Relationships Among Virus Spread, Cytopathogenicity, and Virulence as Revealed by the Noncytopathic Mutants of Newcastle Disease Virus

CHARLES H. MADANSKY^{1,2} AND MICHAEL A. BRATT^{2*}

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115,¹ and Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605²

Received 4 May 1981/Accepted 13 July 1981

We have studied protein synthesis in cultured cells infected with the six noncytopathic (*nc*) mutants of the Australia-Victoria strain (AV-WT) of Newcastle disease virus and their plaque-forming revertants. Virus-specific polypeptides accumulated at 30 to 63% of wild-type levels in *nc* mutant-infected cells and between 66 and 175% of wild-type levels in revertant-infected cells. An exception was the L polypeptide, which accumulated in *nc* mutant-infected cells at only 5 to 20% of the levels found in wild-type infection. The reduced accumulation of the L polypeptide did not appear to be due to increased degradation of that polypeptide. A new polypeptide (X) accumulated instead of polypeptide P in cells infected with mutants *nc4* or *nc16* and in virions released from them. Peptide mapping identified X as an altered form of P. A revertant of mutant *nc4* (*nc4S1*), which forms larger hemadsorbing spots, but still does not form plaques, accumulated P instead of the X polypeptide. Thus, a lesion in P can affect virus spread without affecting cytopathogenicity. Virions of mutant *nc7* and two naturally occurring avirulent strains of Newcastle disease virus (NJ LaSota and B1-Hitchner) contained polypeptides (F_7 and F_A , respectively) related to, but migrating more rapidly than, F_0 in sodium dodecyl sulfate-polyacrylamide gels. As previously reported for avirulent strains, a brief treatment of *nc7* virions with trypsin converted F_7 to F and increased infectivity. Similarly, culturing *nc7*-infected cells in the presence of trypsin facilitated fusion from within and viral spread from cell to cell. A plaque-forming revertant of *nc7* still accumulated F_7 in virions, indicating that the lesions responsible for the F_7 and noncytopathic phenotypes are genetically separable. The virulent parental strain, AV-WT, exhibited a mean embryo death time of 42 h. Both the larger-spot-forming revertant of *nc4* (*nc4S1*) and the small-plaque-forming revertant of *nc7* exhibited a decrease in mean embryo death time (increase in virulence) from 74 to 63 h. A second-step, plaque-forming revertant derived from *nc4S1* (*nc4S1R1*) exhibited a further decrease in mean embryo death time from 63 to 44 h. The results suggest that the F_A - F_7 and X lesions affect the ability of virus to spread from cell to cell. In addition, these lesions appear to be genetically separable from those responsible for the noncytopathic phenotype. However, both types of lesions cause an extension of mean embryo death time and, thus, may be relevant to virulence in vivo.

Unlike their virulent parental strain, the noncytopathic (*nc*) mutants of Newcastle disease virus (NDV) do not form plaques after 2 days of incubation; nevertheless, they produce nearly normal amounts of infectious virus (20, 21). These mutants were originally screened for, and are titrated by, their ability to form hemadsorbing spots on monolayers of chicken embryo cells. Thus, whereas they do not form plaques, they do grow and spread from cell to cell. All of the mutants exhibited extended mean embryo death

times (MDT), suggesting they may be reduced in virulence for their natural host, chickens.

We have previously presented evidence for a causal relationship between the noncytopathic phenotype of each mutant and a reduced accumulation of virus-specific RNA (22, 23). Herein we show that these phenotypes correlate with reduced intracellular accumulation of the L polypeptide. Low levels of virus-specific RNA accumulation are also characteristic of naturally occurring avirulent strains of NDV (3, 19). Avir-

ulent strains also resemble the *nc* mutants because they are less cytopathic than are virulent strains both in tissue culture and in vivo (12, 20).

Three of the *nc* mutants (*nc4*, *nc7*, and *nc16*) form hemadsorbing spots considerably smaller (reduced ability to spread) than those of the other mutants or the plaques of wild-type virus (20–22). These three mutants also grow to the lowest titers and exhibit the longest MDTs. What are the relationships, if any, between these phenotypes and the noncytopathic phenotype? In the present study, we show that reduced ability to spread as well as reduced virus production and very long MDTs correlate with altered polypeptides accumulating in virions or in cells infected by mutants *nc4*, *nc16*, and *nc7*. Furthermore, the lesions responsible for these altered polypeptides can be genetically separated from that responsible for the noncytopathic phenotype. These lesions also contribute to virulence as measured by MDT, but do so independently of the lesions resulting in the noncytopathic phenotype.

MATERIALS AND METHODS

Cell culture. Details of the preparation of the secondary cultures of chicken embryo cells used in all experiments have been described previously (20).

Virus strains. 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 previously (20).

Spontaneous plaque-forming revertants appeared at a frequency of 10^{-2} . For mutants *nc7* and *nc17*, revertants were isolated from mutant stocks. Plaque-forming revertants of mutants *nc9* and *nc12* were isolated from individual clones which had been amplified by growth in chicken embryo cell cultures for 12 to 24 h. The larger-hemadsorbing-spot-forming revertant of mutant *nc4*, *nc4S1*, was isolated from a stock of *nc4* by the method described for the original isolation of the *nc* mutants (20). The spontaneous plaque-forming revertant of *nc4S1*, *nc4S1R1*, was isolated as a second-step revertant from an individual clone of *nc4S1* which had been amplified by growth for 12 h in a chicken embryo cell culture.

Two naturally occurring avirulent strains of NDV were employed for comparative purposes: NJ LaSota and B1-Hitchner. Titers for the avirulent strains were obtained using a plaque assay in the presence of 0.3 μ g of *N*-acetyl trypsin (Sigma Chemical Co.) per ml (25).

All stocks of virus were prepared and purified as described by Hightower and Bratt (13).

Chemicals and solutions. Standard medium consisted of Eagle minimal essential medium supplemented with 2.5% calf serum, 2.5% tryptose phosphate broth, and 0.07% NaHCO_3 . Protein labeling medium consisted of Hanks balanced salts solution (pH 7.4) supplemented with 0.07% NaHCO_3 and 50 μ Ci of [^{35}S]methionine (New England Nuclear Corp.;

1,370 μ Ci/mmol) per ml. Virion labeling medium consisted of Hanks balanced salts solution (pH 7.4) supplemented with essential amino acids minus methionine and with minimal essential vitamins, 0.07% NaHCO_3 , 5% dialyzed calf serum, and 100 μ Ci of [^{35}S]methionine per ml. Chymotrypsin was obtained from Sigma.

Protein accumulation. In all experiments, mutant-infected cells were pulse-labeled at 12 h postinfection, whereas AV-WT- and revertant-infected cells were pulsed at 9 h postinfection. At these times, cells exhibited maximal rates of virus-specific protein accumulation and minimal cytopathic effects (Madansky and Bratt, unpublished observations). As described by Bratt and Gallaher (4, 5), fusion from within (FFWI) by AV-WT is suppressed under the conditions used here (multiplicity of infection, 5; pH 7.2). Cultures incubated at 39.5°C were infected with AV-WT, the *nc* mutants, or revertants derived from *nc* mutants, washed with prewarmed Hanks balanced salts solution (pH 7.4), and labeled as follows.

(i) **Steady-state protein accumulation.** Protein labeling medium was added for 30 min. Cultures were then washed twice with ice-cold Hanks balanced salts solution and extracted with gel sample buffer as described previously (8).

(ii) **Pulse-chase experiments.** Protein labeling medium was added for 4 min (pulsed), and replicate cultures were either washed with ice-cold Hanks balanced salts solution (pH 7.4) and extracted with gel sample buffer or washed twice with prewarmed standard medium and incubated with standard medium for 30 min (chased) and then extracted with gel sample buffer.

(iii) **Radiolabeled virions.** Growth and purification of radioactive virions have been described previously (8).

(iv) **Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (17) described previously (8). Note that in the experiments illustrated in Fig. 1, 3, 7, and 9, 7% gels were run for 2.5 to 4 h at 120 V constant voltage, and polypeptide P migrated slower than X (which migrated slower than NP). In contrast, in the experiments illustrated in Fig. 2, 5, and 6, 15% gels were run at 15 mA constant current, resulting in both P and X migrating faster than NP, and P migrating fastest. This behavior of P has been reported previously and is apparently due to its phosphorylation (28).

Difference analysis. Autoradiograms of dried slab gels were scanned with an Ortec densitometer. Several exposure times were employed to insure that each band could be quantified in a linear range of X-ray film response. The method of Hightower and Bratt (13) was employed to determine virus-specific protein accumulation above a normalized uninfected cell background. However, individual densitometer tracings of infected and uninfected cell polypeptide patterns were used rather than counts per minute of radioactivity obtained from a double-labeled gel. The difference analysis insures that our quantification of the levels of virus-specific polypeptides is independent of the level of accumulation of host-specific polypeptides, assuming that the inhibition of host protein accumulation is relatively uniform for all protein species.

Polypeptide mapping. Identification of polypeptides by polyacrylamide gel electrophoresis of fragments generated by limited digestion with chymotrypsin was performed by a modification of the method of Cleveland et al. (6).

Radiolabeled polypeptides were sliced from dried sodium dodecyl sulfate (SDS)-polyacrylamide gels and rehydrated in 1 ml of a solution containing 0.125 M Tris-hydrochloride (pH 6.8), 0.1% SDS, 1 mM EDTA, and 100 μ g of chymotrypsin per ml for 30 min at room temperature. The rehydrated gel slices were applied to a 15% discontinuous SDS-polyacrylamide gel (containing 1 mM EDTA) run at 30 mA constant current as described by Cleveland et al. (6). The gel was impregnated with 2,5-diphenyloxazole as described by Bonner and Laskey (2). Autoradiograms of the dried slab gels were made using X-Omat X-ray film.

MDT. Measurements of MDT were determined as described previously (20).

Trypsin treatments. Trypsin treatments were as follows.

(i) Radiolabeled virions were treated with 5 μ g of trypsin per ml in phosphate-buffered saline (pH 7.2) for 5 min at 37°C. Soybean trypsin inhibitor (500 μ g/ml; Sigma) was added, and the reaction was terminated in an ice bath. Virions were then centrifuged for 1 h at 24,000 rpm in a Beckman SW27 rotor at 4°C, and the pelleted material was processed for electrophoresis.

(ii) FFWI was assayed at pH 8.4 in the presence or absence of 10 μ g of trypsin per ml, added at 6 h postinfection, in Eagle minimal essential medium supplemented with 1% calf serum.

(iii) Hemadsorbing spot size was measured after incubating infected cell cultures for 2 days in the presence of 0.3 μ g of trypsin per ml.

RESULTS

Host protein metabolism in *nc* mutant-infected cells. Polypeptides synthesized in chicken embryo cells either uninfected or infected with AV-WT, *nc* mutants, or plaque-forming revertants are compared in the electropherograms shown in Fig. 1A and 2. A comparison of densitometer tracings of regions of the electropherogram in Fig. 1A in which only host-specific polypeptides migrate (13) showed that the incorporation of [³⁵S]methionine into host polypeptides in *nc* mutant infection was 48 to 118% of that observed in uninfected cultures, whereas that in infection by AV-WT was only 6%. This result is consistent with our previous report of diminished inhibition of total protein accumulation by the *nc* mutants (20). In contrast (Fig. 2), the plaque-forming revertants of the *nc* mutants showed decreased incorporation into host proteins. (Although not apparent in this gel, *nc*7R1 showed a significant decrease in incorporation into host proteins in other experiments.) These data are summarized in Table 1.

Virus-specific protein metabolism in *nc* mutant-infected cells. Table 1 also summa-

rizes the levels of individual virus-specific polypeptides synthesized in mutant and revertant infection based on a difference analysis of densitometer tracings of autoradiograms like those shown in Fig. 1. Virus-specific polypeptides accumulated in revertant infection at essentially wild-type levels, whereas in mutant-infected cells they accumulated at only 30 to 63% of the levels seen in AV-WT infection. The only exception was the L polypeptide, which accumulated at levels only 5 to 20% of those seen in AV-WT infection.

To test the possibility that the low levels of the L polypeptide in mutant-infected cells resulted from an increased rate of degradation, a pulse-chase experiment was done. Duplicate cell cultures either uninfected or infected with AV-WT or the *nc* mutants were pulse-labeled with [³⁵S]methionine and either extracted for electrophoresis or incubated with an excess of unlabeled methionine and then extracted. Whereas the L polypeptide was somewhat unstable in AV-WT infection, no greater instability of the L polypeptide was detected in cells infected with the *nc* mutants (Fig. 3). Thus, the low levels of the L polypeptide in mutant-infected cultures do not appear to result from increased rates of degradation.

Identity of the X polypeptide. It is evident from Fig. 1A that cells infected by four of the six *nc* mutants accumulated the same virus-specific polypeptides as did cells infected with AV-WT. Mutants *nc*4 and *nc*16, however, accumulated a new polypeptide (X) which migrated slightly slower than NP under the gel conditions employed in Fig. 1A and considerably faster than NP under the conditions employed in Fig. 2. It is clear from Fig. 2 that mutants *nc*4 and *nc*16 failed to accumulate the P polypeptide, recently identified as an NDV phosphopolypeptide whose function is not yet known (9, 24, 28). Thus, mutants *nc*4 and *nc*16 appeared to have a mutation which affects the migration of P in polyacrylamide gels.

This was confirmed when the appropriate [³⁵S]methionine-labeled polypeptides derived from SDS-polyacrylamide gels like those in Fig. 1 were subjected to limited chymotrypsin digestion and then electrophoresed in 15% SDS-polyacrylamide gels. The results (Fig. 4) show the X polypeptide to be most similar to the P polypeptides of AV-WT, NJ LaSota, and B1-Hitchner (Fig. 1B). A similar result with the NJ LaSota strain has been reported (10).

X affects viral spread: a larger-spot-forming revertant of *nc*4. Because mutants *nc*4 and *nc*16 make small hemadsorbing spots, we tested the hypothesis that the accumulation of X rather than P results in reduced viral

FIG. 1. Intracellular viral polypeptides, 7% gels run at 120 V constant voltage. *A* and *B* represent separate experiments. All samples were extracts of chicken embryo cells, either uninfected (*U*) or infected with AV-WT (*AV*), the *nc* mutants, or the avirulent strains NJ LaSota (*N*) and B1-Hitchner (*B1*). Host-specific region used in protein inhibition determinations (see text).

FIG. 2. Intracellular polypeptides of plaque-forming revertants of the *nc* mutants; 15% gel run at 15 mA constant current for 18 h. *U*, Uninfected cell extracts; *AV*, AV-WT; *R*, plaque-forming revertant; *4R*, *nc4S1R1*.

spread. Accordingly, we isolated *nc4S1*, a large-spot-forming revertant of mutant *nc4* which still does not form plaques. Analysis of the polypeptides in cells infected by this revertant indicated

that the revertant no longer accumulated the X polypeptide, but accumulated the P polypeptide (Fig. 5). Thus the spot size (virus spread) of *nc4* and accumulation of P corevert, suggesting that, at least in tissue culture, lesions in the P polypeptide can affect the ability of virus to spread from cell to cell.

The selection of a larger-spot-forming revertant of mutant *nc4* indicated that the phenotype of limited viral spread was genetically separable from the noncytopathic phenotype. As further proof of this hypothesis, we isolated a second-step, plaque-forming revertant from *nc4S1* (*nc4S1R1*). This revertant, like *nc4S1*, accumulated P (Fig. 5).

We have previously reported that, like the other plaque-forming revertants of the *nc* mutants, *nc4S1R1* accumulates nearly wild-type levels of virus-specific RNA, whereas *nc4S1* accumulates virus-specific RNA at levels equivalent to those of *nc4* (23). Thus, the noncytopathic phenotype correlated with reduced virus-specific RNA accumulation, but not with reduced cell-to-cell spread of the virus.

nc4S1 exhibited a decrease in MDT from 74 h for mutant *nc4* (20) to 63 h (Table 2), suggesting that the X phenotype contributes to the extended MDT of mutants *nc4* and *nc16*. The plaque-forming revertant of *nc4S1*, *nc4S1R1*, showed a further decrease in MDT to 44 h and thus approached the MDT of AV-WT.

Polypeptides in virions of the *nc* mutants: identity of the *F*₇ polypeptide. To compare the polypeptides in AV-WT and mutant virions, extracts of [³⁵S]methionine-labeled

TABLE 1. *Virus-specific and host-specific polypeptides in infected cell extracts*

Virus	% of AV-WT levels of viral polypeptides ^a :						Host polypeptide (% uninfected) ^b
	L	HN	F ₀	P	NP-F	M	
<i>nc9</i>	5	49	34	37	35	40	96
<i>nc7</i>	7.5	44	49	34	39	30	71
<i>nc12</i>	5	42	32	52	59	52	118
<i>nc17</i>	5	41	40	57	56	63	84
<i>nc4</i>	20	55	42	0	59	43	48
<i>nc16</i>	7.5	55	43	0	30	37	67
AV-WT	100	100	100	100	100	100	6
<i>nc9R1</i>	97	78	81	134	113	107	8
<i>nc7R1</i>	113	72	104	175	98	93	75
<i>nc12R1</i>	123	95	128	110	87	140	33
<i>nc17R1</i>	153	66	170	103	108	77	50
<i>nc4S1R1</i>	102	86	125	100	100	85	7

^a The data were derived from densitometer tracings of autoradiograms of 7% SDS-polyacrylamide gels such as those shown in Fig. 1 and 2. Each tracing of *nc* mutant-infected extracts or AV-WT-infected extracts was normalized to a tracing of an uninfected cell extract. The level of incorporation of label into particular virus-specific polypeptides was determined by cutting out individual peaks from the normalized tracings and weighing them. The data given above represent the weight of each *nc* mutant polypeptide from normalized tracings divided by the weight of the corresponding polypeptide in normalized tracings of AV-WT-infected extracts. Autoradiograms exposed for several time periods were employed for individual polypeptides so that their exposure fell in the linear range of response of the X-ray film.

^b The data represent the weights of tracings of infected cell extracts divided by the weight of the tracing of an uninfected cell extract. All tracings were derived from a region of an electropherogram (as shown in Fig. 1) which contained only host polypeptides (13).

virions released from AV-WT or *nc* mutant-infected cells were electrophoresed in SDS-polyacrylamide gels. Figure 6 shows an autoradiogram resulting from such an experiment. In contrast to the low levels of L in cells infected by the *nc* mutants, virions of each mutant contained levels of the L polypeptide equivalent to those found in virions of AV-WT; all of the other NDV polypeptides were also present in normal proportions.

Virions of mutants *nc4* and *nc16* contained a polypeptide which comigrated with, and yielded peptide maps identical to, intracellular X (data not shown). Finally, Figure 6 shows that virions of mutant *nc7* also contained a polypeptide (F₇) that migrated between HN and NP-P.

To further explore the origin of the F₇ polypeptide, [³⁵S]methionine-labeled extracts of infected cells were compared with extracts of virions released from those cells. The cells had either been pulse-labeled for 4 min with [³⁵S]-methionine or pulsed and then chased for 30 min. In contrast to AV-WT and *nc4*, virions of mutant *nc7* and the NJ LaSota and B1-Hitchner strains each accumulated a polypeptide which migrated between the HN and NP-P polypeptides (Fig. 7). This result is consistent with the report of Nagai et al. (25) that a more slowly migrating glycosylated polypeptide accumulated in virions of avirulent strains of NDV grown in certain cell types, including chicken embryo cell cultures. These authors concluded that the F₀

polypeptide accumulated in virions instead of the F polypeptide. The data in Fig. 7 suggest that it is not F₀, but a more rapidly migrating polypeptide, here designated F_A (for the avirulent strains) or F₇ (for *nc7*), which actually is incorporated into virions.

Figure 7 also shows that in extracts of avirulent strain-infected cells, F₀ (but not F_A) was present after a 4-min pulse. However, after a 30-min chase, the radioactivity in the F₀ band appeared to be quantitatively transferred to the F_A band. In contrast, only polypeptides with equal or slightly more rapid migration rates than F₀ accumulated in the chased samples of cells infected with AV-WT, *nc4*, or *nc7*. Thus, no significant amounts of a polypeptide comparable to F₇ is seen in *nc7*-infected cells.

Chymotryptic peptide mapping showed that the F₇ polypeptide was indeed related to the F₀ polypeptide of AV-WT (Fig. 8). The chymotryptic peptide patterns derived from the F_A polypeptides appeared identical to those derived from the F₀ polypeptides of the avirulent strains, except that each band is shifted to a somewhat smaller size. Therefore, F_A and F₇ probably represent incomplete or aberrant cleavage products of the F₀ polypeptide.

F₇ affects virus spread. Nagai et al. (25) showed that in cell types presumably lacking the appropriate protease to facilitate cleavage of F₀ to F, avirulent strains of NDV produce noninfectious virions and are therefore unable to

FIG. 3. Pulse-chase analysis of intracellular polypeptides. A, Detail of the L polypeptide region of the 7% gel shown in B. The gel was run at 120 V constant voltage; cell extracts were prepared as described in the text. P, Pulsed for 4 min with [35 S]methionine; C, pulsed for 4 min with [35 S]methionine and chased for 30 min with an excess of unlabeled methionine.

FIG. 4. Chymotryptic peptide mapping of the X polypeptides. A, X polypeptides derived from strains nc4 [X(4)] and nc16 [X(16)] were compared with the P polypeptides derived from cells infected with AV-WT (P), NJ LaSota [P(N)], or B1-Hitchner [P(B)]. Radiolabeled polypeptides were cut from dried SDS-polyacrylamide gels, subjected to limited digestion with chymotrypsin, and electrophoresed in a 15% SDS-polyacrylamide gel as described in the text. B, Separate experiment in which the X polypeptides were compared with the HN, F₀, NP, 46K, and M polypeptides derived from AV-WT-infected cells and the P polypeptides derived from cells infected with NJ LaSota [P(N)] or B1-Hitchner [P(B)].

FIG. 5. Polypeptides of *nc4* and its revertants. The autoradiogram shown resulted from a 15% SDS-polyacrylamide gel run for 16 h at 15 mA constant current. U, Uninfected; AV, AV-WT; 9, *nc9*; 4, *nc4*; 4S, *nc4S1*; 4SR, *nc4S1R1*.

spread from cell to cell and form plaques. Failure to convert F_0 into F was also correlated with the avirulent strain's inability to induce FFWI in those cell types, since the addition of trypsin to the culture medium facilitated FFWI. Similarly, virions of avirulent strains were rendered more infectious after a brief treatment with trypsin, and plaque formation was possible only when trypsin was present in the agar overlay. Embryonated hen eggs appear to be a permissive system for the avirulent strains, presumably because the virus grows in chorioallantoic membrane cells which are also permissive in tissue culture (1, 25). Mutant *nc7*, but not *nc4* or *nc16*, behaved similarly to the avirulent strains in all of these respects (Table 3). Mutant *nc7* was previously shown to grow to approximately 10-fold-lower titers than AV-WT in chicken embryo cells, but to equivalent titers in embryonated hen eggs (20). A brief trypsin treatment increased by sevenfold (relative to AV-WT) the infectivity of a chicken embryo cell-grown stock of mutant *nc7*. The presence of trypsin in the culture medium also facilitated FFWI by *nc7*. In

addition, the presence of trypsin in the agar overlay allowed mutant *nc7* to form larger hemadsorbing spots, i.e., facilitated virus spread from cell to cell. Finally, Fig. 9A shows the disappearance of the F_7 polypeptide after a brief treatment with trypsin of *nc7* virions produced in tissue cultures. (Under the gel conditions used here, the F_1 polypeptide does not separate from NP.)

Levels of the F_1 polypeptide in radiolabeled virions of both *nc7* and AV-WT were determined before and after trypsin treatment by extracting such virions in nonreducing buffer, subjecting the extracts to polyacrylamide gel electrophoresis, cutting out the bands corresponding to the F_0 - F_7 region of the dried gel (Fig. 10A) and re-electrophoresing these bands after rehydration in a reducing buffer (Fig. 10B). Virions of *nc7* did contain detectable levels of F_1 , but less than were found in virions of AV-WT (Fig. 10B). Trypsin treatment appears to convert F_7 into F_1 . Thus, like the avirulent strains of NDV, mutant *nc7* appears to contain a lesion, suppressible by the addition of exogenous protease, which results in defective cleavage of F_0 into F_1 and, thus, affects viral spread.

A small-plaque-forming revertant of *nc7*. To determine whether the accumulation of the F_7 polypeptide and either the noncytopathic or the small-spot-size phenotypes were causally related, we isolated a small-plaque-forming revertant (*nc7R1*) from a stock of *nc7* and analyzed the polypeptides in radiolabeled virions of this revertant (Figure 9B). Like mutant *nc7*, *nc7R1* accumulated F_7 in virions. This revertant also remained unable to cause FFWI in infected cell cultures (data not shown). Thus, the F_7 and noncytopathic phenotypes appeared to be genetically separable.

Analysis of the MDT of *nc7R1* revealed a decrease in MDT from 74 h for *nc7* (20) to 63 h (Table 2), suggesting that the remaining F_7 phenotype may account, in part, for the extended MDT which is characteristic of *nc7*.

DISCUSSION

Protein metabolism in *nc* mutant-infected cells. We have previously shown that all of the *nc* mutants are less cytopathic by virtue of lesions which affect their ability to accumulate virus-specific RNA without affecting their ability to produce wild-type levels of infectious virus (20, 23). The present study suggests that their reduced accumulation of RNA results in reduced accumulation of all virus-specific proteins. Although virus-specific RNA accumulation is reduced to 14 to 34% in mutant infection (23), virus-specific protein accumulation was reduced to 30 to 50%, suggesting that in AV-WT

TABLE 2. Phenotypes of the *nc* mutants and their revertants

Virus	Plaques ^a	F _A -F ₇ ^b	P or X ^c	Relative plaque or spot size ^d	MDT ^e
AV-WT	+	No	P	○	42
<i>nc</i> 9	—	No	P	●	63
<i>nc</i> 9R1	+	No	P	○	40
<i>nc</i> 7	—	Yes	P	●	74
<i>nc</i> 7R1	+	Yes	P	○	63
<i>nc</i> 12	—	No	P	●	63
<i>nc</i> 12R1	+	No	P	○	40
<i>nc</i> 17	—	No	P	●	63
<i>nc</i> 17R1	+	No	P	○	48
<i>nc</i> 4	—	No	X	●	74
<i>nc</i> 4S1	—	No	P	●	63
<i>nc</i> 4S1R1	+	No	P	○	44
<i>nc</i> 16	—	No	X	●	86
NJ LaSota	—	Yes	P		90
B1-Hitchner	—	Yes	P		95

^a Plus (+) designates the ability and minus (—) designates the inability to form plaques at 39.5°C after 2 days of incubation on chicken embryo cell cultures.

^b "No" expresses the absence and "yes" expresses the presence of the F_A or F₇ polypeptides in extracts of radiolabeled virions and infected cells as determined by SDS-polyacrylamide gel electrophoresis.

^c "P" or "X" expresses the presence of the P or X polypeptide in extracts of radiolabeled virions and infected cells as determined by SDS-polyacrylamide gel electrophoresis.

^d Relative size of the hemadsorbing spots produced after 2 days of incubation at 39.5°C on chicken embryo cell cultures (20). ○, Plaques; ●, spots.

^e Mean embryo death times were determined as described in the text. Values represent the average of several experiments.

FIG. 6. Virion polypeptides. Radiolabeled virions were prepared as described in the text. Extracts of these virions were electrophoresed in a 15% SDS-polyacrylamide gel run for 18 h at 15 mA constant current. The figure is a composite of two exposure times.

infection, mRNA may be produced in excess of what is required for maximum protein synthesis and virus production. Clinkscales and Bratt have presented evidence suggesting that an excess of

virus-specific mRNA per se may be responsible for an inhibition of host protein synthesis by AV-WT (7). In fact, our study has shown that there is less inhibition of host protein accumulation by the *nc* mutants. Results obtained with the plaque-forming revertants of the *nc* mutants suggest that plaque formation, accumulation of wild-type levels of RNA (23), and inhibition of protein synthesis (Table 1) all corevert. This evidence strengthens the case for a causal relationship between these phenotypes.

Each of the plaque-forming revertants exhibited a decrease in MDT (Table 2), suggesting that the noncytopathic phenotype was at least in part responsible for the extended MDT characteristic of the *nc* mutants—a result consistent with a causal relationship between cytopathogenicity and virulence in vivo.

The only virus-specific polypeptide which differed in its accumulation in mutant-infected cells was the L polypeptide, which accumulated to only 5 to 20% of the levels in AV-WT-infected cells. A pulse-chase experiment provided no evidence for increased degradation of this polypeptide in mutant-infected cells over that seen in AV-WT-infected cells. Thus, the low levels of L observed in mutant infection are likely to result from a decreased rate of synthesis. Alternatively, it is possible that the degradation occurs immediately after synthesis and is already completed in a 4-min pulse.

FIG. 7. Comparison of *nc* mutant and avirulent strain polypeptides. Extracts of radiolabeled virions (V) were compared by SDS-polyacrylamide gel electrophoresis with extracts of infected cell cultures either (P) pulsed for 4 min with [35 S]methionine or (C) pulsed and then chased as described in the text. The 7% gel was run at 120 V constant voltage. U, Uninfected; AV, AV-WT; NJ, NJ LaSota; B, B1-Hitchner. The figure is a composite of two exposure times.

FIG. 8. Chymotryptic peptide mapping of the F and F_A polypeptides. A, Comparison of F_7 with the F_0 polypeptide derived from AV-WT-infected cells. Radiolabeled polypeptides were cut from dried SDS-polyacrylamide gels, subjected to limited digestion with chymotrypsin, and electrophoresed as described in the legend to Fig. 3. B, Comparison by limited digestion with chymotrypsin of F_7 with the F_0 and F_A polypeptides derived from avirulent strain infection and the P polypeptide from AV-WT infection. LaSota, NJ LaSota; B, B1-Hitchner.

The L polypeptide has been implicated in the transcription of virus-specific mRNA from genomic 50S RNA (10). Clearly, the low levels of L could cause the reduced accumulation of virus-specific RNA by the *nc* mutants. The fact that plaque-forming revertants of the *nc* mutants appeared to accumulate nearly wild-type levels of intracellular L is consistent with this interpretation.

Virions of the *nc* mutants contained amounts of the L polypeptide equivalent to those found in virions of AV-WT. Thus, relative abundance of L in virions was not limited by its abundance in infected cells. This finding of equivalent amounts of L in virions of mutants and AV-WT is also consistent with the finding that the *nc* mutants accumulate RNA during in vivo primary transcription at levels equivalent to those accumulated by AV-WT (23). Thus, lowered amounts of L accumulating in mutant infection rather than a lower enzymatic activity may be responsible for the lowered RNA accumulation in mutant-infected cells (23).

The X polypeptide and viral spread. In addition to their lesions in RNA accumulation and their reduced accumulation of the L polypeptide in infected cells, two of the *nc* mutants accumulated altered polypeptide species both in infected cells and in virions. Mutants *nc4* and *nc16* accumulated significant amounts of a poly-

TABLE 3. *Effects of trypsin on mutant nc7*

Virus	Infectivity (PFU/ml or SFU/ml) ^a		FFWI ^b		Relative spot size ^c	
	-Trypsin	+Trypsin	-Trypsin	+Trypsin	-Trypsin	+Trypsin
AV-WT	1×10^7	2×10^7	12	12		
nc7	1.2×10^6	1.6×10^7	4	12	•	●
nc4			8	8	●	●
nc16			9	8	●	●
Uninfected			1	1		

^a Infectivity of virus released from infected chicken embryo cell cultures before and after treatment of virus with 5 μ g of trypsin per ml for 5 min at 37°C. Titers were determined as described previously (20) and are expressed as PFU per milliliter (for AV-WT) or as hemadsorbing spot-forming units (SFU) per milliliter (for nc7).

^b The extent of FFWI was determined in chicken embryo cell cultures infected at a multiplicity of infection of 0.5 and incubated at pH 8.4 for 12 h at 39.5°C. Trypsin (10 μ g/ml) was added at 6 h postinfection in medium containing 1% calf serum. Cultures fixed and stained as described in the text were scored blind by several individuals for the amount of FFWI on an arbitrary scale of 0 to 12. The results represent the average of several experiments.

^c Relative size of hemadsorbing spots was determined after 2 days of incubation at 37.5°C as described previously (20). Infected cultures were overlaid with agar containing either no trypsin or 0.3 μ g of trypsin per ml, medium 199, and 1% NaHCO₃.

FIG. 9. A, Virion polypeptides of nc7. Electrophoretic comparison of the polypeptides contained in virions of nc7 incubated for 5 min at 37°C in the presence (+T) or absence (-T) of 5 μ g of trypsin per ml. as described in the text. The 7% gel was run at 120 V constant voltage. B, Comparison of polypeptides in radiolabeled virions of nc7 and its small plaque-forming revertant, nc7R1, in a 10% gel run at 30 mA constant current.

peptide, X, related by peptide mapping to the P polypeptide of AV-WT (and the avirulent strains, NJ LaSota and B1-Hitchner), but migrating more slowly on SDS-polyacrylamide gels. Neither nc4 nor nc16 accumulated P in

FIG. 10. Trypsin treatment of nc7 virions. A, Radiolabeled virions of AV-WT and nc7 were prepared and incubated either with or without trypsin as described in the text. Virions thus treated were concentrated by centrifugation for 2 h at 18K in a Beckman SW41 rotor at 4°C. The pelleted virus was extracted in gel sample buffer (17) lacking β -mercaptoethanol (nonreducing buffer) and electrophoresed in a 10% SDS-polyacrylamide gel at 30 mA constant current. The F₀-F₇ containing bands were cut out of the dried gel, and the gel was then subjected to autoradiography as shown in the figure. B, F₀-F₇ bands cut out of the gel in (A) were rehydrated in 0.5 M Tris (pH 6.8) containing 0.4% SDS and 2% β -mercaptoethanol (reducing buffer) and re-electrophoresed on a 7% SDS-polyacrylamide gel at 120 V constant voltage.

infected cells or virions. Thus, mutants nc4 and nc16 appear to contain lesions in the gene for P.

We have previously discussed the evidence for an identity between X in the nc mutants and the P polypeptides in the avirulent strains (also termed 'X' at that time; 21). We also noted a

nonidentity between X and the L, HN, F₀, NP, 46K and M polypeptides of AV-WT. However, at that time we erroneously suggested that X might be an aberrant product of the F₀ gene, because the polypeptide since identified as P in AV-WT (9, 24, 28), but previously suggested to be F (13, 14), appeared to be related by peptide mapping to X (21).

Our selection and analysis of a larger-spot-forming revertant from mutant *nc4* support the notions (i) that a lesion in P can affect virus spread, (ii) that virus spread and cytopathogenicity are genetically separable phenotypes, and (iii) that lesions reducing virus spread are also responsible for measurable extensions of MDT. It is likely that mutants *nc4* and *nc16* contain lesions in two genes, one affecting cytopathogenicity and RNA accumulation (in common with the other *nc* mutants) and one affecting virus spread. In support of this hypothesis, none of the other *nc* mutants exhibits alterations in their P polypeptides, and none complements for RNA accumulation with mutants *nc4* and *nc16* (23). Moreover, none of the *nc* mutants complements for RNA accumulation with the group A, RNA⁻ temperature-sensitive mutants of NDV derived from the same parental strain (30), suggesting that they all share a lesion in the A gene (23). The fact that mutants *nc4* and *nc16* spread less well than AV-WT and the other *nc* mutants could reflect reduced growth in single cycles of infection (20, 21). As discussed previously (20), spot size appears to correlate with virus yield. Support for this hypothesis is provided by the demonstration that *nc4S1* grows to titers equivalent to those achieved by AV-WT (data not shown).

Mutant *nc7*, viral spread, and the avirulent strains of NDV. Mutant *nc7* also contains a lesion affecting its spread from cell to cell. This lesion resulted in phenotypes similar to those of the avirulent strains of NDV. First, a form of the F₀ polypeptide is incorporated into virions; the F_A and F₇ polypeptides appeared similar to each other by peptide mapping, migration on SDS-polyacrylamide gels, incorporation into virions, and susceptibility to cleavage by trypsin with a concomitant increase in virion infectivity (Table 1) (25). Furthermore, like the avirulent strains of NDV (25), mutant *nc7* is activated by trypsin for FFWI as well as virus spread (i.e., the formation of larger hemadsorbing spots). The fact that mutant *nc7* makes hemadsorbing spots at all is probably the result of the accumulation of amounts of the F₁ polypeptide in *nc7*-infected cells or *nc7* virions which are sufficient to allow limited cell-to-cell spread. This contrasts with avirulent strains of NDV, which appear to be more restricted in their ability to spread in chicken embryo cells in tissue culture (25).

The selection of *nc7R1*, a small-plaque-forming revertant of *nc7* which still accumulates F₇ in virions and remains unable to cause FFWI, suggests that the noncytopathic and F₇ phenotypes are genetically separable. Revertant *nc7R1* accumulates virus-specific RNA at nearly wild-type levels (23), supporting the hypothesis that viral mRNA accumulation alone can modulate cytopathogenicity. Finally, *nc7R1* exhibited an MDT of 63 h, compared with 74 h for *nc7* (Table 2), suggesting that the lesion resulting in decreased accumulation of virus-specific RNA accounts for some, but not all, of the increase in MDT for mutant *nc7*. Presumably, the F₇ phenotype also contributes to the increase in MDT in this mutant. Supporting this notion is the isolation by Klenk et al. (16) of a mutant of the avirulent NJ LaSota strain of NDV which no longer accumulates a polypeptide like F_A in its virions. This mutant was reported to exhibit a decrease in MDT from 142 to 99 h.

Schloer and Hanson (27) and others (11, 29) have presented ample evidence for a correlation between the plaque size and virulence of variants and mutants of NDV, although the correlation may not be absolute. Certainly a reduction in the ability to spread in one particular cell type need not result in a concomitant reduction in ability to spread in all other cell types. As Granoff has demonstrated, (11), several small-plaque mutants of NDV isolated in chicken embryo cell cultures were increased in their neuropathogenicity for mice. Our studies with the noncytopathic mutants suggest that virulent and avirulent strains of NDV may differ from each other in at least two phenotypes relevant to virulence in vivo: (i) accumulation of virus-specific RNA, which can modulate cytopathogenicity without affecting infectious virus production (20, 23), and (ii) ability of the virus to spread from cell to cell in chicken embryo fibroblast cultures. These phenotypes may affect the balance between virus growth and the immune response (15) or restrict virus growth to certain tissues, cell types, or developmental stages of the host (18, 25, 26).

ACKNOWLEDGMENTS

We are grateful to Rhona Glickman, Judy Brackett, and Shirley Carnegie for excellent technical assistance, to Larry Hightower, Trudy Morrison, and Mark Peebles for helpful discussions, and to Pam Chatis and Anne Chojnicki for help in the preparation of this manuscript.

This study was supported by National Science Foundation grant PCM 76-84135 and Public Health Service grant AI 12467 from the National Institute of Allergy and Infectious Diseases. C.H.M. was a predoctoral trainee of the National Institute of General Medical Sciences.

LITERATURE CITED

1. Bang, F. B. 1953. The development of Newcastle disease virus in cells of the chorioallantoic membrane as studied by thin sections. *J. Exp. Med.* 88:251-266.

2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled protein and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
3. **Bratt, M. A.** 1969. RNA synthesis in chick embryo cells infected with different strains of NDV. *Virology* **38**: 485-488.
4. **Bratt, M. A., and W. R. Gallaher.** 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus. *Proc. Natl. Acad. Sci. U.S.A.* **64**:536-543.
5. **Bratt, M. A., and W. R. Gallaher.** 1970. Comparison of fusion from within and fusion from without by Newcastle disease virus. *In Vitro* **6**:3-14.
6. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102-1106.
7. **Clinkscales, C. W., and M. A. Bratt.** 1978. Inhibition of protein synthesis by Newcastle disease virus, p. 617-627. *In* B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press, Inc., London.
8. **Clinkscales, C. W., M. A. Bratt, and T. G. Morrison.** 1977. Synthesis of Newcastle disease virus polypeptides in a wheat germ cell-free system. *J. Virol.* **22**:97-101.
9. **Collins, P. L., L. E. Hightower, and L. A. Ball.** 1980. Transcriptional map for Newcastle disease virus. *J. Virol.* **35**:682-693.
10. **Colonno, R. J., and H. O. Stone.** 1976. Isolation of a transcriptive complex from NDV virions. *J. Virol.* **19**: 1080-1089.
11. **Granoff, A.** 1961. Induction of Newcastle disease virus mutants with nitrous acid. *Virology* **9**:636-645.
12. **Hanson, R. P.** 1964. Newcastle disease virus: an evolving pathogen. University of Wisconsin Press, Madison.
13. **Hightower, L. E., and M. A. Bratt.** 1974. Protein synthesis in Newcastle disease virus-infected chicken embryo cells. *J. Virol.* **13**:788-800.
14. **Hightower, L. E., T. G. Morrison, and M. A. Bratt.** 1975. Relationships among the polypeptides of Newcastle disease virus. *J. Virol.* **16**:1599-1607.
15. **Karzon, D. T., and F. B. Bang.** 1951. The pathogenesis of infection with a virulent (CG 179) and an avirulent (B) strain of Newcastle disease virus in the chicken. I. Comparative rates of viral multiplication. *J. Exp. Med.* **93**:267-284.
16. **Klenk, H. D., R. Rott, Y. Nagai, and W. Berk.** 1978. The pathogenicity of Newcastle disease virus as depending on the structure of the viral glycoproteins, p. 607-616. *In* B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press, Inc., London.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
18. **Liu, C., and F. B. Bang.** 1953. An analysis of the difference between a destructive and a vaccine strain of NDV (Newcastle disease virus) in the chick embryo. *J. Immunol.* **70**:538-548.
19. **Lomniczi, B., A. Meager, and D. C. Burke.** 1971. Virus RNA and protein synthesis in cells infected with different strains of Newcastle disease virus. *J. Gen. Virol.* **13**: 111-120.
20. **Madansky, C. H., and M. A. Bratt.** 1978. Noncytopathic mutants of Newcastle disease virus. *J. Virol.* **26**:724-729.
21. **Madansky, C. H., and M. A. Bratt.** 1978. Noncytopathic mutants of Newcastle disease virus: comparison with naturally-occurring avirulent strains, p. 709-720. *In* B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press, Inc., London.
22. **Madansky, C. H., and M. A. Bratt.** 1980. A genetic approach to cytopathogenicity, virus spread, and virulence of Newcastle disease virus (NDV), p. 673-684. *In* B. Fields, R. Jaenisch, and C. F. Fox (ed.), *Animal virus genetics*, ICN-UCLA Symposia on Molecular and Cellular Biology XVIII. Academic Press, Inc., New York.
23. **Madansky, C. H., and M. A. Bratt.** 1981. Noncytopathic mutants of Newcastle disease virus are defective in virus-specific RNA synthesis. *J. Virol.* **37**:317-327.
24. **Morrison, T. G., and D. Simpson.** 1980. Synthesis, stability, and cleavage of Newcastle disease virus glycoproteins in the absence of glycosylation. *J. Virol.* **36**: 171-180.
25. **Nagai, Y., H. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoprotein and its significance for the virulence of Newcastle disease virus. *Virology* **72**:494-508.
26. **Nagai, Y., K. Shimokata, T. Yoshida, M. Hamaguchi, M. Iinuma, K. Maeno, T. Matsumoto, H. D. Klenk, and R. Rott.** 1979. The spread of a pathogenic and an apathogenic strain of Newcastle disease virus in the chicken embryo as depending on the protease sensitivity of the virus glycoproteins. *J. Gen. Virol.* **45**:263-272.
27. **Schloer, G. M., and R. P. Hanson.** 1968. Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. *J. Virol.* **2**:40-47.
28. **Smith, G. W., and L. E. Hightower.** 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. *J. Virol.* **37**:256-267.
29. **Thiry, L.** 1964. Some properties of chemically induced small-plaque mutants of Newcastle disease virus. *Virology* **24**:6-15.
30. **Tsipis, J. E., and M. A. Bratt.** 1976. Isolation and preliminary characterization of temperature-sensitive mutants of Newcastle disease virus. *J. Virol.* **18**:848-855.