Temperature-Sensitive Mutants Isolated from L Cells Persistently Infected with Newcastle Disease Virus

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Virus mutants (NDV_{pi}) isolated from L cells persistently infected with the Herts strain of Newcastle disease virus have been previously reported by this laboratory to differ from the wild-type virus (NDV_o) in several physical and biological properties. It has now been determined that, in addition to these differences, the NDV_{pi} mutants are also spontaneously selected temperature-sensitive mutants. The temperature sensitivity of 10 NDV_{pi} clones was confirmed by temperature inhibition, plaquing efficiency, and single-cycle yield experiments. The cut-off temperature, at which more than 90% of virus replication is inhibited was between 41 and 42 C. All 10 NDV_{pi} clones were also found to be defective in virus-specific ribonucleic acid (RNA) synthesis in infected chick embryo cells at 42 C and are tentatively classified as RNA⁻. The possible relationships of the temperature sensitivity, the other NDV_{pi} properties, and the maintenance of the persistently infected state are discussed.

Temperature-sensitive (*ts*) mutants, which provide a useful tool for the study of virus genetics and replication, have been described for ribonucleic acid (RNA)-containing, enveloped animal viruses such as Sindbis (2), Semliki Forest (9), influenza (7), and vesicular stomatitis viruses (6, 8). The functional defects of some of these mutants at the nonpermissive temperature have been investigated (4, 8, 9, 13), and genetic analysis has been done using complementation tests (3, 5, 7, 9, 13). The *ts* mutants employed in the studies cited were obtained by mutagen treatment of wild-type virus, since spontaneous mutation rates are very low.

In 1967 in this laboratory, a line of L cells (L_{NDV}) was established which was persistently infected with the Herts strain (NDV_o) of Newcastle disease virus (10). Virus (NDV_{pi}) isolated from the L_{NDV} cell cultures differed from the wild-type in the following characteristics: plaque size in permissive primary chick embryo cell cultures, thermostability of infectivity and neuraminidase activity, and ability to replicate in L cells (10, 12). This paper reports that, in addition to these differences, NDV_{pi} clones isolated from the persistent infection are spontaneously selected, temperature-sensitive mutants.

MATERIALS AND METHODS

Cell cultures. Primary chick embryo (CE) cells and mouse L cells (clone 929) were propagated and

maintained in Eagle's minimal essential medium containing 4% calf serum as described previously (14).

Viruses. The Herts strain of NDV (NDV_o) and NDV_{pi} clone 20 described in this communication have been characterized previously (10, 11, 12). NDV_{pi} clone 20 was isolated from L_{NDV} cells a few months after the persistent infection was established in 1967. NDV_{pi} clones 25 to 38 were separated in 1969 from an uncloned stock of NDV_{pi} isolated from L_{NDV} cells; this stock was passed four times in CE cells prior to cloning. The yield from the fourth passage was plated on CE cell monolayers at a dilution sufficient to provide 5 to 10 plaques per plate, and the monolayers were washed twice before addition of the nutrient agar overlay. The agar above 15 well-isolated plaques was transferred to CE cell monolayers in 2-oz (60 ml) prescription bottles. Infected fluids harvested at 24 or 48 hr served as seed stock for the growth of virus in the allantoic cavity of 9-day-old embryonated eggs. With NDV_o, eggs dead at 48 hr were harvested; for NDV_{pi}, only eggs alive at 48 hr were harvested (10).

Fifteen NDV_{pi} clones isolated in 1969 and three NDV_{pi} clones previously studied were grown in eggs as described. Infected allantoic fluid pools were assayed for infectivity and examined for large plaque revertants (10). Several NDV_{pi} clones (clones 22, 33, 34) were discarded at this point; only NDV_{pi} clones yielding 100% small plaques in CE cell cultures were used for further study. Subsequently, NDV_{pi} clones 19 and 27 were discarded due to a high reversion rate of the *ts* property.

Virus in allantoic fluid was clarified by low-speed

centrifugation and then concentrated by centrifugation at $78,000 \times g$ for 1 hr. The supernatant fluid was discarded, and the pellet was suspended in phosphate-buffered saline (PBS, pH 7.2) with the aid of sonic oscillation to give a 20-fold concentration of virus. Virus prepared in this manner was stored at -70°C .

Assays. Infectivity, hemagglutinin, and neuraminidase assays were performed as previously described (12). In all infectivity assays, adsorption was carried out for 1 hr at 37°C . Interferon was assayed by a plaque reduction technique with vesicular stomatitis virus as the challenge virus (14).

Thermal inactivation of virus activities. Concentrated virus preparations were diluted at least 100-fold in PBS and heated by using procedures which have been described in detail elsewhere (10, 12).

Infection of CE cells. Virus was diluted in medium to a concentration sufficient to provide a calculated input multiplicity of infection (MOI) of 10 to 20 plaque-forming units (PFU) per cell when 0.5 ml of virus suspension was added to a 60-mm petri dish ($\sim 3 \times 10^6$ cells). After adsorption of virus for 60 min at 37°C , the monolayers were washed twice with warm medium, and 3 ml of fresh medium was added to each culture. Incubation was then continued at either 37 or 42°C in humidified incubators with an atmosphere of 5% CO_2 . At appropriate intervals, culture fluids were harvested for assay of released virus (RV). To determine cell-associated virus (CAV), the cells were scraped from the glass into 3 ml of fresh medium and disrupted by 3 min of sonic oscillation. Infectivity assays of these samples were performed in CE cell cultures at 37°C .

Cut-off temperature. Virus yield at different temperatures was determined in the following manner. CE cell monolayers ($\sim 4 \times 10^6$ cells) in 2-oz prescription bottles with rubber-lined caps were infected for 1 hr at 37°C with NDV_o or NDV_{pi} (MOI = ~ 20 PFU/cell). The monolayers were rinsed twice to remove unadsorbed virus, and 7 ml of warm medium was added per bottle. The bottles were immersed in thermostatically controlled water baths maintained at the following temperatures ($\pm 0.05^\circ\text{C}$): 37 , 38.5 , 40 , 41 , and 42°C . Infected fluids and cells were harvested 24 hr after infection, as described above, for assay of released and cell-associated virus.

Virus-specific RNA synthesis. Cumulative virus-specific RNA synthesis in CE cells at 37 and 42°C was determined as follows. Cell monolayers ($\sim 3 \times 10^6$ cells) were infected for 1 hr at 37°C with 20 PFU of either NDV_o or NDV_{pi} per cell, as described above. After the adsorption period, replicate cultures were incubated at 37 or 42°C . Control cultures were mock-infected and equilibrated at each temperature. At 2.5 hr postinfection, the culture fluids were replaced with medium containing $2 \mu\text{g}$ of actinomycin D/ml, and incubation was continued at 37 or 42°C . One hour later, the medium was again replaced with medium containing actinomycin D (a gift from H. B. Woodruff of the Merck & Co., Inc.) and $1 \mu\text{Ci}$ of ^3H -uridine (specific activity 2.0 Ci/mmol , Schwarz BioResearch, Inc.) per ml. At appropriate times, the medium was removed, and the monolayers were

washed twice with cold Hanks salt solution and disrupted by addition of 2 ml of 1% sodium dodecyl sulfate (SDS). Cold 10% trichloroacetic acid was added to the disrupted cell suspension; the precipitate was collected on nitrocellulose filters and washed twice with cold 5% trichloroacetic acid. The filters were allowed to air-dry under gentle suction and the acid-insoluble radioactivity was counted in 10 ml of Bray's (1) scintillation fluid in a Packard 3375 Tri-Carb liquid scintillation spectrometer.

RESULTS

Properties of NDV_{pi} mutants. Previous reports from this laboratory have summarized the physical and biological properties of NDV_o and NDV_{pi} (10, 11, 12). The infectivity of NDV_o is stable for 1 hr at 50°C , whereas the infectivity of NDV_{pi} is not. The reverse situation is true with regard to thermal stability of hemagglutinin and neuraminidase activities: 30 min at 55°C completely destroys the hemagglutinin and significantly reduces the neuraminidase activity of NDV_o, whereas these virion-associated activities of NDV_{pi} are stable under the same conditions. NDV_o is also much more lethal for eggs than the NDV_{pi} clones; 10^4 PFU of NDV_o inoculated into the allantoic cavity of 9-day-old embryonated eggs produces 100% mortality by 48 hr, whereas the mortality produced by the same inoculum of NDV_{pi} clones ranges from 6 to 70%. Although both NDV_o and NDV_{pi} induce production of significant amounts of interferon in mouse L cell cultures, NDV_o produces a covert infection, whereas NDV_{pi} undergoes a normal growth cycle with a latent period of 6 to 8 hr (11).

Since NDV_{pi} clones 25 to 38 were isolated from L_{NDV} cell cultures more than 2 years after the isolation of the original NDV_{pi} clones, an examination of the properties of NDV_{pi} clones 25 to 38 was undertaken to determine whether they were similar to the NDV_{pi} clones previously studied. The results indicated that the properties of the more recently isolated NDV_{pi} clones were consistent with those previously published. Subtle differences, however, were observed between NDV_{pi} clone 20 and NDV_{pi} clones 25 to 38. The plaques formed in permissive primary CE cell cultures by clones 25 to 38 were smaller than the plaques formed by NDV_{pi} clone 20 under the same conditions, and the lethality for eggs was lower (6 to 60% dead at 48 hr) for clones 25 to 38 than for clone 20 ($\sim 75\%$ dead at 48 hr). More recently isolated NDV_{pi} clones also plaqued more efficiently on L cells than did clone 20; the L cell-CE cell titer ratios for clones 25 to 38 and for clone 20 were 0.5 to 1.0 and ~ 0.01 , respectively. Small differences were also observed in the ther-

mostability experiments: the hemagglutinin and neuraminidase activities of clones 25 to 38 were more stable to heating than were the same activities of NDV_{pi} clone 20, whereas the infectivity was less stable to heating than the infectivity of NDV_{pi} clone 20. These differences are discussed below.

Temperature sensitivity of NDV_{pi}. Preliminary experiments with NDV_o and NDV_{pi} indicated that the NDV_{pi} mutants characterized above were also temperature-sensitive. CE cell monolayers infected with 20 PFU of NDV_o or NDV_{pi} per cell were incubated at 37, 38.5, 40, 41, or 42 C for 24 hr. RV and CAV were assayed in CE cell cultures at 37 C. The total yield/ml and the fractional yield at each temperature are shown in Table 1. The yield of NDV_o dropped only slightly over this temperature range, whereas the yield of all NDV_{pi} clones was significantly decreased at 42 C. At this temperature, there was at least 95% inhibition of NDV_{pi} virus yield, and, in most cases, there was an abrupt inhibition of virus production between 41 and 42 C. The lowered yield of NDV_{pi} at 42 C was not due to thermal inactivation of virus; no significant difference was found in the rates of thermal inactivation of infectivity of NDV_o and NDV_{pi} at this temperature.

In addition to the cut-off temperature experiments, the plaquing efficiency of the NDV_{pi} clones at 42 C was determined by performing duplicate titrations of virus stocks at 37 and 42 C. The plaquing efficiency was calculated by dividing the titer at 42 C by the titer obtained at 37 C. With

NDV_o, the plaquing efficiency was 0.76; with the NDV_{pi} mutants, the plaquing efficiency ranged from 10⁻³ to 10⁻⁸. These results are consistent with the temperature inhibition data (Table 1) and indicate that all of the NDV_{pi} clones are temperature-sensitive.

The reversion frequency of the *ts* mutation of 12 NDV_{pi} clones was determined as follows. CE cell cultures were infected with 5 to 10 PFU/monolayer and incubated at 37 C. The agar above single, well isolated plaques was transferred to CE cell cultures at 37 C in 2-oz prescription bottles, and the 24-hr yield of virus was assayed in CE cell cultures at 37 and 42 C. The PFU/ml at 42 C divided by the PFU/ml at 37 C was considered a measure of the reversion frequency. The reversion frequencies (Table 2) varied from 1.3 × 10⁻³ (clone 27) to <10⁻⁵ (clones 29, 32, and 37) and depended more on the 37 C titer than on the number of plaques obtained at 42 C. NDV_{pi} clones 19 and 27 were discarded due to their high reversion rates.

The temperature sensitivity of NDV_{pi} was confirmed by one-cycle growth experiments in CE cell cultures at 37 and 42 C. CE cell monolayers were infected with 10 PFU of NDV_o or NDV_{pi} per cell for 1 hr at 37 C and washed to remove unadsorbed virus; duplicate samples were incubated at 37 and 42 C. At various times after infection, RV and CAV were determined by assay in CE cell cultures at 37 C (Fig. 1). The growth curve of NDV_o (Fig. 1A) at 42 C was very similar to that obtained at 37 C. The latent period (2 to

TABLE 1. Replication of NDV_o and NDV_{pi} clones at different temperatures^a

Virus	Total PFU/ml and fractional yield				
	37 C	38.5 C	40 C	41 C	42 C
NDV _o	9.0 × 10 ⁷ (1.0)	7.6 × 10 ⁷ (0.84)	6.7 × 10 ⁷ (0.74)	7.4 × 10 ⁷ (0.83)	3.1 × 10 ⁷ (0.29)
NDV _{pi} Cl. 20	9.5 × 10 ⁶ (1.0)	7.7 × 10 ⁶ (0.81)	1.3 × 10 ⁶ (0.13)	1.0 × 10 ⁶ (0.05)	5.5 × 10 ⁴ (5.8 × 10 ⁻³)
NDV _{pi} Cl. 25	2.7 × 10 ⁷ (1.0)	2.1 × 10 ⁷ (0.81)	6.5 × 10 ⁶ (0.24)	5.8 × 10 ⁶ (0.22)	1.2 × 10 ³ (4.4 × 10 ⁻⁶)
NDV _{pi} Cl. 29	9.3 × 10 ⁵ (1.0)	5.8 × 10 ⁵ (0.62)	3.8 × 10 ⁵ (0.41)	3.3 × 10 ⁵ (0.35)	6 × 10 ¹ (6.5 × 10 ⁻⁵)
NDV _{pi} Cl. 30	3.3 × 10 ⁶ (1.0)	9.9 × 10 ⁵ (0.3)	2.4 × 10 ⁵ (0.07)	2.1 × 10 ⁵ (0.06)	1.0 × 10 ⁵ (0.03)
NDV _{pi} Cl. 35	5.0 × 10 ⁶ (1.0)	2.5 × 10 ⁶ (0.5)	1.1 × 10 ⁶ (0.22)	6.9 × 10 ⁴ (1.4 × 10 ⁻²)	1.4 × 10 ³ (2.8 × 10 ⁻⁴)
NDV _{pi} Cl. 38	3.5 × 10 ⁷ (1.0)	3.0 × 10 ⁷ (0.86)	8.0 × 10 ⁶ (0.23)	3.0 × 10 ⁶ (0.08)	7.0 × 10 ² (2.0 × 10 ⁻⁵)

^a CE cell cultures (~4 × 10⁶ cells) in 2-oz prescription bottles were infected with NDV_o or NDV_{pi} (MOI = 20) for 1 hr at 37 C and washed twice to remove unadsorbed virus; replicate cultures were incubated in water baths maintained at the appropriate temperatures (± 0.05 C). At 24 hr after infection, fluids and cells were harvested. Total plaque-forming units (PFU)/ml and the fractional virus yield at each temperature were calculated with the 37 C virus yield as standard.

TABLE 2. Reversion frequencies of temperature-sensitivity of NDV_{pi}^a

Virus	PFU/ml		Reversion frequency (42 C/37 C)
	37 C	42 C	
NDV _{pi} clone 19	1.4×10^5	5.8×10^2	4.1×10^{-3}
20	1.3×10^5	8.2×10^2	6×10^{-4}
25	2.1×10^5	$<10^1$	$<5 \times 10^{-5}$
27	6.7×10^4	1×10^2	1.3×10^{-3}
29	5.7×10^5	$<10^1$	$<1.8 \times 10^{-5}$
30	2.7×10^4	1×10^1	3.7×10^{-4}
31	1.3×10^5	2×10^1	1.5×10^{-4}
32	1.8×10^5	$<10^1$	$<5 \times 10^{-5}$
35	1.1×10^5	1.4×10^2	1.2×10^{-4}
36	8×10^5	2×10^1	2.5×10^{-5}
37	1.2×10^5	$<10^1$	$<8.3 \times 10^{-5}$
38	4.5×10^4	$<10^1$	$<2.2 \times 10^{-4}$

^a CE cell monolayers were infected at 37 C with 5 to 10 plaque-forming units (PFU)/monolayer with virus grown in CE cells. The monolayers were rinsed to remove unadsorbed virus, and nutrient agar overlay was added. Single, well isolated plaques were transferred to CE cells in 2-oz prescription bottles at 37 C. Infected culture fluids were harvested at 24 hr and assayed for infectivity at 37 and 42 C. The reversion frequency was calculated by dividing the titer at 42 C by the titer at 37 C.

4 hr) was followed by a rapid increase in both RV and CAV. The peak of the growth cycle was reached slightly later at 42 C than at 37 C, but the maximum yields were almost identical. The decline in both RV and CAV at 42 C between 12 and 24 hr probably was due to slow inactivation of the virus at this temperature.

The replication pattern of the NDV_{pi} clones is presented in Fig. 1B. The data shown were obtained with NDV_{pi} clone 25, but the results with the other clones were very similar. At 37 C, a latent period of 6 to 8 hr was followed by a rise in virus production until about 12 hr postinfection. No production of infective virus occurred at 42 C, as evidenced by a steady decline in both RV and CAV at this temperature. The low level of revertants present in the NDV_{pi} populations (Table 2) did not influence the single-cycle yield experiments.

Virus-specific RNA synthesis in infected CE cells at 37 and 42 C. To determine whether NDV_{pi} mutants direct virus-specific RNA synthesis in CE cells at 42 C, the following experiment was performed. CE cell monolayers were infected with NDV_o or NDV_{pi} (MOI = 20), and replicate monolayers were incubated at 37 and 42 C. The infected cultures were treated with actinomycin D for 1 hr and then exposed to 1 μ Ci of tritiated uridine (³H-U)/ml. Uninfected CE cell monolayers with and without actinomycin D incubated at each temperature served as controls. At various times from 4.5 to 9.5 hr postinfection, samples were assayed for trichloroacetic acid-insoluble radioactivity.

Actinomycin D inhibited over 98% of host-cell RNA synthesis at 37 and 42 C by 1 hr. Untreated cultures exposed to 1 μ Ci of ³H-U/ml for 1 hr incorporated over 2,500 counts per min per 10⁶ cells into acid-insoluble material, whereas actinomycin D-treated cultures incorporated an average of 30 counts per min per 10⁶ cells under similar conditions. This level of inhibition of host-cell RNA synthesis was maintained throughout the experiment.

At 42 C, NDV_o-specific RNA synthesis was 71% of that at 37 C (850 to 1,300 counts per min per 10⁶ cells at 9.5 hr after infection). At both temperatures, viral RNA synthesis was first detected between 5 and 6 hr after infection. This finding correlates with the slightly decreased virus yield observed at 42 C (Table 1). Uninfected, untreated CE cell cultures also exhibited a slightly decreased cumulative synthesis of host-cell RNA at 42 C; after 6 hr, the incorporation of ³H-U at 42 C was 73% of that obtained at 37 C. It is interesting to note that both host-cell RNA and NDV_o-RNA synthesis were similarly reduced at 42 C; this reduction may represent an inherent inefficiency of RNA synthesis at this temperature.

In cells infected with NDV_{pi}, viral RNA synthesis at 37 C was on the average 50% lower than in NDV_o-infected cells. Cumulative virus-specific RNA synthesis in CE cells infected with NDV_o or NDV_{pi} at 42 C is shown in Fig. 2. Very little NDV_{pi}-specific RNA was synthesized at any time during infection at the nonpermissive temperature. The possible exception is NDV_{pi} clone 29, which showed a slight increase in incorporation of

FIG. 1. Single-cycle growth curves of NDV_o and NDV_{pi} in chicken embryo (CE) cells at 37 and 42 C. CE cell cultures were infected for 1 hr at 37 C with NDV_o or NDV_{pi} (MOI = 10) and washed to remove excess virus; duplicate cultures were incubated at 37 and 42 C. At appropriate intervals, the culture fluids were collected for determination of released virus (RV), and the cells were disrupted by 3 min of sonic oscillation for determination of cell-associated virus (CAV). Solid symbols, 37 C; open symbols, 42 C; $\blacktriangle$, $\triangle$, CAV; $\bullet$, $\circ$, RV; a, NDV_o ; b, NDV_{pi} clone 25.

3H -U between 7.5 and 8.5 hr postinfection. Cells infected with NDV_{pi} clones 31, 35, 37, or 38 had low levels of acid-insoluble radioactivity present at all times later than 4.5 hr postinfection.

Table 3 lists the NDV_{pi} clones examined to date and their tentative classification with regard to RNA phenotype at 42 C. Two criteria were used to establish the RNA phenotype of a partic-

ular NDV_{pi} clone: (i) the per cent of wild-type RNA synthesis at 42 C and (ii) the shape of the cumulative RNA synthesis curve (Fig. 2). Classification of NDV_{pi} clones 20, 25, 30, 32, and 36 is unambiguous: no virus-specific RNA synthesis could be detected at any time during the growth cycle at 42 C. NDV_{pi} clone 29 was classified RNA⁻ with some reservation because of the borderline significance of the viral RNA synthesis detected at 42 C and the small rise in cumulative RNA synthesis seen between 7.5 and 8.5 hr after infection. NDV_{pi} clones 31, 35, 37, and 38 are all tentatively classified as RNA⁻, even though there is a low level of acid-insoluble radioactivity present during incubation at 42 C. However, since the cumulative RNA synthesis curves are essentially flat, one cannot assume that this really represents significant RNA synthesis. The possibility that the small amount of incorporation of 3H -U is due to the virion-associated RNA-polymerase is currently under study.

TABLE 3. RNA phenotype of NDV_{pi} mutants in CE cell cultures at 42 C^a

Virus	Per cent of wild-type RNA synthesis at 42 C	RNA phenotype (42 C)
NDV_{pi} Cl. 20	0 ^b	—
NDV_{pi} Cl. 25	0 ^b	—
NDV_{pi} Cl. 29	12.5	(—) ^c
NDV_{pi} Cl. 30	1.3	—
NDV_{pi} Cl. 31	7.9	—
NDV_{pi} Cl. 32	5.5	—
NDV_{pi} Cl. 35	9.1	—
NDV_{pi} Cl. 36	0 ^a	—
NDV_{pi} Cl. 37	9.5	—
NDV_{pi} Cl. 38	6.4	—

^a CE cell cultures ($\sim 3 \times 10^6$ cells) were infected with NDV_o or NDV_{pi} (MOI = 20) for 1 hr at 37 C, washed twice to remove unadsorbed virus, and incubated at 42 C. Control cultures were mock-infected and equilibrated at each temperature. At 2.5 hr after infection, 2 μ g of actinomycin D/ml was added to each culture, and 1 hr later all cultures were exposed to 1 μ Ci of 3H -U/ml. At various times, the culture fluids were removed and the cells were disrupted with 1% sodium dodecyl sulfate. 10% trichloroacetic acid-insoluble radioactivity was counted in 10 ml of Bray's scintillation fluid. The per cent of wild-type RNA synthesis was calculated by dividing the counts per minute per 10^6 cells obtained from NDV_{pi} -infected cultures by the counts per minute per 10^6 cells obtained from NDV_o -infected cells at 9.5 hr after infection.

^b No incorporation of 3H -U above that in uninfected, actinomycin D-treated CE cells.

^c Parentheses indicate uncertainty.

FIG. 2. Cumulative virus-specific RNA synthesis at 42 C in chicken embryo (CE) cell cultures infected with NDV_o or NDV_{pi} clones. CE cell monolayers were infected for 1 hr at 37 C with 20 PFU of NDV_o or NDV_{pi}/cell. Cultures were incubated at 42 C. At 2.5 hr postinfection, the cultures were treated with 2 μ g of actinomycin D/ml and 1 hour later were exposed to 1 μ Ci of ³H-U/ml. At the intervals indicated, the medium was discarded, the monolayers were rinsed with cold Hanks solution, and the cells were disrupted with 1% SDS. The 10% trichloroacetic acid-insoluble radioactivity was counted in a liquid scintillation counter. Background ($\sim$ 20 counts/min) and actinomycin D control ($\sim$ 40 counts/min) have been subtracted. (a) $\bullet$, NDV_o; Δ , NDV_{pi} clones 20, 25, 30, 36; $\circ$, NDV_{pi} clone 29; $\blacktriangle$, NDV_{pi} clone 31. (b) $\bullet$, NDV_o; $\blacksquare$, NDV_{pi} clone 32; $\circ$, NDV_{pi} clone 35; $\blacktriangle$, NDV_{pi} clone 37; $\triangle$, NDV_{pi} clone 38.

DISCUSSION

Analysis of NDV_o and 10 randomly chosen NDV_{pi} clones indicates that all 10 mutants are temperature-sensitive at 42 C. The cut-off temperature, at which more than 90% of virus replication is inhibited, is between 41 and 42 C.

Growth curves of NDV_{pi} mutants at 42 C confirm that virus replication is markedly defective at this temperature. Experiments with radioactive precursor also indicate that all 10 NDV_{pi} clones are probably RNA⁻ at 42 C; they do not produce significant amounts of virus-specific RNA in infected CE cells at the nonpermissive temperature.

Taken together, however, these results are surprising in several ways. The NDV_{pi} mutants were isolated from a persistent infection of L cells begun with wild-type NDV_o. The mechanism of selection of the mutants by the persistent infection remains unknown, but it appears to select for temperature sensitivity as well as for other altered physical and biological properties. All 18 NDV_{pi} clones tested so far have displayed varying degrees of temperature sensitivity, although only those clones with low reversion frequencies were employed in the experiments described above. The genetic relationship of the *ts* mutation and the other NDV_{pi} properties is complex. The mutations seem to be co-selected, but experiments now in progress with *ts*⁺ revertants, obtained by purification of plaques formed on CE cell monolayers at 42 C, indicate that the *ts* mutation can revert independent of the mutation(s) responsible for small plaque size and altered thermostability of virion-associated activities, but may be linked to the ability to replicate in mouse L cells.

The second puzzling phenomenon is that NDV_{pi} clones 25 to 38, isolated from L_{NDV} cell cultures in 1969, seem to be "more mutant" than NDV_{pi} clone 20, isolated from L_{NDV} ancestor cell cultures in 1967. NDV_{pi} clones 25 to 38 plaque equally well in L cells and CE cells, whereas clone 20 has a much lower ratio of L cell titer-CE cell titer. Plaques formed on permissive primary CE cells by the clones isolated in 1969 are much smaller than the plaques formed by clone 20 under identical conditions. Hemagglutinin and neuraminidase activities are more stable, and infectivity is less stable for NDV_{pi} clones 25 to 38 than for NDV_{pi} clone 20. These data suggest that genetic studies with these mutants, as well as with mutants obtained from newly established persistently infected L cell cultures, may help elucidate the "conversion" of NDV_o to NDV_{pi} via the selection pressures within the persistent infection.

The third surprising result is that all of the NDV_{pi} mutants apparently exhibit RNA⁻ phenotypes at the nonpermissive temperature. Perhaps in the persistent infection, the *ts* mutations are not random and may play a role in the maintenance of the persistent infection or in the adaptation of NDV to L cells. Preliminary complementation studies with the NDV_{pi} mutants at the nonpermissive temperature indicate that all of the

mutants probably belong to the same complementation group, and therefore are presumably deficient in the same function. The nature of that function is currently under investigation.

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