JOURNAL OF VIROLOGY, June, 1975, p. 1432-1439

Vol. 15, No. 6
Printed in U.S.A.

Polyadenylic Acid on Poliovirus RNA

III. In Vitro Addition of Polyadenylic Acid to Poliovirus RNAs

DEBORAH H. SPECTOR AND DAVID BALTIMORE*

Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received for publication 10 March 1975

A crude RNA polymerase preparation was made from HeLa cells infected for 3 h with poliovirus. All virus-specific RNA species labeled in vitro (35S RNA, replicative intermediate RNA [RI], and double-stranded RNA [dsRNA]) would bind to poly(U) filters and contained RNase-resistant stretches of poly(A) which could be analyzed by electrophoresis in polyacrylamide gels. After incubation for 45 min with [^{3}H]ATP in the presence of the other three nucleoside triphosphates, the labeled poly(A) on the RI and dsRNA migrated on gels as relatively homogenous peaks approximately 200 nucleotides in length. In contrast, the poly(A) from the 35S RNA had a heterogeneous size distribution ranging from 50 to 250 nucleotides. In the absence of UTP, CTP, and GTP, the size of the newly labeled poly(A) on the dsRNA and RI RNA was the same as it was in the presence of all four nucleoside triphosphates. However the poly(A) on the 35S RNA lacked the larger sequences seen when the other three nucleoside triphosphates were present. When [^{3}H]ATP was used as the label in infected and uninfected extracts, heterogeneous single-stranded RNA sedimenting at less than 28S was also labeled. This heterogeneous RNA probably represents HeLa cytoplasmic RNA to which small lengths of poly(A) (approximately 15 nucleotides) had been added. These results indicate that in the in vitro system poly(A) can be added to both newly synthesized and preexisting RNA molecules. Furthermore, an enzyme capable of terminal addition of poly(A) exists in both infected and uninfected extracts.

The mRNA's of eukaryotic cells and many viruses have been shown to contain 3'-terminal poly(A) sequences (see 14 for review). The single-stranded RNA genome of poliovirus also has a sequence of poly(A) at its 3' terminus (1, 17). In a previous paper (12) we examined the content, size, and mechanism of synthesis of the 3'-terminal poly(A) on the various intracellular species of poliovirus RNA: virion RNA, replicative intermediate (RI) RNA, double-stranded "replicative form" (dsRNA), polyribosomal RNA, and total 35S plus strand cytoplasmic RNA. The evidence presented in that paper demonstrated that all species of poliovirus RNA contained poly(A) and suggested that poly(A) is added to 35S RNA molecules in the replication complex.

To analyze further the mechanism of synthesis of poliovirus poly(A), we prepared a crude poliovirus RNA polymerase from cells infected for 3 h (8). In this preparation, the polymerase is bound to RNA and therefore does not respond to or require the addition of exogenous RNA for further nucleotide incorporation. In the presence of four ribonucleoside triphosphates, three species of poliovirus-specific RNA are labeled in this crude polymerase preparation: 35S single-stranded RNA, dsRNA, and RI RNA (8).

The experiments presented here demonstrate that all species of poliovirus RNA synthesized in vitro contain poly(A) sequences and provide further support for the replication complex as the site of synthesis of poliovirus poly(A). The length of these sequences of poly(A) resembles that on the same species synthesized in the infected cell late in infection (4 to 5 h postinfection). We also present evidence that there is terminal addition of poly(A) to preexisting cellular RNA molecules in the extracts prepared from both infected and uninfected cells.

MATERIALS AND METHODS

Cell culture and infection with poliovirus 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 of actinomycin D per ml has been described (3, 12). Three hours after infection, 8×10^8 infected cells were washed by centrifugation two times in Earle

saline. They were then resuspended in 32 ml of RS buffer (0.01 M Tris, pH 7.4, 0.01 M NaCl, 1.5 mM $MgCl_2$). After 10 min at 0 C the cells were broken with a Dounce homogenizer, made 5% in glycerol, and stored in 1.5-ml aliquots at −90 C to be used in the in vitro reaction mix.

Conditions for enzyme incubation and assay. The procedure was essentially that of Baltimore (2). A 1.5-ml aliquot of the frozen infected cell homogenate was thawed and the nuclei and cell debris were removed by centrifugation (1,250 rpm for 5 min in the PR-6000, International centrifuge). The supernatant was then centrifuged at 130,000 × *g* for 45 min at 4 C in the 65 rotor in a L2-65B Spinco ultracentrifuge. The pellet was resuspended in 1.5 ml of 8 mM Tris-hydrochloride (pH 8.0), 8 mM NaCl, and 5% glycerol. The standard 0.2-ml reaction mixture consisted of 0.14 ml of this crude RNA polymerase preparation (80 to 140 μg of protein as determined by the method of Lowry et al. [10]; approximately 3.5×10^6 infected cells), 50 mM Tris-hydrochloride (pH 8.3), 8 mM magnesium acetate, 15 mM creatine phosphate, 40 μg of creatine phosphokinase, 3 μg of actinomycin D, 0.2 mM CTP, 0.2 mM UTP, 0.2 mM or 0.05 mM GTP or ATP (0.05 mM when used as the label), 10 to 12.5 μCi of [8-^{3}H]GTP (5.28 Ci/mmol) or [2,8-^{3}H]ATP (32.69 Ci/mmol), 1 mM dithiothreitol, and 30 mM KCl. Incubation was at 37 C for the indicated times. Duplicate samples were precipitated with 1 ml of saturated sodium pyrophosphate plus 2 ml of ice cold 10% trichloroacetic acid. The precipitate was collected on glass fiber filters which were dried and counted in a toluene-based scintillant.

Fractionation of in vitro labeled RNA. After incubation at 37 C for 45 min, the assay mixtures were adjusted to 20 mM EDTA (pH 7.0), 1% sodium dodecyl sulfate (SDS), and 0.5 M sodium acetate and extracted two times at room temperature with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2) and two times with an equal volume of chloroform-isoamyl alcohol (96:4). The aqueous phase was precipitated with 2.5 volumes of ethanol. The ethanol precipitate was resuspended in 50 mM Tris-hydrochloride (pH 7.3) and 10 mM magnesium acetate and was digested with iodoacetamide-treated DNase (20). This solution was then adjusted to 20 mM EDTA, 1.0% SDS, 10 mM Tris-hydrochloride (pH 7.3), and 2 M LiCl and was placed at −20 C overnight.

From the LiCl precipitate, the RI and single-stranded RNA were purified by chromatography through 2% agarose followed by velocity sedimentation of the single-stranded RNA through a 15 to 30% sucrose gradient in 0.5% SDS buffer (12). From the LiCl supernatant, the dsRNA was purified by chromatography through 2% agarose followed by velocity sedimentation of the excluded RNA through a 15 to 30% sucrose gradient in 0.5% SDS buffer (12).

Binding of viral RNA to poly(U) filters; isolation, acrylamide gel electrophoresis, and determination of chain lengths of poly(A). These procedures have been described in detail previously (11, 12).

Materials. Actinomycin D was a generous gift from Merck Sharp and Dohme. [8-^{3}H]GTP (5.28 Ci/mmol) and [2,8-^{3}H]ATP (32.69 Ci/mmol) were purchased from New England Nuclear; RNase-free DNase was obtained from Worthington Biochemical Corp.; CTP, UTP, GTP, and ATP were from P.L. Biochemicals; creatine phosphate and creatine phosphokinase were from Calbiochem and iodoacetamide was from Sigma Chemical Co. The source of all other materials has been detailed previously (12).

RESULTS

Characteristics of the in vitro assay system. In the experiments reported here, the crude poliovirus RNA polymerase was prepared from cells infected for 3 h. At this time of the infection the rate of viral RNA synthesis has reached its maximum and become constant (3). The crude polymerase is bound to its RNA template and therefore does not require the addition of exogenous viral RNA for further nucleotide incorporation. As has been shown previously, in this crude RNA polymerase system three species of poliovirus-specific RNA are labeled: 35*S* single-stranded RNA, 20*S* dsRNA, and partially double-stranded RI consisting of complementary RNA hydrogen bonded to several growing strands of viral RNA.

In a standard reaction, [^{3}H]GMP incorporation proceeded for at least 50 min at a slowly decreasing rate. The kinetics of the reaction were such that 160 pmol of GMP were incorporated in 10 min per mg of protein. There was little incorporation of [^{3}H]GMP into acid-insoluble material in uninfected extracts (Fig. 1A) and there was insignificant incorporation in infected extracts in the absence of the other three triphosphates (Fig. 2A). Thus it appeared that when [^{3}H]GTP was used as the label only newly synthesized poliovirus RNA was being

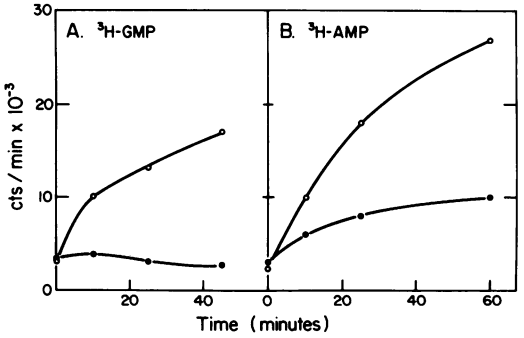

FIG. 1. *Incorporation of [^{3}H]GMP (A) and [^{3}H]AMP (B) in infected and uninfected extracts. Each point represents an average of duplicate 0.2-ml reaction mixtures containing 80 μg of protein incubated at 37 C for the indicated length of time in the presence of [^{3}H]GTP or [^{3}H]ATP plus the other three nucleoside triphosphates. Symbols: ●, extract from uninfected cells; ○, extract from infected cells.*

labeled as suggested previously (8). Most of the single-stranded RNA labeled in vitro sedimented at the same rate as authentic 35S [^{3}H]uridine-labeled virion (Fig. 3). However the in vitro product has some single-stranded RNA sedimenting ahead of 35S which has not been characterized.

Most of the RI labeled in vitro sedimented between 18S and 28S which is smaller than the average size of the in vivo RI at 3 h after infection, but similar to the size of the RI after 6 h of infection (D. H. Spector, unpublished data). The smaller size of the RI is probably a reflection of the absence of the initiation of new RNA chains during in vitro incubation. Apparently, some small number of nascent RNA chains cannot be displaced from the RI so some RI remains even after extended incubation in vitro.

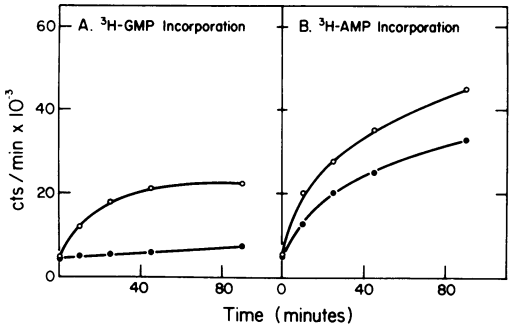

FIG. 2. *Incorporation of [^{3}H]GMP (A) and [^{3}H]AMP (B) in the presence or absence of the other three nucleoside triphosphates. Each point represents an average of duplicate 0.2-ml reaction mixtures containing 140 μg of protein incubated at 37 C for the indicated length of time in the presence of [^{3}H]GTP or [^{3}H]ATP plus (O) or minus (●) the other three nucleoside triphosphates.*

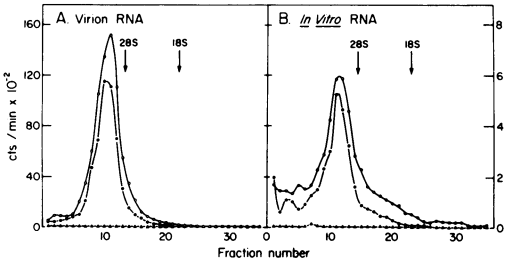

FIG. 3. *Sedimentation of heat-denatured virion RNA and in vitro-labeled single-stranded RNA. [^{3}H]uridine-labeled virion RNA was prepared as described previously (12). [^{3}H]GMP-labeled single-stranded RNA was isolated by two cycles of agarose chromatography from an infected extract incubated for 45 min in the presence of all four nucleoside triphosphates. The RNA samples were dissolved in 0.01% SDS buffer plus 20 mM EDTA and heated to 80 C for 3 min. The samples were then sedimented through 15 to 30% sucrose gradients in 0.01% SDS buffer. Samples from each fraction were either precipitated with trichloroacetic acid (O), bound to poly(U) filters (●), or digested with T_1 plus pancreatic RNases (▲), as described in the Materials and Methods.*

To investigate whether the in vitro RNA products contained poly(A) the capacity of both [^{3}H]GMP and [^{3}H]AMP-labeled 35S and RI RNA to bind to poly(U) filters was studied (Table 1). Both RI and 35S RNA bound to poly(U) filters with efficiencies from 54 to 68%, values similar to the efficiency of binding of [^{3}H]adenosine-labeled virion RNA (12). [^{3}H]adenine- and [^{3}H]uracil-labeled phage f_2 RNA, which contain no poly(A), were bound less than 1% by the filters.

To investigate whether poly(A) was only loosely associated with 35S RNA, both [^{3}H]GMP-labeled in vitro 35S RNA and [^{3}H]uridine-labeled virion RNA were denatured with heat and sedimented through sucrose gradients. Samples of the fractions from the gradient were assayed for acid-precipitable radioactivity, ability to bind to poly(U) filters, or digestability by T_1 plus pancreatic RNases (Fig. 3). The heat denatured in vitro and viral 35S RNA bound to poly(U) with equal efficiency suggesting that both contained covalently bound poly(A). Furthermore, as expected, there was no [^{3}H]GMP- or [^{3}H]uridine-labeled RNase-resistant RNA in either type of RNA.

TABLE 1. *Binding to poly(U) filters of RNAs labeled in vitro*[a]

Sample	Label	% binding to poly(U) filters
In vitro 35S RNA	[^{3}H]ATP	60
In vitro 35S RNA	[^{3}H]GTP	54
In vitro replicative intermediate	[^{3}H]ATP	68
In vitro replicative intermediate	[^{3}H]GTP	60
Virion RNA	[^{3}H]adenosine	55
f_2 Bacteriophage	[^{3}H]adenine	0.55
f_2 Bacteriophage	[^{3}H]uracil	0.35
Poly(A)		100

[a] Labeled 35S and RI RNA were isolated from an infected extract incubated for 45 min with [^{3}H]ATP or [^{3}H]GTP in the presence of the other three nucleoside triphosphates. Virion RNA labeled from [^{3}H]adenosine and [^{3}H]adenine- and [^{3}H]uracil-labeled f_2 bacteriophage RNA were prepared as described previously (12). Values of the percentage of binding to poly(U) filters represent the average of two independent determinations.

Polyadenylate sequences in the RNA product synthesized in vitro. To study further the polyadenylate sequences on the in vitro product, we used [^{3}H]ATP as the label. Figure 1B shows the incorporation of [^{3}H]AMP into both infected and uninfected extracts. In contrast to the results with [^{3}H]GTP, there was considerable incorporation of [^{3}H]AMP into acid-insoluble material in the uninfected in vitro extract. Furthermore, there was considerable [^{3}H]AMP incorporation in the absence of the other three triphosphates (Fig. 2B).

The distribution of sedimentation rates of the [^{3}H]AMP-labeled single-stranded RNA ranged from 35*S* to less than 18*S* but included a peak at 35*S* (Fig. 4A). This was in contrast to the sharp 35*S* peak of [^{3}H]GMP-labeled single-stranded RNA (Fig. 3B). The [^{3}H]AMP-labeled RI displayed the same sedimentation pattern as seen for the [^{3}H]GMP-labeled RI and the dsRNA labeled with [^{3}H]AMP in vitro has the same size as the dsRNA isolated from infected cells.

To physically identify the poly(A) made in vitro, we utilized the fact that poly(A) sequences are resistant to digestion by pancreatic plus T_1 RNases. The 35*S* single-stranded RNA (see Fig. 4A), less than 28*S* RNA (see Fig. 4A), RI RNA, and dsRNA, were purified from an in vitro preparation labeled for 45 min with [^{3}H]ATP in the presence of the other nucleoside triphosphates. The samples were denatured by brief treatment with alkali and digested with pancreatic plus T_1 RNases, and the resistant fraction was assayed by determining the remaining acid-precipitable radioactivity (Table 2, column 1). Under these conditions, authentic [^{3}H]poly(A) was completely RNase resistant whereas the 35*S* RNA labeled in vitro with [^{3}H]GTP was completely susceptible to digestion. The RI, dsRNA, and 35*S* RNA were found to have an acid-precipitable RNase-resistant fraction representing from 9 to 11% of the total [^{3}H]AMP label. In contrast, 51% of the less than 28*S* RNA was RNase resistant, indicating that this RNA contained a higher percentage of labeled poly(A) than the RI, dsRNA, or 35*S* RNA.

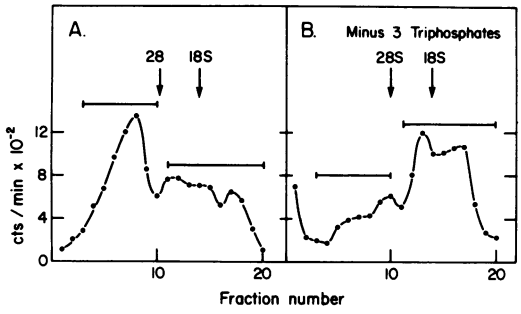

FIG. 4. *Sedimentation of single-stranded RNA labeled in vitro with [^{3}H]ATP. Single-stranded RNA was isolated as in Fig. 3 from an infected extract incubated for 45 min with [^{3}H]ATP plus (A) or minus (B) the other three nucleoside triphosphates after two cycles of agarose chromatography. The RNA samples were dissolved in 0.5% SDS/SDS buffer and sedimented through 15 to 30% sucrose gradients in 0.5% SDS buffer. Samples from each fraction were counted in a xylene-based scintillant. The brackets indicate those fractions which were pooled as 35S RNA and as less than 28S RNA for further analysis.*

To investigate the size of the poly(A) sequences on the RNA species labeled in vitro, the poly(A) which resisted digestion by pancreatic plus T_1 RNases was bound to poly(U) filters, eluted, and subjected to electrophoresis in 10% polyacrylamide gels (Fig. 5). The poly(A) from the 35*S* RNA and less than 28*S* RNA both had heterogeneous size distributions ranging from 50 to 250 nucleotides. In contrast to this heterogeneous distribution, the poly(A) from both the RI and the dsRNA migrated as relatively homogenous peaks approximately 200 nucleotides in length. The distribution of the poly(A) on the in vitro labeled 35*S*, RI, and dsRNA is very similar to that seen on the same species labeled in the infected cell with [^{3}H]adenosine late during the infection cycle (4 to 5 h) (12).

Size of poly(A) on the in vitro product as a function of time. In the preceding paper (12), we showed that the size of the poly(A) on the RI at the time the polymerase extracts were made (3 h postinfection) was 50 to 125 nucleotides whereas that on the RI isolated after 4 h of

TABLE 2. *Resistance of RNAs labeled in vitro to digestion by RNase*[a]

Sample	% Resistance	
	4 Nucleoside triphosphates present	[^{3}H]ATP alone
In vitro 35*S* RNA	11	93
In vitro replicative intermediate	11	79
In vitro dsRNA	9	88
In vitro <28*S* RNA	51	100
[^{3}H]GMP-labeled in vitro 35*S* RNA	<1	
Poly(A)	100	

[a] The various in vitro RNAs were prepared from infected extracts incubated for 45 min with [^{3}H]ATP plus or minus the other three nucleoside triphosphates. Values of percentage of resistance to T_1 plus pancreatic RNase represent the average of at least two independent determinations.

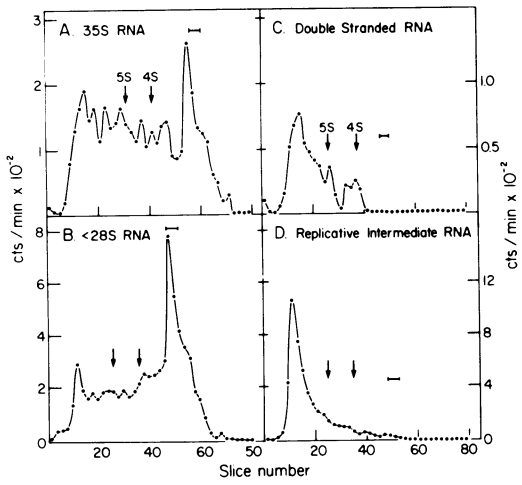

FIG. 5. *Polyacrylamide gel electrophoresis of poly(A) sequences on RNA labeled with [³H]ATP in the presence of the other three nucleoside triphosphates. The RNA species were isolated from an infected extract incubated for 45 min. The 35S RNA and less than 28S RNA were isolated from pooled regions shown in Fig. 4A. The RNA was treated with 0.1 M NaOH at room temperature for 1.5 min, neutralized with 0.5 M Tris-hydrochloride, pH 7.5, digested with T_1 plus pancreatic RNases, bound to poly(U) filters, and eluted as described in the Materials and Methods. The ethanol-precipitated poly(A) was dissolved in 100 μl of 50% formamide, 25% glycerol, 0.04 M Tris, pH 7.2, 0.02 M sodium acetate, 0.001 M EDTA, and 0.2% SDS and was analyzed by electrophoresis through 10% polyacrylamide gels at 7.5 mA/gel for 5.5 h. Slices (2 mm) were placed in 1 ml of a solution containing 0.5 M sodium acetate, 0.001 M EDTA, and 0.2% SDS (pH 7.0) and were shaken for 12 h at 37 C after which samples from odd numbered fractions were counted in xylene based scintillant. (A) 35S RNA; (B) <28S RNA; (C) dsRNA; and (D) RI RNA.*

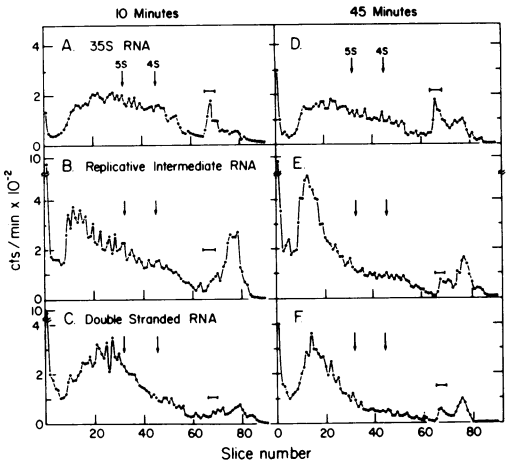

FIG. 6. *Polyacrylamide gel electrophoresis of poly(A) sequences on RNA labeled for 10 or 45 min. The RNA species were isolated and treated as in Fig. 5 except that slices (2 mm) of the gel were directly counted in a toluene-based scintillant containing 3.5% Nuclear Chicago Solubilizer. (A) 35S RNA, 10 min; RI RNA, 10 min; (C) dsRNA, 10 min; (D) 35S RNA, 45 min; (E) RI RNA, 45 min; (F) dsRNA, 45 min.*

infection was at least 200 nucleotides. After 4 h of infection the in vivo 35*S* cytoplasmic RNA also showed the presence of larger sequences of poly(A). To determine whether a size transition occurred in vitro, we compared the size of [³H]AMP-labeled poly(A) on the 35*S* RNA, RI, and dsRNA isolated from crude polymerase extracts after 10 and 45 min of labeling (Fig. 6). The poly(A) on the 35*S* and dsRNA when isolated at either 10 or 45 min of incubation showed approximately the same distribution but there was a slight shift to larger sequences for the poly(A) isolated from the 45-min dsRNA or 35*S* RNA. In contrast, the difference between the size of the poly(A) in the 10- and 45-min RI is considerable. Although at 10 min of incubation the poly(A) on the RI shows a heterogeneous distribution from 50 to 250 nucleotides in length, after 45 min of incubation most of the poly(A) on the RI is at least 200 nucleotides long. From this result we conclude that the switch to the synthesis of larger poly(A) sequences on the RI occurs immediately in the in vitro extract, but that for some reason the newly synthesized molecules with the largest poly(A) remain attached to the RI during the isolation procedure.

Size of the poly(A) synthesized in the absence of UTP, GTP, and CTP. Because the less than 28*S* RNA labeled with [³H]ATP in the presence of the other three nucleoside triphosphates displayed a high degree of RNase resistance (Table 2) and because there was considerable incorporation of [³H]AMP into acid-insoluble material in the absence of the other three triphosphates (Fig. 2B), we investigated whether there was terminal addition of poly(A) to preexisting RNA molecules in the in vitro extract. From an extract incubated for 45 min with [³H]ATP in the absence of UTP, CTP, and GTP, the RI, dsRNA, and single-stranded RNA were isolated. The single-stranded RNA was displayed on a sucrose gradient (Fig. 4B) and the 35*S* RNA and less than 28*S* RNA were pooled as indicated. Comparing Fig. 4A and B, it appears that in the absence of three nucleoside triphosphates there was very little [³H]AMP-labeled 35*S* single-stranded RNA.

However, there was at least as much [^{3}H]AMP label in the less than 28S RNA in the absence of the other three nucleoside triphosphates as there was in their presence. Furthermore, there was considerable [^{3}H]AMP labeling of the RI and the dsRNA (data not shown). These results suggest that there was terminal addition of poly(A) to preexisting RNA molecules in the extract.

To investigate further the possibility of terminal addition, we denatured and digested with pancreatic plus T_1 RNases the RI, dsRNA, 35S RNA and less than 28S RNA labeled with [^{3}H]AMP in the absence of the other three nucleoside triphosphates (Table 2, column 2). All of these species displayed greater than 79% RNase resistance. Most of the material which was RNase resistant bound to and eluted from poly(U) filters indicating that it was truly poly(A) and not just [^{3}H]AMP-labeled dsRNA (data not shown). The size of the poly(A) on the 35S and less than 28S RNA synthesized in the absence of three nucleoside triphosphates (Fig. 7A and B) was virtually identical to that seen on the less than 28S RNA synthesized in the presence of four nucleoside triphosphates (Fig. 5B). The larger sequences seen when the other three nucleoside triphosphates were present were lacking from the 35S RNA poly(A). The size of the poly(A) on the dsRNA and RI (Fig. 7C and D) was the same both in the presence and absence of the other three nucleoside triphosphates.

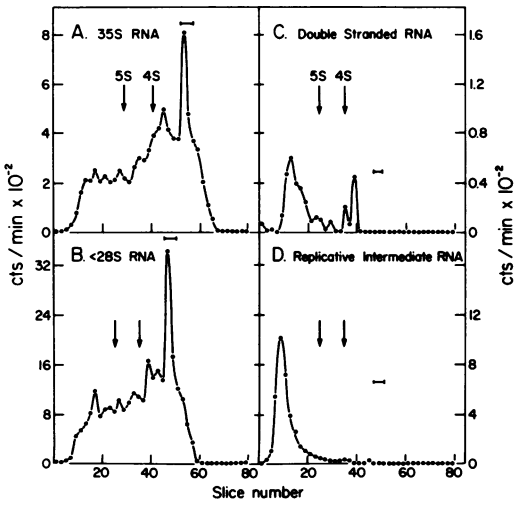

FIG. 7. *Polyacrylamide gel electrophoresis of poly(A) sequences on RNA labeled for 45 min in an infected extract containing only [^{3}H]ATP. The RNA was isolated and treated as in Fig. 5. (A) 35S RNA; (B) <28S RNA; (C) dsRNA; (D) RI RNA.*

As a direct test for terminal addition of poly(A), we isolated the less than 28S RNA poly(A) from gels similar to those seen in Fig. 5B and 7B, digested the material with KOH and subjected it to electrophoresis to determine the amount of radioactivity in adenosine and AMP (Table 3). The ratio of amount of radioactivity in AMP to adenosine is a direct measure of the chain length of newly synthesized 3'-terminal poly(A). From the migration of this poly(A) on gels we would have expected an average chain length of 100 nucleotides. However, the adenosine/AMP ratio of the poly(A) on less than 28S RNA synthesized in the presence of the other three nucleoside triphosphates was 1/15 and in their absence was 1/25. As a control, we determined that the adenosine/AMP ratio of the poly(A) found on virion RNA was 1/71 which agreed with the estimated chain length from the migration of the poly(A) in 10% polyacrylamide gels (12). The adenosine/AMP ratio of the poly(A) on the other species of RNA was not determined because there was too little radioactivity in the poly(A) isolated from the gels to

TABLE 3. *Length of poly(A) on less than 28S RNA labeled in vitro*

Sample	Counts/ min in adenosine	Counts/ min in AMP	Adenosine/ AMP
<28S RNA (plus three triphosphates)			
Trial 1	157	2,351	1/15
Trial 2	53	876	1/18
<28S RNA ([^{3}H] ATP alone)	230	5,706	1/25
Virion RNA			
Trial 1	170	12,065	1/71
Trial 2	173	14,304	1/82
Trial 3	395	23,923	1/61

[a] In vitro less than 28S RNA was prepared from infected extracts incubated for 45 min with [^{3}H]ATP in the presence or absence of the other three nucleoside triphosphates. Poly(A) was isolated from gels similar to those seen in Fig. 5B and 7B. The solution containing gel slices 10 to 60 in 0.5 M sodium acetate, 0.001 M EDTA, and 0.2% SDS (pH 7.0) was centrifuged to remove any pieces of gel. The eluate was precipitated with ethanol, hydrolyzed with 1 M KOH, and subjected to electrophoresis as previously described (12). The value for adenosine and AMP of virion RNA are taken from Table 3 of reference 12.

permit an accurate analysis. From these results, we conclude that, at least in the case of the poly(A) on the less than 28S RNA, much of AMP incorporation represents the addition of small lengths of poly(A) to preexisting larger poly(A) sequences on the RNA.

Test for poly(A) polymerase in uninfected extracts. The finding that uninfected extracts incorporated [^{3}H]AMP (Fig. 1) but not [^{3}H]GMP into acid-insoluble material suggested that the poly(A) adding activity observed in the absence of new poliovirus RNA synthesis might be present in uninfected extracts. When an extract prepared from uninfected cells was incubated with [^{3}H]ATP in the presence of the other three nucleoside triphosphates, as expected there was no radioactivity incorporated into RNA with the properties of poliovirus RI or dsRNA. However a considerable amount of the radioactivity was incorporated into RNA which was partially included on a 2% agarose column. The sedimentation of this RNA on sucrose gradients (Fig. 8) was virtually identical to that of the single-stranded RNA labeled with [^{3}H]AMP in the absence of the other three nucleoside triphosphates (Fig. 4B). Almost all of this RNA bound to poly(U) filters and was resistant to T_1 plus pancreatic RNases, indicating that terminal addition of poly(A) occurred in uninfected extracts. Since the sedimentation pattern of this RNA resembled that of HeLa cell mRNA, it is probable that the [^{3}H]AMP labeled less than 28S RNA found in both infected and uninfected extracts is HeLa cell mRNA to which small lengths of poly(A) have been added.

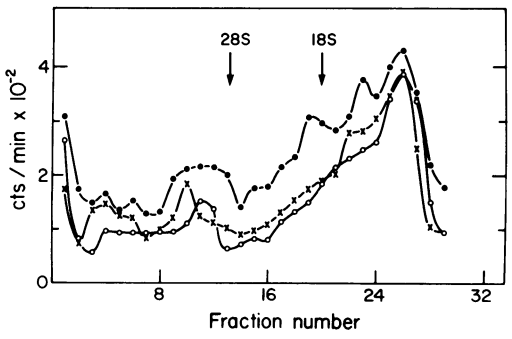

FIG. 8. *Sedimentation of single-stranded RNA labeled with [^{3}H]ATP in extracts of uninfected cells. Single-stranded RNA was isolated from an uninfected extract incubated for 45 min with [^{3}H]ATP plus the other three nucleoside triphosphates after one cycle of agarose chromatography. The RNA was dissolved in 0.01% SDS buffer and sedimented through 15 to 30% sucrose gradients in 0.01% SDS buffer. Samples from each fraction were either precipitated with trichloroacetic acid (●), bound to poly(U) filters (×), or digested with T_1 plus pancreatic RNases (○) as described in the Materials and Methods.*

DISCUSSION

The 35S RNA, RI RNA, and dsRNA labeled in crude cell-free extracts of poliovirus-infected HeLa cells all bind as efficiently to poly(U) filters as does poliovirion RNA. Direct analysis for poly(A) by RNase resistance revealed [^{3}H]AMP label in poly(A) on 35S RNA, RI RNA, and dsRNA.

The sizes of the poly(A) stretches on the in vitro labeled RI and 35S RNA molecules are not the same as the sizes of poly(A) on the same structures in the infected cell at the time at which the extract is made. At 3 h after infection the poly(A) on the in vivo 35S and RI RNA has a heterogeneous distribution varying from 50 to 125 nucleotides in length. After 45 min of incubation in vitro the poly(A) on RI RNA appears as a homogenous species of approximately 200 nucleotides and the poly(A) on the 35S RNA has a size distribution ranging from 50 to 250 nucleotides in length. The size of the poly(A) on these in vitro labeled poliovirus RNA species is very similar to that seen on the same species labeled in the infected cells with [^{3}H]adenosine late in the infection cycle (4 to 5 h) (12). The experiments showing the size of the poly(A) on the in vitro products as a function of time suggest that the switch to the synthesis of larger poly(A) sequences on the RI occurs immediately in the in vitro extract, but that only newly synthesized molecules with the largest poly(A) remain attached to the RI during the isolation procedure.

In the absence of CTP, UTP, and GTP, the RI RNA, dsRNA, and 35S RNA are labeled with [^{3}H]AMP. Over 79% of this label is resistant to RNases, and the size of the poly(A) on the ds- and RI RNA is the same as in the presence of all four triphosphates. The poly(A) on 35S RNA lacks the larger sequences seen when the other three nucleoside triphosphates are present. From these results, it appears that poly(A) can be added to both newly synthesized and preexisting RNA molecules.

When [^{3}H]ATP was used as the label, in either infected or uninfected extracts and in the presence and absence of the other three nucleoside triphosphates, a heterogeneous species of single-stranded less than 28S RNA is also labeled. This RNA is almost completely resistant to RNases and binds efficiently to poly(U) filters. The size of the poly(A) on this less than 28S RNA is similar to that seen on the in vitro 35S RNA (50 to 250 nucleotides) but lacks some

of the larger sequences. An analysis of the ratio of adenosime to AMP in the poly(A) on this RNA, however, indicates that only 15 to 25 nucleotides of newly synthesized poly(A) have been added. This RNA probably represents HeLa cytoplasmic RNA to which small lengths of poly(A) have been added. In vivo cytoplasmic polyadenylation in the presence of high levels of actinomycin D has been observed in Chinese hamster and mouse sarcoma 180 cells (6).

The replication complex is a membrane-bound structure which is the intracellular site for viral RNA synthesis (9). In the experiments reported here, the 130,000 $\times$ g pellet from cytoplasmic extracts of infected cells was incubated in vitro. Such a preparation contains both the replication complex and other membrane-bound structures. The finding that the RNA made by the extracts is adenylated suggests that the poly(A) adding activity is membrane bound and probably associated with the replication complex.

There are two possible mechanisms by which adenylation of product RNA might occur in vitro. First, virus-specific polymerase may transcribe poly(U) sequences found in the minus strand of the dsRNA (16, 18) and the RI (19; D.H. Spector and D. Baltimore, manuscript in preparation). The appearance of large poly(A) on the RI and dsRNA in the absence of UTP, CTP, and GTP would then arise from the synthesis of poly(A) by slippage of the poly(A) over poly(U) sequences in the minus strand by a mechanism similar to that reported for the *Escherichia coli* DNA-dependent RNA polymerase system (4). Second, AMP residues could be added sequentially to the 3′ ends of RNA chains. An enzyme(s) capable of such action has been described in a variety of mammalian cells (5, 7, 13, 15).

The observation that there are fewer large poly(A) sequences on 35*S* RNA molecules labeled with [^{3}H]AMP in the absence of the other three nucleoside triphosphates suggests that the largest poly(A) sequences are only added to newly synthesized 35*S* RNA molecules on the RI.

ACKNOWLEDGMENTS

The work was supported by Public Health Service grants CA-14051 (from the National Cancer Institute) and AI-08388 (from the National Institute of Allergy and Infectious Diseases). D.H.S. was a predoctoral fellow of the National Science Foundation for part of the work. D.B. is an American Cancer Society Research professor.

LITERATURE CITED

1. Armstrong, J. A., M. Edmonds, H. Nakazato, B. S. Phillips, and M. H. Vaughan. 1972. Polyadenylic acid sequences in the virion RNA of poliovirus and Eastern equine encephalitis virus. Science **176**:526–528.
2. Baltimore, D. 1964. In vitro synthesis of viral RNA by the poliovirus RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **51**:450–456.
3. Baltimore, D., M. Girard, and J. E. Darnell. 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology **29**:179–189.
4. Chamberlin, M., and P. Berg. 1964. Mechanism of RNA polymerase action: characterization of the DNA-dependent synthesis of polyadenylic acid. J. Mol. Biol. **8**:708–726.
5. Coleman, M. S., J. J. Hutton, and F. J. Bollum. 1974. Terminal riboadenylate transferase in human lymphocytes. Nature (London) **248**:407–409.
6. Diez, J., and G. Brawerman. 1974. Elongation of the polyadenylate segment of messenger RNA in the cytoplasm of mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **71**:4091–4095.
7. Edmonds, M., and R. Abrams. 1960. Polynucleotide biosynthesis: formation of a sequence of adenylate units from adenosine triphosphate by an enzyme from thymus nuclei. J. Biol. Chem. **235**:1142–1149.
8. Girard, M. 1969. In vitro synthesis of poliovirus ribonucleic acid. Role of the replicative intermediate. J. Virol. **3**:376–384.
9. Girard, M., D. Baltimore, and J. E. Darnell. 1967. The poliovirus replication complex: site for synthesis of poliovirus RNA. J. Mol. Biol. **24**:59–74.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. Spector, D., and D. Baltimore. 1974. Requirement of 3-terminal polyadenylic acid for the infectivity of poliovirus RNA. Proc. Natl. Acad. Sci. U.S.A. **71**:2983–2987.
12. Spector, D. H., and D. Baltimore. 1975. Polyadenylic acid on poliovirus RNA. II. Poly(A) on intracellular RNAs. J. Virol. **15**:1418–1431.
13. Tsiapalis, C. M., J. W. Dorson, D. M. De Sante, and F. J. Bollum. 1973. Terminal riboadenylate transferase: a polyadenylate polymerase for calf thymus gland. Biochem. Biophys. Res. Commun. **50**:737–743.
14. Weinberg, R. A. 1973. Nuclear RNA metabolism. Ann. Rev. Biochem. **42**:329–354.
15. Winters, M. A., and M. Edmonds. 1973. The poly(A) polymerase from calf thymus. J. Biol. Chem. **248**:4756–4762.
16. Yogo, Y., M. H. Teng, and E. Wimmer. 1974. Poly(U) in poliovirus minus RNA is 5′-terminal. Biochem. Biophys. Res. Commun. **61**:1101–1109.
17. Yogo, Y., and E. Wimmer. 1972. Polyadenylic acid at the 3′-terminus of poliovirus RNA. Proc. Natl. Acad. Sci. U.S.A. **69**:1877–1882.
18. Yogo, Y., and E. Wimmer. 1973. Poly(A) and poly(U) in poliovirus double stranded RNA. Nature (London) New Biol. **242**:171–174.
19. Yogo, Y., and E. Wimmer. 1975. Sequence studies of poliovirus RNA. III. Polyuridylic acid and polyadenylic acid as components of the purified poliovirus replicative intermediate. J. Mol. Biol. **92**:467–477.
20. Zimmerman, S. B., and G. Sandeen. 1966. The ribonuclease activity of crystallized pancreatic deoxyribonuclease. Anal. Biochem. **14**:269–277.