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

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The temperature-sensitive defects of virus mutants isolated from L cells persistently infected with Newcastle disease virus (NDV) were analyzed. Genetic grouping of the mutants by complementation tests was attempted by using several different methods, including yield analysis, RNA synthesis, and heterozygote formation at 42 to 43 C, the nonpermissive temperature. In each case, specific interference prevented detection of complementation. This interference was shown to occur prior to or at the level of virus RNA synthesis. Temperature-shift experiments with five different NDV_{pi} clones showed that virus replication begun at 37 C could not be completed at the nonpermissive temperature. The activity of the NDV-specific RNA-dependent RNA polymerase in the cytoplasm of infected chicken embryo cells was not stable and could not be demonstrated directly. However, indirect measurement of RNA polymerase activity at the nonpermissive temperature was accomplished by studying the kinetics of virus-specific RNA synthesis in infected cells after temperature shift. Two types of response were obtained: with three NDV_{pi} clones, virus-specific RNA synthesis ceased immediately upon transfer of infected cells to 42 to 43 C, whereas in cells infected with two other NDV_{pi} clones, RNA synthesis continued for several hours at this temperature. These results suggested that there may be two types of ts defects in NDV_{pi}, both associated with virus-specific RNA polymerase activity.

Previous reports from this laboratory (28-30) have demonstrated that virus mutants (NDV_{pi}), isolated from a continuous line of mouse L cells persistently infected with Newcastle disease virus (NDV), differ from the wild-type virus (NDV_o) in several phenotypic properties. Recently, we also showed that the NDV_{pi} mutants did not replicate in primary chicken embryo cell cultures at 43 C, whereas NDV_o replicated normally at that temperature (22). The cut-off temperature for virus replication was between 41 and 43 C and none of the 10 NDV_{pi} clones tested synthesized virus-specific RNA at the nonpermissive temperature. Only a few other reports of ts mutants of NDV have been published. Kirvaitis and Simon (17) briefly mentioned attempts to obtain wild-type recombinants from crosses of ts mutants, but gave no details of the properties of the mutants. Similarly, Dahlberg and Simon (9) described complementation due to heterozygote virions produced in cells mixedly infected with two different ts mutants. The study was intended, however, to confirm the

existence of multiploid particles of NDV and no details concerning the mutants were presented.

Because it is difficult to study directly the evolution of NDV_{pi} from NDV_o via the selection pressures within the persistent infection, the temperature-sensitive property of NDV_{pi} was investigated. The objectives in studying these ts mutants were (i) to find the number of complementation groups represented by various NDV_{pi} clones; (ii) to identify the biochemical defect responsible for expression of the ts marker; (iii) to determine the relationship between the ts marker and the other properties which distinguish NDV_o and NDV_{pi}; and (iv) to assess the role of the ts mutation in establishment and maintenance of the persistent infection. The present report deals with the first two objectives, whereas the third and fourth are the subjects of a separate communication (23).

MATERIALS AND METHODS

Viruses. The Herts strain of NDV was used in all experiments. The origins of NDV_{pi} clone 20, isolated

from persistently infected L cells (L_{NDV}) in 1967, and NDV_{PI} clones 25 to 38, isolated from L_{NDV} cells in 1969, were described previously (22, 28). Virus stocks were grown in 9-day-old fertile eggs. With NDV_0 , allantoic fluids from dead eggs were harvested at 48 h, whereas with NDV_{PI} only eggs still alive 48 h after infection were harvested. Virus was sedimented by centrifugation at $78,000 \times g$ for 1 h, suspended in phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.2) to 1/20 the original volume, and stored at $-70^\circ C$.

Cell cultures. Primary chicken embryo (CE) cell cultures were propagated and maintained in Eagle minimal essential medium (MEM) plus 4% calf serum.

Infection of CE cells with NDV. Virus was diluted in MEM to provide a calculated input multiplicity of infection (MOI) of 10 to 20 PFU per cell when 0.5 ml of virus inoculum was added to a 60-mm Petri dish (3×10^6 to 4×10^6 cells) or when 1 ml of inoculum was added to a 2-oz prescription bottle (4×10^6 to 5×10^6 cells). In all experiments, virus adsorption was for 60 min at $37^\circ C$. The infected cultures were washed twice with warm MEM to remove unadsorbed virus, and 3 ml of fresh MEM was added. Incubation was continued at the appropriate temperature in humidified incubators flushed with 5% CO_2 or in thermostatically controlled water baths when rapid temperature equilibration was required. During the course of this investigation, it was discovered that, although the thermometer taped inside the door of the incubator used for the nonpermissive temperature registered $42^\circ C$, the average internal temperature of the incubator compartment was actually $43^\circ C$. In all experiments, infection with the wild-type virus was carried out as a control; NDV_0 consistently replicated normally at $43^\circ C$.

At appropriate intervals, culture fluids were harvested for assay of released virus (RV) and the cells were scraped from the glass and disrupted with sonic oscillation to determine cell-associated virus (CAV).

Measurement of virus-specific RNA synthesis. CE cells were infected for 1 h at $37^\circ C$, washed, fed with fresh MEM, and incubated at 37 or $43^\circ C$. At 2.5 h after infection, the cultures were treated with $2 \mu g$ of actinomycin D per ml in MEM for 1 h and then exposed to 3 ml containing $1 \mu Ci$ of tritiated uridine per ml (3H -U, specific activity ~ 25 Ci/mmol). Incubation was continued at the appropriate temperature. At different times, the monolayers were washed twice with cold Hanks balanced salt solution (BSS) and disrupted by addition of 2 ml of 1% sodium dodecyl sulfate (SDS). Material precipitated by 10% trichloroacetic acid was collected on nitrocellulose filters and the acid-insoluble radioactivity was determined in 10 ml of Bray's solution in a Packard 3375 Tricarb liquid scintillation spectrometer.

RESULTS

Complementation analysis. To determine if the different NDV_{PI} clones were temperature-sensitive due to genetic defects in different viral cistrons, complementation analysis was undertaken. The results of repeated attempts

employing several different methods to obtain complementation are presented here as evidence that, if complementation does occur between NDV_{PI} clones in CE cells at $43^\circ C$, it cannot be detected by conventional methods.

CE cell monolayers were infected for 1 h at $37^\circ C$ with a mixture of 5 to 10 PFU per cell of each of two NDV_{PI} mutants or with 10 to 20 PFU per cell of each mutant alone. The monolayers were washed, fed, and incubated at $43^\circ C$. At 3-h postinfection, the monolayers were again washed twice to remove any reversibly adsorbed, eluted virus that might obscure complementation. At 14 h after infection, infected culture fluids were harvested and assayed for infectivity on CE cell monolayers at $37^\circ C$. The complementation index (C_i) was calculated according to Burge and Pfefferkorn (7) and was equal to the mixed yield divided by the sum of the single virus control yields. Table 1 shows the results from four typical mixed infection experiments. Reproducible complementation results could not be obtained; the cross between clone 37 and clone 25 was performed four different times, with C_i values ranging from 0.29 to 2.4. The results from these four experiments were not compatible with any non-overlapping complementation map. The consistently low C_i values for most crosses, however, suggested that the failure of the experiments might be due to interference between viruses infecting the same cell.

Interference between NDV_{PI} clones. To test this possibility, the experiment summarized in Table 2 was performed. CE cell monolayers were doubly infected, as described above, with NDV_0 and NDV_{PI} or with two NDV_{PI} clones, washed to remove reversibly adsorbed virus, and incubated at $37^\circ C$. Total virus yield, harvested 12 to 14 h after infection, was assayed

TABLE 1. Lack of complementation in four experiments using yield analysis of mixed NDV_{PI} infections of CE cells at $43^\circ C$

Cross ($V_1 \times V_2$)	C_i^a			Exp. 4	
	Exp. 1	Exp. 2	Exp. 3	Cross ($V_1 \times V_2$)	C_i
37 $\times$ 20	1.0	4.4	0.66	25 $\times$ 20	0.50
37 $\times$ 25	2.4	0.29	0.39	25 $\times$ 29	0.44
37 $\times$ 29	0.50	2.2	0.40	25 $\times$ 30	0.60
37 $\times$ 30	0.70	0.79	0.03	25 $\times$ 31	0.65
37 $\times$ 31	0.02	1.1	0.61	25 $\times$ 32	0.40
37 $\times$ 32	1.0	0.65	0.41	25 $\times$ 35	0.42
37 $\times$ 35	0.31	0.70	0.32	25 $\times$ 36	0.18
37 $\times$ 38	0.36	0.85	0.79	25 $\times$ 37	0.58
				25 $\times$ 38	0.34

^a C_i = yield ($V_1 \times V_2$) at $43^\circ C$ / yield V_1 + yield V_2 at $43^\circ C$.

TABLE 2. *Interference between NDV clones in mixed infections in CE cells at 37 C*

Cross $V_1 \times V_2$	V_1 PFU/ml $\times 10^6$	V_2 PFU/ml $\times 10^6$	$(V_1 \times V_2)$ PFU/ml $\times 10^6$		I_1^a	
			NDV _o	NDV _{pi}	NDV _o ^b	NDV _{pi} ^b
NDV _o $\times$ 20	50	4.6	10	4.8	0.20	1.1
NDV _o $\times$ 25	50	22.0	9.2	6.8	0.184	0.31
NDV _o $\times$ 29	50	0.24	3.0	0.6	0.06	2.5
NDV _o $\times$ 30	50	0.25	42.0	0.6	0.84	2.4
NDV _o $\times$ 32	50	2.6	10.0	3.6	0.20	1.4
NDV _o $\times$ 35	50	10.0	19.0	1.9	0.38	0.19
NDV _o $\times$ 38	50	4.0	8.0	6.2	0.124	1.5
20 $\times$ 25	4.6	22.0		13.0		0.48 ^c
20 $\times$ 29	4.6	0.24		4.4		0.92 ^c
20 $\times$ 30	4.6	0.25		5.2		1.1 ^c
20 $\times$ 32	4.6	2.6		8.6		1.2 ^c
20 $\times$ 35	4.6	10.0		6.4		0.43 ^c
20 $\times$ 38	4.6	4.0		3.6		0.42 ^c

^a I_1 , interference index.^b Yield in mixed infection $\div$ yield in single infection.^c $(V_1 \times V_2)/(V_1 + V_2)$.

in CE cells at 37 C. In NDV_o $\times$ NDV_{pi} crosses, NDV_o (>2 mm) and NDV_{pi} (<1.5 mm) plaques could be distinguished and were tallied separately.

Specific interference occurred between NDV_o and NDV_{pi} (Table 2). In mixed NDV_o and NDV_{pi} infections, the yield of NDV_o was reduced from 50×10^6 PFU per ml to as little as 3×10^6 PFU per ml, representing a 94% decrease. The yield of NDV_{pi} was less affected by the mixed infection with NDV_o. In cultures infected with two different NDV_{pi} clones, the total virus yield was less than or equal to the sum of the control yields from singly infected cultures; the tendency of virus yields to plateau with increasing MOI might account for these results at 37 C. However, as will be shown later (Table 3), lack of complementation between NDV_{pi} clones at 43 C may be due to mutual interference at the level of RNA synthesis.

Alternative methods of complementation.

Several other methods of detecting complementation were investigated. Granoff (15) demonstrated that heterozygous particles could result from mixed infection of CE cells with mutants of two strains of NDV. Dahlberg and Simon (9) confirmed this finding and reported that heterozygotes also could be formed during mixed infection of CE cells with a complementing pair of ts mutants. These heterozygotes were then able to produce plaques at the nonpermissive temperature. Viral yields from CE cells mixedly infected at 37 C with various NDV_{pi} clones were harvested and assayed at 37 and 43 C to determine total yield and percentage of complementing heterozy-

gotes, respectively. No increase in plaquing efficiency at 43 C above that found with yields from self-cross controls was observed with the yields from mixed infections, indicating the absence of complementing heterozygotes.

Another alternative method was explored which did not require high MOI. In the "complementation on a plate" technique (21; R. Chanock, personal communication) virus samples containing either one NDV_{pi} clone alone, or a mixture of equal concentrations of two NDV_{pi} clones, were diluted serially in twofold steps. Virus dilutions ranging from the equivalent of 1 total PFU per cell to 0.01 PFU per cell (0.5–0.007 PFU/cell of each mutant) were plated on CE cell monolayers ($\sim 3 \times 10^6$ cells) and the cells were overlaid with nutrient agar. The plates were incubated at 43 C and plaques were counted after 5 to 7 days when NDV_o control plaques were easily counted. If complementation occurs between two NDV_{pi} clones, then plaques should be obtained on plates inoculated with more dilute virus mixtures. Also, if more than one virus particle is required to produce a plaque (i.e., complementation), then the number of plaques observed on the plates inoculated with the virus mixtures should decrease more steeply than the MOI with increasing virus dilution.

Several crosses were conducted by this procedure. In each case, within the error of the assay, the average number of plaques per plate in each dilution series decreased as expected for a twofold dilution and the plaque counts on mixed inoculum plates could be accounted for by each mutant replicating alone.

As shown previously, all NDV_{pi} clones were RNA⁻ at the nonpermissive temperature (22). Consequently, another alternative procedure for complementation analysis involved measurement of virus-specific RNA synthesis in infected cells at 43 C. CE cells were infected for 1 h at 37 C with NDV_o or with NDV_{pi} clone 25 or 29 separately (MOI = 20 PFU/cell) or with equal mixtures of NDV_o and NDV_{pi} (MOI = 10 PFU/cell of each virus). Replicate cultures were incubated at 37 and 43 C. The cultures were treated with actinomycin D and exposed to ³H-U. At 9 h after infection, the cultures were washed with BSS and disrupted with SDS. Trichloroacetic acid-insoluble radioactivity was counted in 10 ml of Bray's fluid.

Fewer counts than expected were obtained from mixed infection samples at both 37 and 43 C (Table 3). These results suggest that at least some of the interference obtained in mixed infections (Table 2) occurred prior to virus-specific RNA synthesis or was at the level of RNA synthesis. NDV_{pi} consistently interfered with NDV_o at both temperatures, although the interference was more dramatic at 43 C. These data indicated that complementation between NDV_{pi} clones in mixed infection was obscured under all conditions by interference and could not be detected by conventional methods. The possibility exists, however, that all NDV_{pi} clones examined belong to the same complementation group and are deficient in the same function.

Biochemical analyses of ts mutants are usually performed with a representative mutant from each complementation group. Since complementation analysis could not be used to

group the mutants according to functional defects, the following experiments on the functional defect(s) of NDV_{pi} were usually performed with four or five different NDV_{pi} clones.

Temperature-shift experiments. To temporally localize the ts block in the replication of NDV_{pi} at 43 C, the replication of several NDV_{pi} mutants under conditions of temperature shift was analyzed. CE cell monolayers in 2-oz prescription bottles were infected for 1 h at 37 C with NDV_o or NDV_{pi} (MOI = 10 PFU/cell in 1.0 ml of inoculum). The monolayers were washed twice to remove unadsorbed virus, fed with 4 ml of warm MEM, and immersed in a water bath maintained at 37 C (± 0.05 C). At various times after infection, duplicate samples were either harvested to determine the amount of virus present, or immersed in another water bath maintained at 42 C (± 0.05 C). All shifted samples were harvested 12 h after infection.

The results for NDV_o and NDV_{pi} clone 35 are shown in Fig. 1. With NDV_o, a normal yield of virus was obtained after all temperature shifts. In two replicate experiments, however, NDV_{pi} clone 35 was unable to complete at 42 C replication begun at 37 C; the data for NDV_{pi} clones 20, 25, 29, and 32 were essentially identical to those shown for clone 35. Regardless of the length of incubation at the permissive temperature before incubation at 42 C, the total virus per ml obtained from shifted samples harvested at 12 h postinfection was not significantly different from the amount of virus present at the time of the temperature shift. Virus replication apparently ceased very soon after transfer of infected cell cultures to 42 C.

Shift-down. To determine the effect of a shift

TABLE 3. Virus-specific RNA synthesis in mixedly infected CE cells at 37 and 43 C^a

Cross (V ₁ × V ₂)	Temp. (C)	Counts per min per 10 ⁶ cells			I _i ^b	C _f ^c
		V ₁	V ₂	V ₁ × V ₂		
NDV _o × Cl 25	37	827	386	1065	0.88	
NDV _o × Cl 25	43	386	127	274		0.43
NDV _o × Cl 29	37	827	421	973	0.79	
NDV _o × Cl 29	43	386	44.2	10		0.02
Cl 25 × Cl 29	37	386	421	729	0.90	
Cl 25 × Cl 29	43	127	44.2	0 ^d		0
Cl 25 × Cl 32	37	386	318	176	0.25	
Cl 25 × Cl 32	43	127	2.8	0 ^d		0
Cl 29 × Cl 32	37	421	318	471	0.64	
Cl 29 × Cl 32	43	44.2	2.8	69.2		1.4

^a CE cells were infected with pairs of virus clones or with each clone separately and incubated at 37 or 43 C. Infected monolayers were exposed to actinomycin D and ³H-U (1 μ Ci/ml, specific activity 2 Ci/mmol). At 10 h after infection, acid-precipitable radioactivity was determined.

^b (V₁ × V₂)/(V₁ + V₂) at 37 C.

^c (V₁ × V₂)/(V₁ + V₂) at 43 C.

^d No counts per minute in excess of those in uninfected, control cells.

FIG. 1. Effect of transfer from 37 to 42 C on yield of NDV₀ and NDV_{pi} clone 35. CE cells were infected with NDV₀ or NDV_{pi} clone 35, and at various times infected cultures were either harvested (●, ▲) or transferred to 42 C (○, △). All shifted samples were harvested at 12 h. Symbols: ▲, △, NDV₀; ●, ○, NDV_{pi} clone 35.

FIG. 2. Effect of transfer from 42 to 37 C on yield of NDV_{pi} clone 35. CE cells were infected with NDV_{pi} clone 35 and incubated in a water bath maintained at 42 C. At various times, samples were either harvested (●) or transferred to a water bath at 37 C (○). Shifted samples were harvested at 12 h after infection.

from the nonpermissive to the permissive temperature on virus yield, CE cell monolayers infected with NDV₀ or NDV_{pi} clone 35 were transferred from 42 to 37 C at various times. Virus replication in NDV_{pi}-infected cells did not begin until the cultures were transferred to 37 C (Fig. 2). The data in Fig. 2 were obtained with NDV_{pi} clone 35, but the results with the other NDV_{pi} clones were essentially identical. The data for NDV₀ are not shown since, as in the experiment shown in Fig. 1, a normal yield of virus was obtained with all shifted samples. With NDV_{pi}, cultures transferred to 37 C at 2, 4, or 6 h after infection produced normal or near normal viral yields, whereas cultures transferred at 8 h postinfection produced very low yields (~2% of control yields) by 12 h after infection. Virus replication did occur, however, after the temperature shift since the sample shifted at 8 h and harvested at 12 h contained significantly more virus than the sample harvested at 8 h. If the virus were simply inactivated at 42 C, then one would expect the two values to be very similar. Since with NDV_{pi} at least a 4-h lag period was necessary for virus replication at 37 C even after 8 h at 42 C, the temperature-sensitive block must involve a very early step in virus replication.

RNA polymerase. The data from the temperature-shift experiments (Fig. 1 and 2) and the lack of virus-specific RNA synthesis in infected cells at 42 to 43 C (22) suggested that the ts defect of NDV_{pi} was in a viral function that began early in infection but continued to operate throughout infection. Virus-specific RNA polymerase would fit this description. Two NDV-specific RNA polymerase activities have been reported: an RNA-dependent RNA polymerase activity associated with detergent-treated purified virions (16), and an RNA synthesizing activity detectable in the cytoplasm of infected cells (25). The *in vitro* reaction catalyzed by the virion polymerase has an optimum incubation temperature of 32 C (16) so that direct assay of the virion polymerase at 43 C is not possible. An *in vivo* method of analyzing the activity of the virion polymerase in the presence of actinomycin D and cycloheximide has been described for vesicular stomatitis virus (VSV) (19). However, the activity of the

NDV polymerase was only about 1 to 3% of that found with VSV (16) and attempts to use an *in vivo* system similar to that described for VSV with NDV-infected CE cells have so far been unsuccessful in this laboratory (Thacore, Kowal, and Preble, unpublished data).

NDV-specific RNA polymerase activity in the cytoplasm of infected cells was described in 1969 by Scholtissek and Rott (25). The optimal conditions for NDV polymerase activity were reported to be identical to those for the influenza enzyme (25). A slight modification of their procedure was used to examine CE cells infected with NDV_o, NDV_{pi}, and influenza A virus (strain WSN). Significant polymerase activity was found only in extracts of WSN influenza-infected CE cells; the incorporation of ³H-UTP into acid-insoluble material by NDV-infection extracts was no different from that of uninfected extracts. Although previous results (25) indicated maximum RNA polymerase activity between 5 and 7 h after infection, the possibility existed that differences in virus strain, methods, and conditions resulted in either earlier or delayed activity. Cytoplasmic extracts were therefore prepared at 4, 5, 6, 7, and 8 h after infection, and at no time was significant NDV-specific RNA polymerase activity found in extracts of cells from either 37 or 43 C. Also, no activity was demonstrable in microsomal fractions sedimented by centrifugation.

The activity of the NDV polymerase is not very stable (C. Scholtissek, personal communication) and attempts were made to stabilize the enzyme or the RNA template, or both, by addition either during preparation of the extracts or during the *in vitro* assay, of the following reagents: 10% (final concentration) glycerol; 10% (final concentration) sucrose; 1, 5, or 10 μ g of bentonite per ml; 10 μ g of dextran sulfate per ml; 10 μ g of carrier yeast RNA per ml. None of these manipulations resulted in detection of NDV-specific RNA polymerase activity in cytoplasmic extracts of either NDV_o- or NDV_{pi}-infected CE cells from either temperature. Consequently, indirect methods had to be used to determine whether the cytoplasmic RNA polymerase was defective in NDV_{pi}-infected cells at 43 C.

RNA synthesis under conditions of temperature shift. To determine if RNA synthesis begun at 37 C could be continued at the nonpermissive temperature, CE cell monolayers in 2-oz prescription bottles were infected for 1 h at 37 C with 25 PFU per cell of NDV_o or NDV_{pi}, treated for 1 h with actinomycin (2.5–3.5 h after infection) and exposed to 1 μ Ci/ml of ³H-U

(specific activity 25.8 Ci/mmol). At 3.5, 4.5, 6.0, and 7.5 h after infection, infected monolayers were either transferred to a water bath at 42 C (± 0.05 C) or harvested with 1% SDS. At 9.0 h after infection, all shifted samples and 37 C controls were harvested and trichloroacetic acid-insoluble radioactivity was determined.

The data shown in Fig. 3 are typical data for NDV_o and NDV_{pi} clones 20, 25, 29, 32, and 35). In three replicate experiments, transfer from 37 to 42 C had no effect on NDV_o-specific RNA synthesis (Fig. 3A). The NDV_{pi} clones appeared to fall into two groups. The first included NDV_{pi} clones 20, 25, and 32, and representative data for clone 25 is shown in Fig. 3B. With these virus clones, RNA synthesis ceased almost immediately upon transfer of the infected cultures to 42 C. In samples shifted at any time after infection and harvested with SDS at 10 h after infection, the amount of acid-insoluble radioactivity present at 10 h was the same or less than that present at the time of the temperature shift. These data are in agreement with the yield data from the similar shift-up experiment shown in Fig. 1.

The results for NDV_{pi} clones 29 and 35 are shown in Fig. 3C. With these NDV_{pi} clones, virus-specific RNA synthesis appeared to continue for several hours after infected cultures were transferred to 42 C. For example, the amount of acid-precipitable virus-specific RNA present in samples shifted to 42 C at 4.5 h and harvested at 10 h was equivalent to the amount present in samples incubated for 6 h at 37 C. RNA synthesis after transfer to the nonpermissive temperature ceased after 1 to 2 h and did not reach control levels as in NDV_o-infected shifted cultures (Fig. 3A). The RNA synthesized at the nonpermissive temperature was not processed to mature virus, however, since previous data (Fig. 1) indicated that no infectious virus was produced in infected cells after transfer to 42 C. This result may indicate that some NDV_{pi} clones are double ts mutants, i.e., an early block in RNA synthesis can be overcome by 3 to 6 h at 37 C, but a later maturation defect prevents the RNA synthesized after a temperature-shift from being packaged into mature virions. In an ordinary yield analysis or RNA synthesis experiment at the nonpermissive temperature, the second ts defect would be masked by the first.

Kinetics of RNA synthesis after temperature shift. To confirm the two types of RNA synthesis in response to a temperature shift, the above experiment was repeated in a slightly different manner. CE cells infected with NDV_o or NDV_{pi} were treated at 37 C with actinomycin

FIG. 3. RNA synthesis in infected CE cells under conditions of temperature shift. CE cells were infected with NDV₀ or NDV_{pi} and incubated at 37 C. The infected cultures and mock-infected controls were treated with actinomycin and exposed to ³H-U. At various times, replicate cultures were either harvested (●—●) or shifted to 42 C (●---●). Trichloroacetic acid-insoluble radioactivity was determined. Panel A: NDV₀; panel B: NDV_{pi} clone 25; panel C: NDV_{pi} clone 29.

D, exposed to ³H-U, and incubated at 37 C until 5 h after infection when all cultures except 37 C controls were transferred to 42 C. At various times after transfer to 42 C, acid-insoluble radioactivity was determined as before.

The results, shown in Fig. 4, indicated that this treatment did not affect NDV₀-specific RNA synthesis; by 10 h after infection (5 h after shift), RNA synthesis in shifted samples was 68.5% of that obtained in NDV₀-infected control cultures at 37 C. With NDV_{pi} clones 25 and 32, virus-specific RNA synthesis stopped immediately when cultures were transferred to 42 C. However, RNA synthesis in cells infected with NDV_{pi} clone 29 or clone 35 proceeded for at least 2 to 3 h.

DISCUSSION

NDV_{pi} clones isolated from L cells persistently infected with NDV differ from wild-type NDV₀ in several different physical and biological properties (28-30) and in the ability to replicate in CE cells at 42 to 43 C (22). Ts mutants of several other RNA-containing viruses, including Sindbis virus (6), Semliki Forest virus (27), and VSV (12, 24), have been described. In each case, characterization of the mutants with regard to functional defect has revealed both RNA⁺ and RNA⁻ mutants (6, 8, 24, 27) and it has been possible to place the mutants into genetic groups using complementation tests (7, 12, 13, 27). Complementation temperature-sensitive mutants of NDV have also been reported (9) but details concerning the origins and functional defects of the mutants have never been published.

The experiments discussed here indicate that genetic analysis of NDV_{pi} clones by complementation grouping was frustrated by inter-

FIG. 4. Kinetics of virus-specific RNA synthesis after transfer of infected CE cells to 42 C. Infected CE cells were exposed to actinomycin D and ³H-U. At 5 h after infection, all infected cultures were transferred to 42 C. At various times, acid-insoluble radioactivity was determined. Inset is expanded scale representation of the NDV_{pi} clones. Symbols: ●, NDV₀; ○, NDV_{pi} clone 25; △, NDV_{pi} clone 29; ▽, NDV_{pi} clone 32; □, NDV_{pi} clone 35.

ference which decreased virus yields under all conditions of mixed infection tested. The interference was evident between NDV_o and NDV_{pi} as well as between different NDV_{pi} clones, and occurred at both 37 and 43 C. Several possible mechanisms could be used to explain this interference. Inactive or uninfecious virus is known to interfere with active virus of the same strain (1-4) and all NDV populations contain noninfectious "incomplete" hemagglutinating particles (14). However, no significant difference in the PFU/HA ratio was observed between NDV_o and NDV_{pi} clones (Preble and Youngner, unpublished data). Also, no difference was apparent between NDV_o and NDV_{pi} in the ratio of complete to incomplete particles separated on sucrose density gradients (LaMontagne and Youngner, unpublished data). These findings suggest that the interference observed in mixed infections was probably not caused by an excess of inactive particles in NDV_{pi} preparations. The hypothesis that each cell can adsorb a specific number of virus particles, and that the amount of virus in the mixed inocula exceeded that amount, can also be ruled out since the same total inoculum of one clone alone produced a normal yield of virus.

Competition between viruses in mixed infection for replicative sites or substrates is the most probable explanation of the interference observed in these studies. Durand (10) and Bratt and Rubin (4, 5) concluded that interference by an avirulent NDV with the multiplication of a virulent strain of virus was caused by this mechanism, and also that replication of the interfering avirulent virus was necessary for development of maximum interference. Virus-specific RNA synthesis was reduced in doubly-infected CE cells exhibiting interference, suggesting that competition between interfering viruses occurred at a stage in virus replication prior to RNA synthesis (Table 3).

The biochemical defect responsible for the temperature sensitivity of NDV_{pi} was investigated by using five different NDV_{pi} clones. Previous results (22) indicated that all NDV_{pi} clones were RNA⁻ at the nonpermissive temperature. In addition, the shift-up and shift-down experiments suggested that the virus-specific RNA polymerase of NDV_{pi} may be temperature sensitive. Several interpretations are possible, however, in view of the two types of responses obtained when RNA synthesis was examined after temperature shift. Two levels of sensitivity of one defect could exist. With NDV_{pi} clones 29 and 35, the polymerase may be more resistant to 43 C than the polymerase of clones 20, 25, and 32, and several hours at the higher temperature are needed to inactivate the

enzyme activity. One might expect that the more resistant clones would have a higher cut-off temperature, but as shown previously, this was not the case (22).

Alternatively, two separate defects may exist, both affecting the RNA polymerase. With clones 20, 25, and 32, transfer to 43 C would cause immediate loss of conformation of the polymerase molecule and loss of enzyme function. With NDV_{pi} clones 29 or 35, however, the synthesis or assembly of the polymerase protein would be blocked at 43 C. Once formed at 37 C, the molecules would be stable at 43 C, but synthesis of new polymerase molecules to replace ones that have "turned over" would be prevented. This possibility was also discussed by Martin (20) with respect to ts mutants of Semliki Forest virus.

Evidence for any of these hypotheses, however, would have to come from *in vitro* assays of cytoplasmic viral RNA polymerase. The *in vitro* assay of virus-specific RNA polymerase in infected cell extracts has been described for other paramyxoviruses, including Sendai virus (18, 26) and mumps virus (11). Scholtissek and Rott (25) also reported a low and relatively unstable RNA polymerase activity in NDV-infected CE cells. However, all attempts to assay this activity in Herts NDV-infected CE cells in this study were unsuccessful. The reason for this failure is not clear at this time, since the conditions used appear to be very similar to conditions used successfully by others. Until the *in vitro* assay of RNA polymerase from infected cells incubated at 37 and 43 C can be achieved, no further conclusions can be made concerning the exact biochemical defect which renders NDV_{pi} temperature sensitive.

Another possibility which cannot be ruled out at the present time is that NDV_{pi} clones 29 and 35 are double ts mutants which also possess a ts block in a maturation step. In this case, in a regular growth or RNA-synthesis experiment, the later ts defect would be masked by the earlier defect. However, it must be noted that neither of these mutants have abnormally low reversion frequencies for the ts mutation (22).

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