polyacrylamide gels have also been

reported (8). These, and strain-specific differences in rates of migration for the polypeptides of avirulent strains of NDV (20, 29; Madansky and Bratt, in preparation), provide diagnostic markers for these strains and support the notion that there may be many genetic differences between virulent and avirulent strains (4). In addition, neither of the avirulent strains complemented the tsE mutant. A possible explanation for this is that protein components may not be interchangeable among these strains. Certainly the evidence suggests that the avirulent strains and the nc mutants differ widely in their genotypes. Thus, whatever the genotype, it appears to be the phenotype of reduced RNA synthetic capacity which is relevant to differences in cytopathogenicity between strains of NDV.

RNA accumulation, FFWI, and cell killing. Is there indeed a causal relationship between the accumulation of viral RNA and cytopathogenicity? The fact that all six of the independently isolated nc mutants—selected only for their loss of plaque-forming ability—exhibit reduced RNA accumulation suggests that this is the case. Moreover, our results with plaque-forming revertants derived from five of the nc mutants show an excellent correlation between RNA synthetic capacity and plaque-forming ability. Blair and Robinson (1) suggested that differences in cytopathogenicity between strains of NDV and Sendai might result from differences in virus-specific RNA accumulation. Based on studies with temperature-sensitive mutants of vesicular stomatitis virus, Marcus and Sekellick (21) suggested that a certain level of primary transcription is necessary for cell killing by that negative-strand virus. Clinkscales and Bratt have presented preliminary evidence supporting a hypothesis for the direct involvement of NDV mRNA in the inhibition of host cell protein synthesis (6).

There are also several reports of reduced RNA synthetic capacity associated with persistent infections, another situation in which cytopathogenicity is reduced. All of the NDV variants isolated from persistent infections of L cells show a defect in RNA synthesis which is detected as temperature sensitivity (25). The same appears to be true of vesicular stomatitis virus variants from persistent infections (35). Reduced 18S RNA accumulation was also observed in persistent infections by measles virus (34). Sarver and Stollar (27) have shown that clones of mosquito cells which are most sensitive to a destructive effect of Sindbis virus are most permissive for viral RNA synthesis. In none of these cases is it clear whether the RNA synthetic capacity is directly involved in the establishment or main-

tenance of the persistent infection, or is simply required as a mechanism for reducing cytopathogenicity which will then permit persistence to occur. Nevertheless, these results are intriguing in light of our present findings that the nc mutants of NDV share a defect in the accumulation of virus-specific RNA and suggest that RNA viruses can modulate their interaction with the host cell through the intracellular accumulation of viral mRNA, without affecting their production of infectious virus.

The data presented here provide no information on which species and/or products of viral RNA might be responsible for cell killing. Portner et al. (26) suggested that the Sendai virus HN polypeptide might be involved in cell killing. In contrast, Holmes and Choppin (15) have cited the susceptibility of the host cell membrane to FFWI as the major determinant of cell killing by simian virus 5. FFWI has also been cited as the cause of "early" cell killing by measles virus (12); measles virus-infected cells incubated in the presence of an inhibitor of FFWI did not die until much later than those incubated in normal medium. The authors postulated another, unidentified, cause of "late" cell death. In the present study we have obtained genetic evidence that FFWI, per se, is not correlated with cell killing by NDV. Several of the nc mutants (nc17, nc4, and nc16) are capable of causing wild-type levels of FFWI, but are still less able to kill cells than wild-type virus. Conversely, several plaque-forming (and cell-killing) revertants of mutants nc9 and nc12 are unable to cause FFWI. Thus FFWI and cell killing vary independently. These results do not, however, rule out some role in cell killing of the F glycoprotein per se, distinct from its cell-fusing activity.

Finally, we have presented evidence suggesting that the ability of one of the nc mutants, nc4, to spread from cell to cell is unrelated to its ability to kill cells. By selecting stepwise revertants of nc4, restoring first its ability to spread (nc4S1), and then its ability to synthesize virus-specific RNA and make plaques (nc4S1R1), we have demonstrated that cytopathogenicity and virus spread are genetically separable phenotypes. The stepwise diminution of MDT of the revertants of nc4 suggests that both viral spread and cytopathogenicity contribute to virulence in vivo. A more detailed account of the viral-spread phenotypes of the nc mutants and their revertants will appear elsewhere (Madansky and Bratt, in preparation).

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