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Viral Ribonucleic Acid Synthesis by Newcastle Disease Virus Mutants Isolated from Persistently Infected L Cells: Effect of Interferon

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The synthesis of different viral ribonucleic acid (RNA) species was studied in chick embryo (CE) and mouse L-cell cultures infected with the Herts strain of Newcastle disease virus (NDV_o) and a mutant isolated from persistently infected L cells (NDV_{pi}). In CE cell cultures, both viruses synthesized significant amounts of 54, 36, and 18*S* RNA. However, in L cells, synthesis of 54*S* virion RNA was markedly reduced. From these results, it seems likely that the low yield of infective virus in L cells is due to a deficient synthesis of 54*S* RNA in this host. On this basis, however, it is apparent that the "covert" replication of NDV_o in L cells is due to factors other than viral RNA synthesis. When low concentrations of interferon were used to pretreat CE cells, a differential effect on the synthesis of various RNA species was observed. The 18*S* RNA of NDV_o was more sensitive to interferon action than the 36 and the 54*S* RNA species. In contrast, the 18*S* RNA of NDV_{pi} was less sensitive than the 36*S* and the 54*S* RNA. The inhibition of 54*S* RNA synthesis correlated with the reduction of viral yield and explained the greater sensitivity of NDV_{pi} to interferon.

Previous papers from this laboratory have described the properties of mutants of Newcastle disease virus (NDV_{pi}) isolated from L cells persistently infected with the Herts strain (NDV_o). NDV_{pi} mutants differed from the wild type in the following characteristics: plaque size in permissive primary chick embryo (CE) cell cultures, thermostability of infectivity and neuraminidase activity, and ability to replicate in L cells (13-15). Whereas the virus yield per cell in CE cell cultures was 320 and 107 plaque-forming units (PFU) for NDV_o and NDV_{pi}, respectively, the yields from L cells were only 11 and 30 PFU per cell for the wild-type and mutant viruses. The infection of L cells by NDV_o was termed a "covert" infection since intracellular maturation of virus occurred, but the infectivity remained cell-associated and disappeared without being detectable in the medium. In contrast, in cells infected with NDV_{pi}, progeny virus was released efficiently upon maturation.

The decreased yield of infective progeny in L cells compared to the yields from CE cells could not be explained by a lack of viral ribonucleic acid (RNA) synthesis since both viruses produced large amounts of viral RNA in L cells (14). Experiments were therefore done to analyze the different RNA species synthesized in CE and L cells infected with wild-type and mutant NDV in an attempt to explain the different virus yields observed in the two cell systems. The present study reports (i) the synthesis of different RNA species by the two viruses in CE and L cells and (ii) the influence of homologous interferon on this synthesis.

MATERIALS AND METHODS

Cell cultures. Primary CE cell cultures and mouse L cells (clone 929) were grown and maintained in Eagle's minimal essential medium containing 4% calf serum as described earlier (18).

Viruses. The isolation, propagation, and concentration of the wild-type Herts strain of NDV (NDV_o, clone 19) and the small plaque mutant (NDV_{pi}, clone 2) have been described in detail in earlier reports (13, 14).

Interferon preparation. Mouse interferon was prepared in L-cell cultures infected with NDV (18). The interferon preparation was treated at *p*H 2 and stored at −20 C. Chicken interferon was prepared from the allantoic fluid of embryonated eggs infected with the WS strain of influenza virus as described in detail elsewhere (9).

Chemicals. ^{3}H-uridine (specific activity, 24.6 Ci/mmole) was purchased from Schwartz BioResearch, Inc., Orangeburg, N.Y. Actinomycin D was made available through the courtesy of H. B. Woodruff of Merck, Sharp and Dohme Co.

Incorporation of ^{3}H-uridine into virus-specific RNA.

Cell cultures (5×10^6 cells per dish) were infected at a multiplicity of infection (MOI) of 350. After adsorption for 60 min at 37 C, the cells were washed twice, medium was added, and the cultures were reincubated at 37 C. The cells were treated with medium containing actinomycin D (3.3 μg/ml) for 1 hr prior to the addition of ^{3}H-uridine at a concentration of 4 μCi/ml.

The cultures were allowed to incubate for 2 hr at 37 C and then were processed for the isolation of RNA as described below.

Isolation of RNA from infected cells. The phenol-sodium dodecyl sulfate procedure for extraction of RNA as described by Rake and Graham (11) was used in this study. The monolayer cultures were washed three times with cold phosphate-buffered saline (PBS), *p*H 7.4, and the cells were suspended in a cold solution (5 ml) of 10^{-2} M acetate buffer (*p*H 5.0) containing 0.14 M LiCl, 10^{-3} M Mg^{2+}, 0.0004% polyvinyl sulphate (PVS), and 0.5% SDS. To this cell suspension an equal volume of phenol (saturated with acetate buffer, *p*H 5.0) was added, and the mixture was vigorously shaken at 4 C for several minutes. The deoxyribonucleic acid (DNA) was first removed by treating the mixture at 60 C for 3 min and then rapidly chilled to 4 C. The two phases were separated by low-speed centrifugation and the top aqueous phase, containing RNA, was subjected to two more phenol extractions as described above. The phenol was removed from the aqueous phase by shaking the mixture with an equal volume of ether, and the residual ether was removed by bubbling nitrogen through the solution. To 5 ml of this solution containing RNA, 30 ml of absolute alcohol was added, and precipitation of RNA was allowed to occur at −20 C for 24 hr. The RNA precipitate was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4) containing 0.1 M NaCl and 0.001 M ethylenediaminetetraacetic acid (EDTA) and stored at − 70 C.

Analysis of RNA. Samples (1 to 2 ml) were layered on a 30-ml linear gradient consisting of 15 to 30% (w/v) sucrose in the Tris buffer described above. L-cell ribosomal RNA (0.5 ml) was used as marker. Experimental samples and marker RNA were centrifuged in the same tube in an SW25.1 rotor at 20,000 rev/min at 4 C for 18 hr and then scanned at 260 nm in a Gilford recording spectrophotometer by pumping the contents through a flow cell. Fractions (1 ml) were collected, and trichloroacetic acid-precipitable radioactivity was determined for each fraction as follows. To each sample (0.2 ml), 4 ml of 10% trichloroacetic acid was added, and the mixture was incubated for 30 min at 4 C. The precipitate was collected onto a membrane filter (Millipore Corp.), washed once with 5% trichloroacetic acid, and dried. The filter was transferred to a scintillation vial, and 10 ml of Bray's fluid (5) was added. The samples were kept at 4 C overnight, and the radioactivity was counted in a Packard Tri-Carb 3375 liquid scintillation spectrometer. Resistance to digestion by ribonuclease was determined by treating samples with 10 μg of ribonuclease in Tris buffer containing 0.004 M $MgCl_2$ (4), at 37 C for 1 hr, after which acid-precipitable counts were determined as described above.

RESULTS

Viral RNA synthesis in CE cell monolayer cultures infected with NDV_o and NDV_{pi}. A study was carried out to compare the synthesis of viral RNA species at different times during the infectious cycle of the two viruses in CE cells. The time for labeling viral RNA was based on one-step growth cycle and total viral RNA studies described in an earlier report from this laboratory (14). Cell cultures were infected with NDV_o or NDV_{pi} at an input MOI of 350, and at appropriate times ^{3}H-uridine was added as described above. With NDV_o-infected cells, the viral RNA was labeled for 2-hr intervals starting at 3 or 6 hr after infection. Because of the longer latent period of NDV_{pi}, cells infected with this virus were labeled for 2 hr at 7 or 10 hr after infection. Uninfected control cultures were treated with actinomycin D and exposed to ^{3}H-uridine for 2 hr at comparable times. The RNA was extracted and analyzed on a sucrose density gradient as described above. The results (Fig. 1) show that three species of RNA, 54, 36, and 18*S*, could be distinguished in CE cells infected with NDV_o and NDV_{pi}. Only the 36*S* RNA of both viruses was partially (16%) resistant to ribonuclease, the 54 and 18*S* RNA peaks were eliminated completely by ribonuclease treatment. Bratt and Robinson (4), Bratt (3), and Blair and Robinson (2) have reported similar results using different strains of NDV although the synthesis of the three viral RNA species at different times of the infectious cycle was not reported. The relative proportions of the different RNA species did not change significantly during the two periods of the infectious cycle we examined. The 18*S* RNA represented 55 to 69% of the total viral RNA synthesized in cells infected with either NDV_o or NDV_{pi}, whereas 36 and 54*S* represented only 15 to 17% and 13 to 23%, respectively (Table 1). Although the actual function(s) of these different macromolecules is not completely understood, the 54*S* RNA has been shown to be present in the virion (6).

Viral RNA synthesis in L-cell monolayer cultures infected with NDV_o and NDV_{pi}. L-cell cultures were infected at an input MOI of 350 in the usual manner. Cells infected with NDV_o were labeled for 2-hr periods beginning at 4 and 7 hr after infection, NDV_{pi}-infected cultures were labeled for 2 hr at 8 and 12 hr after infection. The RNA was extracted and analyzed on sucrose density gradients (Fig. 2). As in CE cells, the majority of the viral RNA synthesized in L cells was 18 and 36*S* species, which represented 54 to 62% and 7 to 12% of the total RNA, respectively (Table 2). In contrast, proportionately less 54*S* RNA (2 to 6%) was synthesized by both viruses

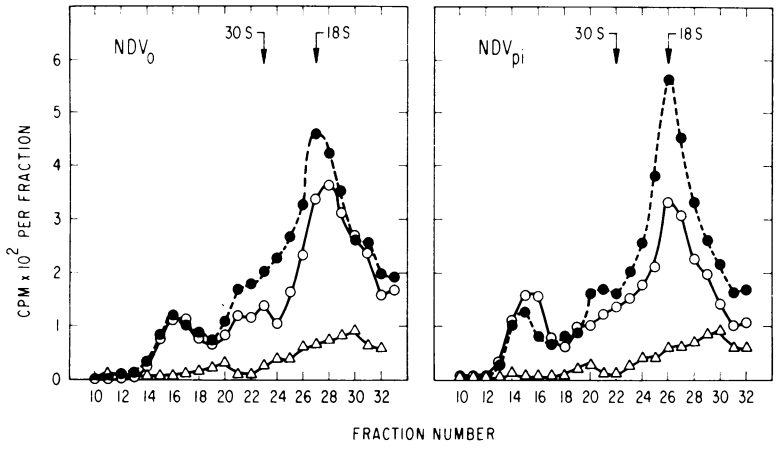

FIG. 1. *Sucrose density gradient analysis of RNA synthesized in CE cells infected with NDV_o and NDV_{pi}. The RNA was extracted from infected cells as described in the text, layered on a sucrose gradient [15 to 30% (w/v)] with 30 and 18S markers, and centrifuged at 18,000 rev/min at 4 C in an SW25.1 rotor. Symbols: the distribution of radioactivity of RNA from actinomycin D-treated, uninfected control cell cultures* (△) *NDV_o- infected cultures between 3 and 5 hr* (●), *6 and 8 hr* (○), *from NDV_{pi}-infected cultures between 7, and 9 hr* (●), *and 10 and 12 hr* (○).

TABLE 1. *Relative amounts of viral RNA species synthesized in chicken embryo cells infected with NDV_o and NDV_{pi}*[a]

Virus	Time of labeling post infection (hr)	Total counts incorporated into viral RNA	54S Region		36S Region		18S Region		Ratios		
			Counts	Fraction of total	Counts	Fraction of total	Counts	Fraction of total	54S/36S	54S/18S	36S/18S
NDV_o	3–5	3,400	465	0.13	588	0.17	2,060	0.60	0.76	0.21	0.28
	6–8	2,515	440	0.17	378	0.15	1,498	0.59	1.1	0.28	0.25
NDV_{pi}	7–9	3,330	375	0.11	513	0.15	2,318	0.69	0.73	0.15	0.21
	10–12	2,480	590	0.23	425	0.17	1,365	0.55	1.3	0.41	0.30

[a] NDV_o, Newcastle disease virus, Herts strain; NDV_{pi}, from persistently infected L cells.

in L cells, compared to the 54S synthesized in CE cells (13 to 23%). Since the 54S RNA represents RNA incorporated into the virion, the decreased synthesis of this species of RNA may be responsible for the low viral yields observed in L cells infected with both viruses.

It is interesting to note that, although the relative amounts of the three RNA species did not change, the total amount of viral RNA synthesized during the later labeling period increased twofold in the case of NDV_o and threefold with NDV_{pi}. This is in contrast to the declining rates of RNA synthesis during the later labeling time in CE cells, a phenomenon we have noted previously (14).

Effect of interferon on viral yield and on viral RNA synthesis in CE cells infected with NDV_o and NDV_{pi}. CE cell cultures (2.5×10^6 cells) were incubated at 37 C with 10-fold serial dilutions of chick interferon or with medium alone. After 20 hr, a plaque reduction assay with VSV as the challenge virus was carried out to determine the number of interferon units present in each dilution. The remainder of the interferon-treated cultures were washed with medium and duplicate cultures were infected in the usual manner with VSV (8), NDV_o, or NDV_{pi}. The medium from one set of cultures was harvested after the time required for one cycle of replication, i. e., 7 hr for VSV and NDV_o and 10 hr for NDV_{pi} (14), to determine inhibition of virus yield. The rest of the cultures were labeled with ^{3}H-uridine (1.5 μCi/ml) for 2 hr in the usual manner starting at 4 and 8 hr after infection with NDV_o and NDV_{pi}, respectively. RNA was extracted and analyzed on sucrose gradients as described above. The effect of interferon on viral RNA synthesis and on viral yield in CE cells is presented in Table 3. A direct correlation was observed with both viruses between the inhibition of viral RNA synthesis and

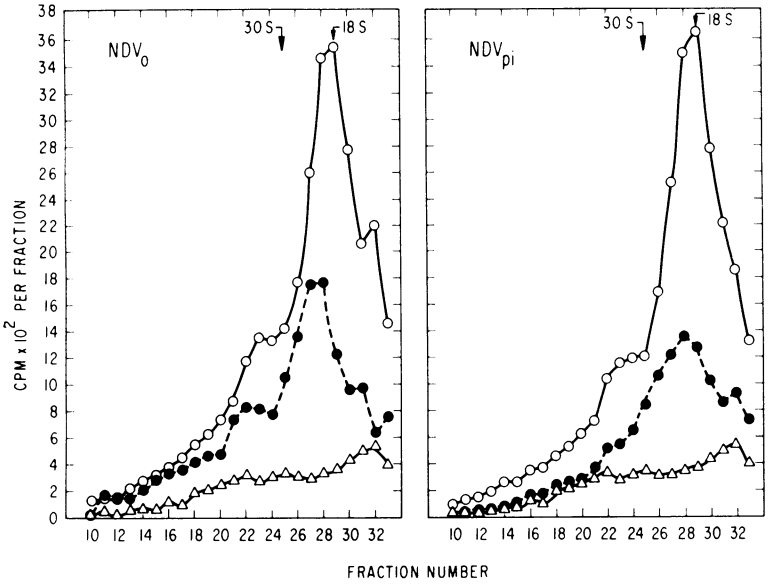

FIG. 2. *Sucrose density gradient analysis of RNA synthesized in L cells infected with NDV_o and NDV_{pi}. The RNA was extracted and analyzed on a sucrose gradient as described in the legend of Fig. 1. Symbols: the distribution of radioactivity of RNA from actoinmycin D-treated, uninfected control cultures (△); NDV_o-infected cultures between 4 and 6 hr (●); 7 and 9 hr (○); from NDV_{pi}-infected cultures between 8 and 10 hr (●) and 12 and 14 hr (○).*

TABLE 2. *Relative amounts of viral RNA species synthesized in L cells infected with NDV_o and NDV_{pi}*[a]

Virus	Time of labeling post-infection (hr)	Total counts incorporated into viral RNA	54S Region		36S Region		18S Region		Ratios		
			Counts	Fraction of total	Counts	Fraction of total	Counts	Fraction of total	54S/36S	54S/18S	36S/18S
NDV_o	4–6	10,930	695	0.06	1,365	0.12	5,990	0.54	0.50	0.11	0.22
	7–9	23,545	960	0.04	2,460	0.10	13,260	0.56	0.40	0.07	0.17
NDV_{pi}	8–10	6,925	140	0.02	535	0.07	4,645	0.67	0.28	0.03	0.10
	12–14	21,420	665	0.03	2,185	0.10	13,445	0.62	0.30	0.04	0.16

[a] See Table 1, footnote *a*.

viral yield at interferon concentrations of eight units per culture or more. In contrast, at low concentration of interferon (0.8 units/culture), although the total RNA synthesis of the two viruses was inhibited to the same extent (about 60%), only 20% of NDV_o yield was inhibited as compared to 90% inhibition of NDV_{pi} yield. This could be explained on the basis of a differential inhibition of viral RNA species at this concentration of interferon (Table 3). The 54S RNA of NDV_o was significantly less sensitive to interferon (36% inhibition) than the 54S RNA of NDV_{pi} (95% inhibition). Also, at this concentration of interferon the inhibition of 54S RNA correlated with the reduction of viral yield, suggesting that the amount of 54S RNA synthesized may determine the formation of infective virus particles. The observation that the degree of inhibition of the different RNA species may fall below or exceed the degree of inhibition of total viral RNA synthesis is explainable on the basis of the fact that only 70 to 90% of the total viral RNA placed on the gradient can be recovered in the three distinct viral RNA peaks (Tables 1 and 2). It should be noted that, at low concentrations of interferon, the difference in sensitivity of NDV_o and NDV_{pi}, as determined by yield inhibition, can be eliminated when a lower MOI is used (Thacore and Youngner, *unpublished data*).

Effect of interferon on viral yield and RNA synthesis in L cells infected with NDV_o and NDV_{pi}. Experiments similar to those described above were

carried out in L cells. Duplicate cultures (4×10^6 cells per culture) were pretreated with 10-fold dilutions of mouse interferon for 20 hr and then infected with NDV_o and NDV_{pi} in the usual manner. For the determination of total viral yield, the cells from one set of cultures were scraped into the medium with a rubber policeman at 7 and 12 hr after infection with NDV_o and NDV_{pi}, respectively. The cell suspensions were disrupted by sonic oscillation for 60 sec, and infectivity assays were performed on CE cell monolayers as described earlier (18). The other set of cultures was labeled with ^{3}H-uridine for 2 hr, beginning at 5 hr in the case of NDV_o and at 9 hr after infection with NDV_{pi}. RNA was extracted and the effect of interferon on viral RNA synthesis and on viral yield of NDV_o and NDV_{pi} was determined (Tables 4 and 5). Both viruses were as sensitive to interferon as VSV (plaque reduction) when compared by either a yield reduction assay or total viral RNA inhibition. With a concentration of interferon less than 1 unit per culture, a differential effect of interferon on the synthesis of viral RNA species in L cells was observed, similar to that seen in CE cells. The 18*S* RNA of both viruses was found to be less sensitive than the 36*S* RNA. In these experiments, the small number of counts incorporated into the 54*S* region was not considered significant.

DISCUSSION

Although the actual mechanism of the defective release of NDV_o from covertly infected L cells is not elucidated, it is apparent that this phenome-

TABLE 3. *Effect of pretreatment with interferon on viral yield and viral RNA synthesis in chicken embryo cell cultures infected with NDV_o or NDV_{pi}*[a]

Interferon used for pretreatment (units/3 ml)	VSV viral yield[b] (per cent inhibited)	NDV_0					NDV_{pi}				
		Viral[b] yield (per cent inhibited)	Total V-RNA (counts/min)	Species (counts/min)			Viral[b] yield (per cent inhibited)	Total V-RNA (counts/min)	Species (counts/min)		
				54*S*	36*S*	18*S*			54*S*	36*S*	18*S*
None	0	0	1,527	240	142	516	0	1,010	71	221	462
0.8	77	20	655	155	98	232	90	386	4	64	222
			(58)[c]	(36)	(31)	(56)		(62)	(95)	(71)	(52)
8	95	78	379	75	33	134	99	115	3	10	20
			(76)	(69)	(77)	(73)		(89)	(96)	(96)	(96)
80	100	95	138	21	18	54	99	0	0	0	0
			(91)	(92)	(88)	(90)		(100)	(100)	(100)	(100)

[a] See Table 1, footnote *a*.

[b] Viral yields in untreated control cultures were 2.4×10^7 and 3.4×10^6 plaque-forming units per ml for NDV_o and NDV_{pi}, respectively, and 1.4×10^8 plaque-forming units per ml for VSV.

[c] Values in parentheses indicate per cent inhibited.

TABLE 4. *Effect of pretreatment with interferon on viral yield and viral RNA synthesis in L-cell cultures infected with NDV_o*[a]

Interferon used for pretreatment (units/3 ml)	VSV plaques (per cent inhibited)	NDV_0				
		Viral yield[b] (per cent inhibited)	Total V-RNA[c] (counts per min)	Species		
				54*S*	36*S*	18*S*
None	0	0	1,595	38	207	732
0.1	22	38	945	0	11	459
			(41)	(100)	(95)	(38)
1	52	80	0	0	0	0
			(100)	(100)	(100)	(100)
10	100	100	0	0	0	0
			(100)	(100)	(100)	(100)

[a] NDV_o, Newcastle disease virus Herts strain. VSV, vesicular stomatitis virus.

[b] Viral yield in untreated cultures was 1.7×10^7 plaque-forming units per ml.

[c] Values in parentheses indicate per cent inhibited.

TABLE 5. *Effect of pretreatment with interferon on viral yield and viral RNA synthesis in L-cell cultures infected with NDV_{pi}*[a]

Interferon used for pretreatment (units/3 ml)	VSV plaques (per cent inhibited)	NDV_{pi}				
		Viral yield[b] (per cent inhibited)	Total V-RNA[c] (counts/min)	Species (counts/min)		
				54S	36S	18S
None	0	0	1,583	35	142	1,068
0.25	28	23	516	0	0	375
			(68)	(100)	(100)	(65)
2.5	97	100	0	0	0	0
			(100)	(100)	(100)	(100)
25	100	100	0	0	0	0
			(100)	(100)	(100)	(100)

[a] NDV_{pi}, Newcastle disease virus from persistently infected L cells. VSV, vesicular stomatitis virus.
[b] Viral yield in untreated cultures was 1.1×10^7 plaque-forming units per ml.
[c] Values in parentheses indicate per cent inhibited.

non does not involve a deficiency in viral RNA synthesis. No significant difference was observed in the patterns of RNA synthesis by NDV_o and the mutant, NDV_{pi}, which causes a productive cytolytic infection in L cells. The low yield of PFU per cell with both viruses in L cells, as compared to the yields obtained in CE cells, may be due to decreased synthesis of 54S RNA in L cells. Approximately 67 and 89% less 54S RNA was synthesized by NDV_o and NDV_{pi}, respectively, in L cells compared to 54S RNA synthesized in CE cells. It is interesting to note that, although the per cent of total counts incorporated into the 54S RNA of the two viruses in CE cells was the same (Table 1), the viral yield of NDV_o was three times greater than that of NDV_{pi} (14). It is conceivable that the incorporation of 54S RNA into infective virus particles is more efficient in NDV_o-infected cells than it is with NDV_{pi}. An altered 54S RNA synthesized by NDV_{pi} in CE cells cannot be ruled out at this time.

The mutant NDV_{pi}, which plaques in L cells, has been shown by the plaque reduction method to be as sensitive to interferon as vesicular stomatitis virus (8). This finding was confirmed when NDV_{pi} was tested for interferon sensitivity by yield reduction and by viral RNA inhibition. Furthermore, NDV_o, a virus which does not produce a cytopathic effect or plaque in L cells, was also found to be as sensitive to interferon as NDV_{pi} and vesicular stomatitis virus when tested by yield reduction and viral RNA inhibition.

A direct correlation was observed between the inhibition of viral yield and the amount of 54S RNA synthesized at all concentrations of interferon tested. In CE cells, the 54S RNA of the mutant NDV_{pi} was found to be more sensitive than the 54S RNA of the wild-type NDV_o (Table 3). However, at concentrations of less than 1 unit per culture, a differential inhibition of viral RNA species of NDV_o and NDV_{pi} was found in CE as well as in L-cell cultures. In CE cells, the 18S RNA of NDV_o was more sensitive to interferon than the 36 and the 54S RNA species. In contrast, the 18S RNA of the NDV_{pi} was less sensitive than the 36 and the 54S RNA. Similar differential inhibition of viral RNA species by interferon has been reported for other viruses (1, 10, 17). In cases where such differential effect of interferon has not been observed (7, 12), a low concentration of interferon (1 unit or less) was not tested. As shown in this report, higher concentrations of interferon mask the differential effect of this inhibitor on the synthesis of viral RNA species (10). Whatever the mechanism of action of interferon, its differential effect on the synthesis of viral RNA species suggests that not all viral functions are equally sensitive to this inhibitor.

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