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Requirement of 3′-Terminal Poly(adenylic acid) for the Infectivity of Poliovirus RNA

[ribonuclease H/removal of poly(A)]

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ABSTRACT **Ribonuclease H (EC 3.1.4.34) has been used to remove selectively much of the 3′-terminal poly(adenylic acid) [poly(A)] from poliovirus RNA by treating the RNA with the enzyme in the presence of poly(dT). Over 80% of the poly(A) could be removed and the residuum was found as oligo(A) stretches attached to many or all of the viral RNA molecules. Reduction of the size of the poly(A) markedly decreased the specific infectivity of poliovirus RNA, indicating that poly(A) is necessary to the infectivity of the RNA. The virions in plaques deriving from infection with treated RNA have a normal amount and size of poly(A), indicating that mechanisms exist in infected cells to regenerate normal length poly(A) from truncated poly(A).**

Sequences of poly(A) are found covalently linked to the messenger RNA and heterogenous nuclear RNA of eukaryotic cells, to mitochondrial RNA of HeLa cells, and to the genome of one plant and several mammalian single-stranded RNA viruses (1–3). The biological function of these 3′-terminal poly(A) sequences, however, is unknown. It has been suggested that they may play some role either in the processing and transport of messenger RNA from the nucleus to the cytoplasm or in the translation of the messenger RNA (4, 5).

The single-stranded RNA genome of poliovirus also has a sequence of poly(A) at its 3′-terminus (4, 6). Whereas the addition of poly(A) to nuclear RNA appears to be post-transcriptional (1), poliovirus poly(A) seems to be transcribed because an appropriate length of poly(U) can be recovered from the viral replicative form (7) and replicative intermediate (D. H. Spector; Y. Yogo and E. Wimmer, unpublished results). The fact that the genome of poliovirus RNA is infectious and serves both as messenger RNA for the synthesis of virus specific protein and as a template in viral RNA replication (8) makes it an excellent source of homogeneous RNA for studies on the mechanism of synthesis and function of poly(A).

In this study we have utilized a highly purified RNase H (EC 3.1.4.34) from *Escherichia coli* (W. Keller, manuscript in preparation), to digest selectively poliovirus 3′-terminal poly(A) that has been hybridized with poly(dT). The evidence suggests that the poly(A) on poliovirus RNA serves a critical biological function, because removal of much of the poly(A) reduces the infectivity of the RNA. Furthermore, a mechanism appears to exist in the infected cell that can regenerate full-size poly(A) on progeny viral RNA molecules.

Abbreviations: RNase H, ribonuclease H (a nuclease only able to degrade the RNA portion of a DNA·RNA hybrid); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate.

MATERIALS AND METHODS

Cell Culture, Infection, and Labeling of Viral RNA. The growth of suspended HeLa cells in Joklik modified minimal essential medium plus 7% horse serum and their infection by type 1 poliovirus in the presence of 10 μg/ml of actinomycin D has been described (9). To label viral RNA, 75 μCi/ml [*2,8-*^{3}H]adenosine was added 1 hr after infection. Five hours later a cytoplasmic extract was prepared by Dounce homogenization (10).

For isolation of labeled virus, the cytoplasmic extract was adjusted to 10 mM ethylenediaminetetraacetate (EDTA), 1% sodium dodecyl sulfate ($NaDodSO_4$), 2 M LiCl, and placed at −20° overnight. Single-stranded and partially double-stranded RNA were removed by centrifugation, and the supernatant containing virions and double-stranded RNA was layered over a 35-ml linear 15–30% sucrose gradient in 0.5% $NaDodSO_4$ buffer (0.1 M NaCl; 0.01 M Tris·HCl, pH 7.5; 1 mM EDTA, 0.5% $NaDodSO_4$) and centrifuged at 22° for 2.5 hr at 95,000 × *g* in the SW 27 rotor in the L2-65 B Spinco ultracentrifuge.

RNA, extracted from the purified virions by the $NaDodSO_4$–acetic acid extraction method (11, 12), was centrifuged through a 35-ml linear 15–30% sucrose gradient in 0.5% $NaDodSO_4$ buffer at 22° for 10.5 hr at 95,000 × *g* in the SW 27 rotor. The fractions containing viral RNA were pooled, made 0.4 M in sodium acetate and 50% in ethanol and placed at −20° overnight. The ethanol-precipitated RNA was collected by centrifugation at 10° for 6 hr at 95,000 × *g* in the SW 27 rotor. The precipitated RNA was resuspended in 0.5 ml of 5.0 mM EDTA, pH 7.0, and 0.1-ml aliquots were stored at −70°. By this procedure poliovirus RNA was obtained with a specific activity of from 5.1 to 6.5 × 10^5 cpm/μg.

RNase H Treatment of Poliovirus RNA. RNase H in 0.01 potassium phosphate pH 7.0, 0.2 M potassium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol was kindly supplied by Walter Keller, Cold Spring Harbor. The concentration of the enzyme was not known, so the necessary quantities were determined empirically as the amount required to produce a limit digest of [^{3}H]poly(A)·poly(dT). The reaction mixture of 1.3 ml contained 50 mM Tris·HCl, pH 7.9, 10 mM Mg acetate, 10% glycerol, 1.0 mM EDTA, 27.2 μg of polio RNA [equivalent to 1.02 nmoles of poly(A)], 340 pmoles of poly(dT), and 20 λ of enzyme (omitted in "untreated" sample). After incubation for 60 min at 27°, reactions were terminated by the addition of 0.7 ml of 0.5% $NaDodSO_4$ buffer, and the RNA was immediately collected by

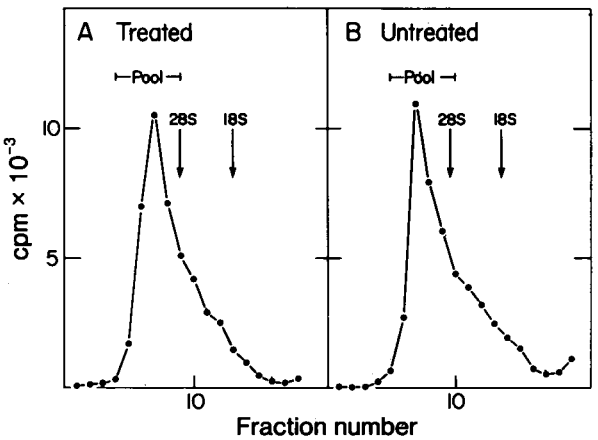

FIG. 1. Sedimentation of RNase H-treated (*A*) and untreated (*B*) [^{3}H]adenosine-labeled poliovirus RNA after denaturation with formamide.

ethanol precipitation. In order to complex the poly(dT), we resuspended the RNA in 2 ml of 90% formamide with 100 μg of poly(A) and 2 mM Tris·HCl, pH 7.3, and incubated it at room temperature for 5–10 min. One milliliter of 0.5% NaDodSO$_4$ buffer was then added and the samples were ethanol-precipitated. The RNA was resuspended in 0.5% NaDodSO$_4$ buffer, layered over a linear 15–30% sucrose gradient in 0.5% NaDodSO$_4$ buffer, and centrifuged at 22° for 10.5 hr at 95,000 × *g* in the SW 27 rotor (Fig. 1). The 35S RNA was collected, pooled, ethanol-precipitated, and resuspended in 5.0 mM EDTA, pH 7.0.

Poly(U) Binding of Viral RNA. Poly(U)-containing glass fiber filters were prepared and used according to a modification of the techniques of Sheldon *et al.* (13). Labeled RNA dissolved in 1 ml of binding buffer (0.3 M NaCl, 0.01 M Tris·HCl, 0.5% NaDodSO$_4$, pH 7.6) was passed through poly(U) filters. The filters were then extensively washed with binding buffer, followed by several washings with a solution of 0.3 M ammonium acetate in 50% ethanol. The filters were dried and counted in toluene-based scintillation fluid.

Infectious RNA Assay. The infectious RNA assay was a combination of the methods of Koch *et al.* (14) and Ellem and Colter (15). The assay is linear with RNA concentration and added excess nonspecific RNA does not compete with infectious RNA (D. Gelfand and D. Baltimore, unpublished results). In the assay, 0.1 μg of viral RNA in 1 ml of PES buffer (0.9 M NaCl, 0.02 M sodium phosphate buffer, pH 7.3) was incubated with 10^7 HeLa cells at 37° for 8 min. The mixture was diluted 10-fold with growth medium containing 2% horse serum and aliquots of 0.1–1 ml were mixed with 10^7 indicator HeLa cells suspended in sufficient medium plus 2% horse serum to give a final volume of 1.5 ml. Then, 1 ml of 1.4% agar medium was added, and the mixture was poured onto plates containing a base layer of 15 ml of agar (0.9% agar in medium plus 5.0% calf serum). Incubation and visualization of plaques was the same as for the poliovirus plaque assay which has been described previously (16).

Isolation of Poly(A). [^{3}H]Adenosine-labeled RNA was treated with 10 μg/ml of pancreatic ribonuclease and 10 units/ml of T$_1$ ribonuclease in 4 ml of 0.3 M NaCl, 0.03 M Na acetate for 60 min at 37°. The reaction was stopped by the addition of 1.6 ml of a 4 mg/ml solution of proteinase K in 0.025 M Tris·HCl pH 7.5. After 10 min at room temperature, the samples were adjusted to 0.5% NaDodSO$_4$ and incubated for 30–60 min at room temperature. Diethylpyrocarbonate was then added to 0.5% and the solution was adjusted to 0.4 M NaCl. The ribonuclease-resistant poly(A) was then isolated by filtering the solution through poly(U) filters. Elution of the poly(A)-containing RNA from poly(U) filters was carried out with two, 1 ml aliquots of 90% formamide containing 2 mM Tris and 100 μg/ml of poly(A) at 65°. The poly(A) was ethanol-precipitated with 100 μg of carrier yeast RNA. The radioactivity remaining on the poly(U)-filters was determined by drying the filters and determining their radioactivity using a toluene-based scintillation fluid.

Acrylamide Gel Electrophoresis of Poly(A). The size of poly(A) samples eluted from poly(U) filters was determined by electrophoresis through 10% acrylamide gels at 7.5 mA per gel for 5.5 hr (17). ^{32}P-labeled *Dictyostelium* 4S and 5S RNA (kindly provided by Bambi Young) were used as markers and bromophenol blue served as the tracking dye. Slices (2 mm) were taken and counted in toluene-based scintillation fluid containing 3.5% NCS (Nuclear Chicago Solubilizer).

Materials. Actinomycin D was a generous gift from Merck, Sharp and Dohme. [*2,8*-^{3}H]Adenosine (34.2 Ci/mmol) was purchased from New England Nuclear Corp.; pancreatic ribonuclease (ribonuclease A) and ribonuclease T$_1$ from Worthington Biochemical Corp.; poly(A) and poly(U) from Miles Laboratories; sodium dodecyl sulfate and formamide from Matheson, Coleman, and Bell; diethylpyrocarbonate, acrylamide and *N,N*-methylene bisacrylamide from Eastman; and proteinase K from EM Laboratories.

RESULTS

RNase H Treatment of Poliovirus RNA. The requirements for RNase H activity as measured by acid solubilization of [^{3}H]poly(A) are summarized in Table 1. Degradation of poly(A) required the presence of both poly(dT) and the enzyme. Furthermore, the presence of a 9-fold excess of poliovirus RNA did not inhibit the solubilization of the poly(A). This RNase H preparation was therefore suitable for removing the poly(A) on poliovirus RNA.

To examine whether the enzyme preparation contained any endonucleolytic activity, we treated [^{3}H]adenosine-labeled poliovirus RNA with RNase H. Treated and untreated RNA

TABLE 1. *Conditions for RNase H digestion*

Reaction system	% Solubilization of [^{3}H]poly(A)
Complete	89
Minus poly(dT)	9
Minus enzyme	3
Plus 87 pmoles of poliovirus RNA	92

Reaction conditions were as described in *Materials and Methods*. The complete mixture (0.2 ml) consisted of 10 pmoles of [^{3}H]-poly(A) (synthesized and kindly provided by Stanley Drost), 3.3 pmoles of poly(dT), and 1 μl of a 1/10 dilution of RNase H. Incubation was for 60 min at 27°.

were then exposed to a denaturing solvent (90% formamide, 2 mM Tris·HCl, pH 7.3) in the presence of 100 μg of poly(A), which would complex the poly(dT) used in the RNase H treatment. The RNA samples were then analyzed in sucrose gradients (Fig. 1). Both preparations show only minimal degradation. The untreated and treated 35S RNA's from this experiment were then pooled (see Fig. 1) and used for further studies.

Poly(U) Binding of RNase H-Treated RNA. To study the fate of the poly(A) sequences on RNase H-treated poliovirus RNA, we assayed binding of the RNA to poly(U) filters (Table 2, *Exp. I*). Untreated RNA bound to the filters with an average efficiency of 60%, while the treated RNA bound with an average efficiency of 8.3%. These values represent minimum estimates of binding because the untreated viral RNA molecules that pass through poly(U) filters contained poly(A) sequences of the same size as those found on RNA molecules that were bound to the filters (D. H. Spector, unpublished results). It is clear that the RNase H treatment markedly reduced the ability of poliovirus RNA to bind to poly(U) filters.

Resistance to Pancreatic plus T_1 Ribonucleases. Further study of the RNase H-treated RNA utilized the resistance of poly(A) sequences to digestion by pancreatic plus T_1 RNases. Treated and untreated, [^{3}H]adenosine-labeled viral RNA molecules were digested with pancreatic plus T_1 RNases and the resistant fraction was assayed by either determining the remaining acid-precipitable radioactivity or by binding the RNase-resistant material to poly(U) filters as described in *Materials and Methods* (Table 2; *Exp. I*). Treatment with RNase H resulted in loss of 83% of the acid-precipitable poly(U)-binding radioactivity remaining on the viral RNA molecules.

Size of the Poly(A) Sequences. To determine whether the 17% remaining poly(A) was due to undigested full-length poly(A) on a few molecules or a residue of short poly(A) on many or all molecules, we studied the approximate length of the poly(A) sequences on the treated and untreated RNA. The poly(A) that resisted treatment with pancreatic plus T_1 RNases and that bound to poly(U) filters was subjected to electrophoresis in 10% polyacrylamide gels (Fig. 2*A* and *B*). The poly(A) from the untreated RNA showed a heterogeneous distribution. From the positions of the 4S and 5S RNA markers, it was estimated that this untreated poly(A) is approximately 50 to 125 nucleotides in length. This estimate agrees with determinations by other methods (ref. 6; and D. H. Spector, unpublished results). The poly(A) from the treated RNA (Fig. 2*B*), however, showed only a sharp peak of oligo(A), less than 40 nucleotides in length. The small peak of radioactivity in fractions 69–75 (Fig. 2*A* and *B*) appears in both RNase H-treated and untreated preparations of poly(A) and probably represents either an oligonucleotide contaminant or a small internal stretch of poly(A).

From the electrophoretic analysis it appears that the pancreatic plus T_1 RNase-resistant [^{3}H]adenosine label in viral RNA that is not degraded by RNase H represents oligo(A) left on many or all of the molecules and not full-length undigested poly(A). The amount of radioactivity in full-length poly(A) is at the very most 300 cpm in the experiment shown in Fig. 2*B*. It is our experience that the minimal re-

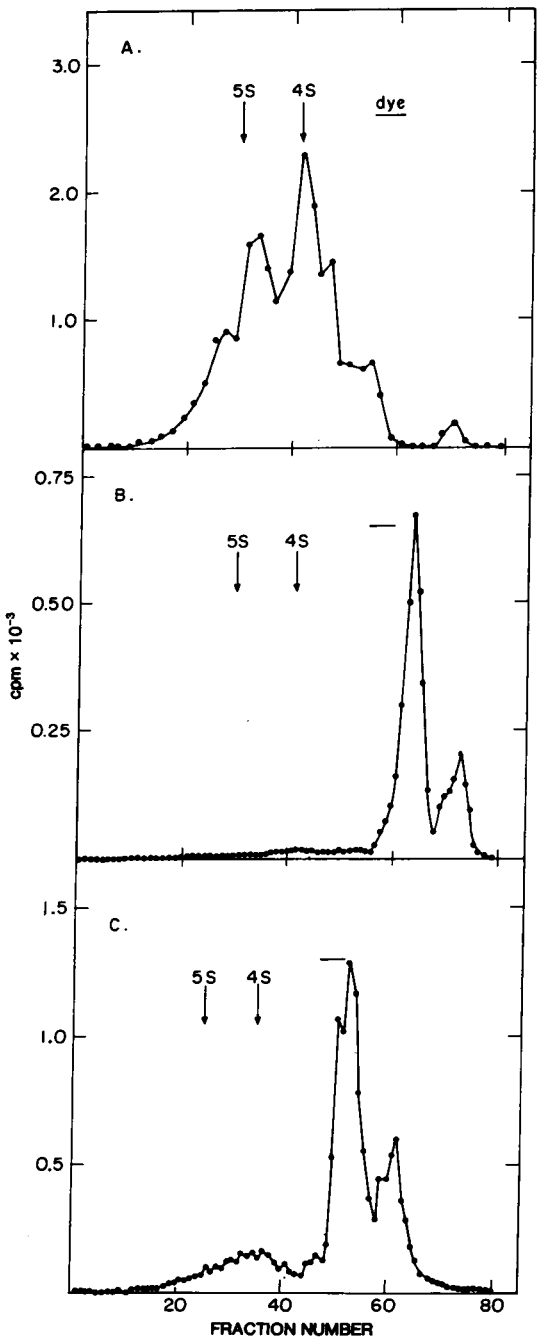

FIG. 2. Polyacrylamide gel electrophoresis of the poly(A) sequences in the RNase H-treated and untreated RNA. [^{3}H]-Adenosine labeled untreated RNA (*A*) and RNase H-treated RNA (*B*) from Experiment I and RNase H-treated RNA (*C*) from Experiment II were digested with pancreatic plus T_1 RNases, bound to poly(U) filters, and eluted as described in *Materials and Methods*. The ethanol-precipitated poly(A) was dissolved in 100 μl of solution of 50% formamide, 25% glycerol, 0.04 M Tris·HCl, pH 7.2, 0.02 M Na acetate, 1 mM EDTA, and 0.2% $NaDodSO_4$ and subjected to electrophoresis in 10% polyacrylamide gels at 7.5 mA per gel for 5.5 hr (*A* and *B*) or 4.5 hr (*C*). Slices (2 mm) were counted in toluene-based scintillation fluid containing 3.5% Nuclear Chicago Solubilizer (*B* and *C*). For gel *A* the 2-mm slices were placed in 1 ml of a solution containing 0.5 M Na acetate, 1 mM EDTA, 0.2% $NaDodSO_4$, pH 7.0, and shaken for 12 hr at 37°, after which 0.2-ml aliquots from odd-numbered fractions were counted in Aquasol.

TABLE 2. *Binding to poly(U) filters and resistance to pancreatic and T_1 RNase of RNase H-treated and untreated poliovirus RNA*

	Experiment I		Experiment II		
	Treated	Untreated	Treated	Untreated	Treated with RNase H minus poly(dT)
Percent of binding to poly(U)	8.3 (4.5–12)	60 (55–72)	20 (16; 25)	64 (50; 78)	62 (47; 78)
Percent RNase-resistant	0.78	4.6	—	—	—
Percent of untreated	17	100	—	—	—
Percent RNase-resistant and poly(U) bound	0.42 (0.35–0.49)	2.4 (2.3–2.5)	0.91 (0.82; 1.0)	2.3 (2.2; 2.3)	2.2 (2.1; 2.3)
Percent of untreated	17.5	100	39.5	100	96

Values in parentheses represent individual determinations or the range of 3 or 4 determinations; each determination involved a separate RNase H digestion.

covery of full-length poly(A) under these conditions is 47%. If 17% of the molecules had had full size poly(A) we would have expected at least 3000 cpm in this region of the gel. Therefore, at most 2% of the treated RNA molecules have full size poly(A). From these results we conclude that RNase H treatment of poliovirus RNA almost completely eliminates the long stretches of poly(A) on poliovirus RNA but leaves a residual stretch of oligo(A).

Infectivity of RNase H-Treated RNA. To assay whether the poly(A) sequences on the poliovirus RNA molecules are necessary to the infectivity of the molecules, we assayed the infectivity of RNase H-treated and untreated 35S RNA, selected from sucrose gradients like those in Fig. 1 (Table 3, *Exp. I*). Treatment of viral RNA with RNase H resulted in more than a 10-fold decrease in infectious titer relative to the untreated sample, indicating that poly(A) is required in order for the molecules to be infectious.

TABLE 3. *Infectious RNA assay*

	Experiment I		Experiment II	
	PFU/ 0.01 μg RNA	% of Untreated	PFU/ 0.01 μg RNA	% of Untreated
Treated				
Trial 1	40		102	16.5
		6.7		
Trial 2	46		—	—
Untreated				
Trial 1	700		620	(100)
		(100)		
Trial 2	600		—	—
Treated with RNase H minus poly(dT)	—	—	660	106
Treated and poly(U)-bound	190	29	—	—
Untreated and poly(U)-bound	725	113	—	—

The conditions for testing the infectivity of the RNase H-treated and untreated RNA and the procedure for the binding and elution of poliovirus RNA from poly(U) filters are described in *Materials and Methods*. Each trial represents the average of eight samples; the two trials represent separate RNase H digestions.

When the RNase H-treated molecules were first preselected on poly(U) filters, the specific infectivity of the untreated RNA was unaffected, while that of the treated RNA increased 4-fold. However, even after preselection on poly(U) filters, the specific infectivity of the treated preparation was still only 29% that of the untreated RNA. Thus it would appear that a sequence of poly(A) of sufficient length to permit poliovirus RNA to bind to a poly(U) filter is not necessarily sufficient to allow the molecule to be infectious.

In a second set of experiments we utilized a different batch of RNase H which removed the poly(A) less efficiently from poliovirus RNA molecules. Pancreatic and T_1 RNase digestion of the RNA treated with this RNase H preparation indicated that only 60% of the poly(A) had been removed (Table 2, *Exp. II*). This RNase H-treated RNA bound to poly(U) filters with an average efficiency of 20% (Table 2, *Exp. II*), and its specific infectivity was 16.5% of that of the untreated RNA (Table 3, *Exp. II*). Electrophoretic analysis of the pancreatic and T_1 RNase-resistant [^{3}H]adenosine label remaining on the RNase H-treated molecules (Fig. 2*C*) indicated that as many as 8% of the molecules had full size poly(A), while the majority of the adenosine label migrated as a relatively homogeneous fraction just ahead of the dye marker. Furthermore, this set of experiments demonstrated that the RNase H treatment of RNA in the absence of poly(dT) caused no detectable change in the RNA. RNA treated with the enzyme alone had the same ability to bind to poly(U) filters, the same percentage of RNase-resistance, and the same specific infectivity as untreated RNA (Tables 2 and 3, *Exp. II*).

The combined data of the two experiments showed a correlation between the amount of residual poly(A) on the RNA molecules after RNase H treatment and the level of residual infectivity of the molecules. In Experiment I, the removal of 82% of the poly(A) radioactivity reduced the specific infectivity to 6.7% of normal; in Experiment II the removal of 60% of the poly(A) radioactivity reduced the infectivity to 16.5% of normal.

Characterization of Virus Resulting from Infection with RNase H-Treated RNA. Five plaques arising from the RNase H-treated RNA in Experiment I were used to prepare [^{3}H]adenosine-labeled viral RNA. The poly(A) from all five preparations was indistinguishable in size from the poly(A) on viral RNA produced by plaques arising from

untreated RNA. Since there was 3-fold greater residual infectivity on the treated molecules than there was full-length poly(A), the chance that all five plaques arose from molecules containing full length poly(A) is 3^{-5} and, therefore, it would appear that mechanisms exist in the infected cell to regenerate full-length poly(A) from truncated molecules.

DISCUSSION

From the data presented here we conclude that the 3'-terminal sequence of adenylic acid residues on poliovirus RNA is important to the infectivity of the molecule. This conclusion rests on the ability of RNase H in the presence of poly(dT) to remove only the poly(A) from the molecules. The nuclease appears to make no endonucleolytic scissions in the viral RNA since, after treatment with the enzyme followed by denaturation, the RNA molecules remain intact (Fig. 1). Furthermore, treatment with RNase H in the absence of poly(dT) does not affect the specific infectivity of 35S RNA molecules, indicating that no other critical alteration of the RNA is produced by the RNase H preparations. The oligo(A) that remains presumably does so because, after the enzyme has degraded most of the poly(A), the poly(dT) can no longer bind to the residual oligo(A) and the oligo(A) is therefore not degraded by the RNase H.

The absolute length of poly(A) needed for infectivity is not known. The fact that even poly(U) selection of RNase H-treated molecules reveals a population of molecules that has a specific infectivity lower than normal suggests that a fairly long stretch of poly(A) is needed. The role of the poly(A) is not known; it could even be that it is needed for naked RNA to be infectious but would not be needed if poly(A)-deficient RNA were encapsidated.

It is apparent from analysis of the data that the percent residual infectivity of the RNase H-treated RNA is two to four times above the percent of RNA molecules with normal size poly(A). Reconstruction experiments have indicated that the treated RNA does not enhance the infectivity of untreated molecules (D. Spector, unpublished results), so the excess infectivity appears to result from molecules deficient in poly(A). When five plaques arising from treated RNA were analyzed, the progeny had normal size poly(A). Apparently the infected cell is able to regenerate full-size poly(A) on some of the treated molecules. Virus-specific or cellular enzymes might be responsible for lengthening the poly(A).

Various hypotheses concerning the role of poly(A) in the processing, selection, or transport to the cytoplasm of nuclear RNA or in the translation of mRNA have been proposed (4, 5). The findings that histone mRNA has no poly(A) (18), that some viruses that replicate solely on the cytoplasm have poly(A) (1), and that removal of poly(A) segments from poliovirus RNA, and from species of mRNA that were previously polyadenylylated, does not affect the *in vitro* translational capacity of the RNA (refs. 19 and 20; D. H. Spector, L. Villa-Komaroff, and D. Baltimore, unpublished results) seem to indicate that in their simplest form these various hypotheses do not explain poly(A) function. The experiments presented in this paper represent the first direct demonstration of a biological function for 3'-terminal poly(A) on an RNA. Since the intracellular steps of poliovirus replication have been well documented (6), the use of deadenylylated poliovirus RNA in one-step growth experiments should help clarify more precisely the function of the poly(A).

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