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Preliminary Observations Pertaining to Polyadenylation of Rhinovirus RNA

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Human rhinovirus type 14 contained polyadenylated RNA. Virus growth in HeLa cells was inhibited by cordycepin or polyuridilic acid and stimulated by polyadenylic acid. Polyadenylic acid also reversed cordycepin inhibition of virus-induced cytopathology of infected HeLa cells.

The presence of polyadenylic acid (poly[A]) sequences in the genome RNA or mRNA of mammalian viruses is well documented (1, 8, 9, 12). Small RNA viruses with adenylated genomes include Eastern equine encephalitis virus, poliovirus (1), Columbia SK virus, and Sindbis virus (10). Posttranscriptional addition is believed to be the mechanism by which the mRNA of viruses maturing in the nucleus (2), and of vaccinia virus maturing in the cytoplasm (4), acquire these sequences. Available evidence is conflicting in regard to how the mRNAs of cytoplasmic RNA viruses are adenylated. The absence of polyuridylic acid (poly[U]) in the genome RNA of several viruses was cited as evidence for nontranscriptive adenylation of the messengers of these viruses (11). However, the finding of poly(U) in poliovirus-specific double-stranded RNA and of poly(A) in the transcripts of the double-stranded RNA by virion polymerase (18) raises the possibility of polyadenylation by transcription. Consistent with the above possibility is the recent observation that in vitro poly(A) synthesis by the cytoplasm of vesicular stomatitis virus-infected L cells was coupled to RNA transcription (6). Our experiments with human rhinovirus type 14 (1059) (HRV-14) indicate that the RNA of this virus has a high poly(A) content, that virus replication is inhibited by cordycepin and enhanced by exogenous poly(A), and, furthermore, that preformed poly(A) might be added to viral RNA.

To determine labeling of poly(A) in viral RNA, confluent monolayer cultures were infected at moderately high multiplicity of infection with purified HRV-14 (see below) for 0.5 h at room temperature. [^{3}H]adenosine was added either immediately or 6 h postinfection (Table 1) to a final concentration of 5 μCi/ml of growth medium (Eagle minimal essential medium with 5% calf serum and antibiotics), and the cultures were incubated at 34 C. At 20 h postinfection, virus was released from the cultures by freeze-thawing three times and forcing the lysate through a syringe needle. The lysate was clarified by low-speed centrifugation, and the virus was pelleted from the supernatant by centrifugation at 90,000 $\times$ g at 4 C for 4 h. The pellet was suspended in 0.02 M Tris-hydrochloride buffer, pH 7.5, and the suspension was sedimented through a 5 to 25% (wt/wt) sucrose gradient in the above buffer at 70,000 $\times$ g at 4 C for 2 h. The virus peak was located by counting radioactivity of fractions in a scintillation spectrometer. Peak fractions were pooled and dialyzed against 0.02 M Tris-hydrochloride, pH 7.5. The dialyzed virus was extracted two times with a 1:1 (vol/vol) mixture of chloroform and phenol (14) in the presence of 0.2% sodium dodecyl sulfate, 0.002 M EDTA, and carrier yeast RNA. The nonaqueous phase was reextracted with 0.1 M Tris-hydrochloride, pH 9.0 (10). RNA was precipitated from the combined aqueous phases as described (13) and was dissolved in 0.01 M Tris-hydrochloride (pH 7.6) containing 0.2 M KCl. Total acid-insoluble radioactivity, membrane filter binding radioactivity (10), and RNase-resistant radioactivity (3) in a sample of the RNA were determined (Table 1). To observe the effect of cordycepin (Sigma Chemical Co., St. Louis, Mo.), poly(A) (P-L Biochemicals, Inc., Milwaukee, Wisc.), and poly(U) (Sigma Chemical Co.) on virus replication, replicate monolayers in petri plates (60 by 15 mm) containing approximately 2 $\times$ 10^6 cells/plate were infected with a suspension of purified HRV-14 in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} at a multiplicity of

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TABLE 1. *Labeling of poly(A) in rhinovirus RNA*[a]

Time of addition of [^{3}H]adenosine	Counts per minute in 200 μliters of viral RNA[b]				
	Acid insoluble[c]	Filter bound[d]	Total (%)	RNase resistant[e]	Total (%)
0 h Postinfection	1,888	821	43.5	140	7.4
6 h Postinfection	3,244[f]	1,654	51.0	150	4.6

[a] Multiplicity of infection was not determined, but was moderately high as judged from the cytopathology of infected culture at 10 h postinfection; virus growth was allowed to take place for 20 h postinfection.

[b] Each value represents the average of two determinations, 3 days apart, on the same preparation of RNA.

[c] Acid-insoluble radioactivity in 200 μliters of [^{3}H]adenosine-viral RNA was precipitated with 10% trichloroacetic acid at 4 C, collected on a membrane filter (Millipore Corp., type HA, 0.45 μm) washed with 5% trichloroacetic acid, dried at 70 C for 0.5 h, and assayed by liquid scintillation counting.

[d] [^{3}H]adenosine-viral RNA (200 μliters) in 0.01 M Tris-hydrochloride (pH 7.6) was made 0.5 M in KCl and 0.002 M in $MgCl_2$, chilled for 10 min at 4 C, filtered through a membrane filter, washed with 10 ml of the above binding buffer, dried, and counted.

[e] [^{3}H]adenosine-viral RNA (200 μliters) in 0.01 M, Tris-hydrochloride (pH 7.6) containing 0.2 M KCl was incubated for 0.5 h at 37 C with T1 and pancreatic RNases (50 units and 10 μg/ml, respectively). Acid-insoluble radioactivity was precipitated with trichloroacetic acid, collected, washed, dried, and counted.

[f] Cell density in the 6-h monolayer was visibly higher than that in the 0-h monolayer.

infection of 2 PFU/cell, and control cultures were mock-infected with PBS. Medium containing cordycepin, poly(A), cordycepin plus poly(A), or poly(U) at the final concentrations indicated (Table 2) was added (2 ml/culture) to one infected and one control culture. One of the infected cultures received 2 ml of normal medium only. After incubation at 34 C for 10 h, the cytopathology of each culture was estimated under the microscope. Virus was released from the cultures as described above, and the virus titer was determined by plaque assay (13).

The results summarized in Table 1 show that, on the basis of membrane filter-binding (10), approximately one-half of the viral RNA molecules contained poly(A) and, as judged by RNase resistance (3), the average poly(A) content per RNA molecule was high. The data also suggest that a higher proportion of viral RNA label was in poly(A) when labeling was initiated immediately after infection than at 6 h after infection.

The data in Table 2 indicate that the adenosine analogue cordycepin at 20 μg/ml inhibited virus replication, and exogenous poly(A) apparently reversed this inhibition. In the absence of cordycepin, poly(A) enhanced, while poly(U) inhibited, virus production. The observed effects of these agents on the cytopathology of infected cultures were reproducible.

The figures for the poly(A)-containing fraction and the poly(A) content of viral RNA, based on membrane filter-binding and RNase resistance, respectively, are only rough estimates, because not all molecules of polyadenylated RNA would have bound to filters, and the RNase-resistant fraction not further purified probably included some oligonucleotide contaminants (17). Even so, it appears that the poly(A) content of the RNA of HRV-14 is higher than that of poliovirus RNA (17). Our results are new and have interesting implications for the mechanism and significance of polyadenylation of a viral RNA. The inhibition by cordycepin and poly(U), the reversal of cordycepin inhibition by poly(A), and the enhancement by poly(A) of virus replication suggest a need for poly(A) for rhinovirus reproduction. The low efficiency of rhinovirus replication (15) may be, at least in part, due to the insufficiency of poly(A) synthesis in the host cell. The mechanism of poly(U) inhibition of virus replication is not known, but might involve hybrid formation between poly(A) and poly(U). It will be noted that poly(A) inhibits rather than stimulates leukemia virus replication in mouse embryo cells (16) and vaccinia virus replication in chicken embryo fibroblasts (F. A. Garver, personal communication). The inhibition of HRV-14 replication by cordycepin might also mean that the poly(A) in viral RNA is synthesized nontranscriptively (5). Our results also suggest that at least some of the poly(A) sequences in viral RNA might be preformed. The following observations support this notion: (i) a higher fraction of viral RNA label appeared to be in poly(A) when labeling was started imme-

TABLE 2. *Effect of cordycepin, poly(A) and poly(U) on rhinovirus replication in HeLa cells*

Treatment of infected cells[a]	Cells showing cytopathology at 10 h postinfection (%)	PFU/ml at 10 h postinfection
Untreated (control)	50	1.9×10^6
+ Cordycepin, 20 μg/ml	25	2.2×10^5
+ Poly(A), 500 μg/ml	75	7.3×10^6
+ Cordycepin, 20 μg/ml + Poly(A), 500 μg/ml	70	Not tested
+ Poly(U), 500 μg/ml	40	0.8×10^6

[a] No cytopathology could be detected in uninfected controls incubated with any of the agents for 10 h.

diately after infection, rather than at 6 h after infection; (ii) cordycepin inhibition required early presence of the drug in the infected culture (unpublished data); and (iii) cordycepin inhibition was reversed by poly(A). A ligase might link preformed poly(A) to viral RNA.

The questions raised by these findings are currently being investigated.

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