Polyadenylic Acid on Poliovirus RNA

II. Poly(A) on Intracellular RNAs

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The content, size, and mechanism of synthesis of 3'-terminal poly(A) on the various intracellular species of poliovirus RNA have been examined. All viral RNA species bound to poly(U) filters and contained RNase-resistant stretches of poly(A) which could be analyzed by electrophoresis in polyacrylamide gels. At 3 h after infection, the poly(A) on virion RNA, replicative intermediate RNA, polyribosomal RNA, and total cytoplasmic 35S RNA was heterogeneous in size with an average length of 75 nucleotides. By 6 h after infection many of the intracellular RNAs had poly(A) of over 150 nucleotides in length, but the poly(A) in virion RNA did not increase in size suggesting that the amount of poly(A) which can be encapsidated is limited. At all times, the double-stranded poliovirus RNA molecules had poly(A) of 150 to 200 nucleotides. Investigation of the kinetics of poly(A) appearance in the replicative intermediate and in finished 35S molecules indicated that poly(A) is the last portion of the 35S RNA to be synthesized; no nascent poly(A) could be detected in the replicative intermediate. Although this result indicates that poliovirus RNA is synthesized 5' → 3' like other RNAs, it also suggests that much of the poly(A) found in the replicative intermediate is an artifact possibly arising from the binding of finished 35S RNA molecules to the replicative intermediate during extraction. The addition of poly(A) to 35S RNA molecules was not sensitive to guanidine.

During a poliovirus infection of HeLa cells, five different species of poliovirus RNA may be isolated from the infected cell (see reference 4 for review): (i) 35S RNA which has been encapsidated into a virion; (ii) replicative intermediate RNA (RI) which contains RNA molecules in the process of synthesis as well as a strand of RNA complementary in base sequence to virion RNA; (iii) completely double-stranded RNA (dsRNA) which sediments at 20S; (iv) polyribosomal 35S RNA which is indistinguishable from virion RNA but is the functional messenger RNA for the synthesis of viral proteins; and (v) total 35S cytoplasmic RNA, the bulk of which is polyribosomal RNA at 3 h of infection but later involves both virion precursor RNA and RNA of uncharacterized (if any) function (14). Every newly synthesized 35S viral RNA molecule has several possible fates: (i) it can serve as a template for the translation of protein; (ii) it can serve as a template for the transcription of minus strand; (iii) it can associate with capsid protein to form a virion; or (iv) it can remain attached to the minus strand to form a dsRNA molecule. Very little is known about the factors governing the choice made by a newly repli-

cated viral RNA molecule except that the fraction of RNA which is encapsidated increases as the infection cycle proceeds (4).

Poly(A) sequences have been found covalently linked to the messenger RNA and heterogeneous 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 (10, 21, 26). The biological function of these 3'-terminal poly(A) sequences, however, is unknown. The single-stranded RNA genome of poliovirus also has a sequence of about 75 nucleotides of poly(A) at its 3'-terminus (1, 29) and the intracellular dsRNA has a sequence at least twice this size (30). This discrepancy, coupled with previous experiments (23) showing that poliovirus poly(A) has a critical biological function since its removal greatly reduced the infectivity of the RNA, suggested that the size of the poly(A) might serve a regulatory role.

In this paper, we present a detailed study of the content, size, and kinetics of addition of the poly(A) on the various intracellular species of poliovirus RNA at different times of infection.

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 the production of type 1 poliovirus has been described (5). For labeling virus-specific RNA, a culture of 8×10^8 cells was washed once in Earle saline and infected by resuspending at more than 5×10^7 cells/ml in medium containing 50 PFU of virus per cell. After adsorption at room temperature for 30 min, cells were diluted to 4×10^6/ml with warm medium containing horse serum (5%) and actinomycin D (10 μg/ml) and incubated at 37 C. To label viral RNA, 50 to 100 μCi of [2,8-³H]adenosine or [5,6-³H]uridine per ml were added 1 h after infection or at the time indicated in the figure legends. To prepare ³²P-labeled RNA, cells at 1 h after infection were exposed to 500 μCi of ³²PO₄ per ml in phosphate-free medium plus 5% dialyzed horse serum. The progress of each infection was monitored on a sample of the culture by [¹⁴C]uridine uptake in the presence of 10 μg of actinomycin D per ml. A cytoplasmic extract was prepared from the infected cells broken with a Dounce homogenizer (19).

Fractionation of RNA. Figure 1 presents a flow sheet of the methods used to isolate the various species of poliovirus RNA. The initial homogenate (cytoplasmic extract) was adjusted to 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 2 M LiCl and was placed at −20 C overnight. Single-stranded cytoplasmic 35S RNA and partially double-stranded RI RNA were removed by centrifugation, and the supernatant containing virions and dsRNA was layered over a 35-ml linear 15 to 30% sucrose gradient in 0.5% SDS buffer (0.1 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M EDTA, 0.5% SDS) and centrifuged at 22 C for 2.5 h at 95,000 × *g* in the SW27 rotor in the L2-65 B Spinco ultracentrifuge. The gradients were collected in 1.5-ml fractions through a recording Gilford spectrophotometer (Fig. 2A). Fractions containing virions, as detected by absorbancy at 260 nm (A_{260}) or radioactivity, were

pooled and diluted with 0.5% SDS buffer, and the virions were harvested by centrifugation at 78,000 × *g* for 6 h in the type 30 Spinco rotor. The fractions at the top of the gradient containing dsRNA were also pooled, made 0.4 M in sodium acetate, and precipitated with 2.5 volumes of ethanol at −20 C overnight.

RNA extracted from the purified virions using the SDS acetic acid extraction method (13, 15) was centrifuged through a 35-ml linear 15 to 30% sucrose gradient in 0.5% SDS buffer at 22 C for 10.5 h at 95,000 × *g* in the SW27 rotor (Fig. 2B). The fractions containing viral RNA were pooled, made 0.4 M in sodium acetate and 50% in ethanol, and placed at −20 C overnight. The ethanol-precipitated RNA was collected by centrifugation at 10 C for 6 h at 95,000 × *g* in the SW27 rotor. The precipitated RNA was resuspended in 0.1% SDS buffer plus 20 mM EDTA and frozen at −70 C.

The precipitate containing dsRNA was resuspended in 1% SDS buffer plus 20 mM EDTA and 0.4 M sodium acetate and was extracted at room temperature twice with an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2) and twice with an equal volume of chloroform:isoamyl alcohol (96:4). The aqueous phase was precipitated with 2.5 volumes of ethanol, and the RNA was dissolved in 1 ml of 0.5% SDS buffer and chromatographed through 2% agarose (Sepharose 2B) in 0.5% SDS buffer as previously described (2, 11). Fractions of 1.5 ml were collected and the dsRNA, which is excluded from the column (Fig. 2C), was pooled and ethanol precipitated. The precipitated dsRNA resuspended in 0.5% SDS buffer was further purified by centrifugation through a linear 35-ml 15 to 30% sucrose gradient in 0.5% SDS buffer at 22 C for 17 h at 95,000 × *g* in the SW27 rotor (Fig. 2D). Fractions containing the 20S dsRNA were pooled, ethanol precipitated, resuspended in 0.1% SDS buffer plus 20 mM EDTA, and frozen at −70 C.

To purify the cytoplasmic 35S and RI RNA, the 2 M LiCl precipitate, either first phenol extracted and ethanol precipitated or directly resuspended in 0.5%
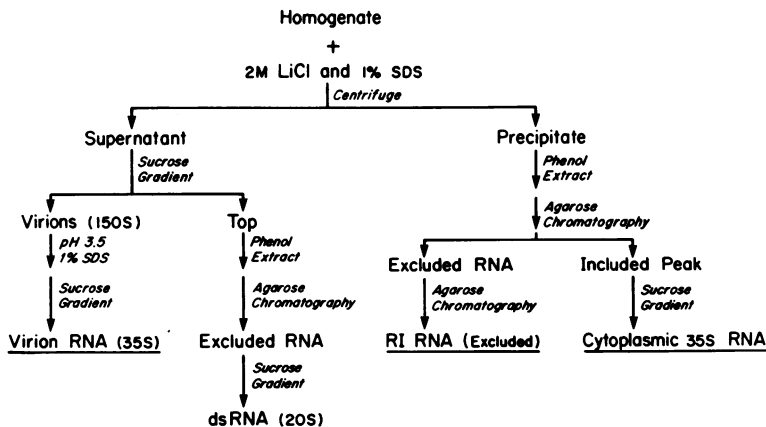

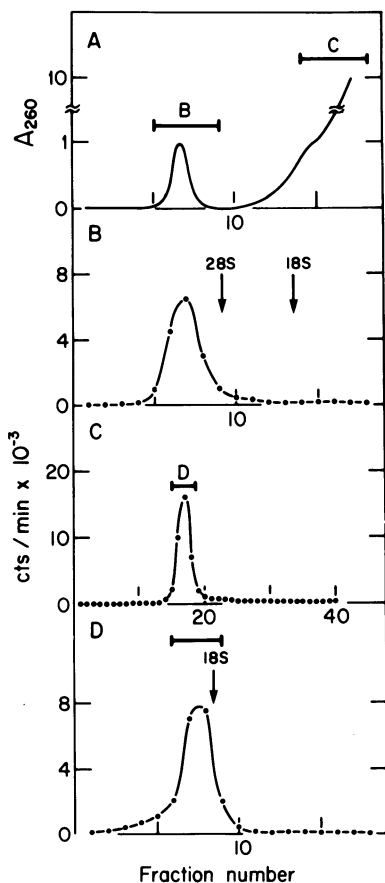FIG. 1. *Fractionation of poliovirus-specific RNA.*

FIG. 2. *Fractionation of 2 M LiCl supernatant from a 6-h infection continuously labeled with [³H]-adenosine. (A) Sedimentation of LiCl supernatant through 15 to 30% sucrose in 0.5% SDS buffer at 22 C for 2.5 h at 95,000 × g in the SW27 rotor. Fractions containing virions indicated by brackets B and containing dsRNA indicated by brackets C were pooled. (B) RNA extracted from virions of pool B in (A) was centrifuged through a 35-ml linear 15 to 30% sucrose gradient in 0.5% SDS buffer at 22 C for 10.5 h at 95,000 × g in the SW27 rotor. HeLa 28S and 18S ribosomal RNA served as markers. Samples from each fraction were counted in a xylene-based scintillant. The fractions containing viral 35S RNA were pooled. (C) dsRNA from pool C of (A) were extracted with phenol-chloroform-isoamyl alcohol and chromatographed through 2% agarose. Fractions of 1.5 ml were collected and the dsRNA which is excluded from the column was pooled. (D) dsRNA from pool D of (C) was sedimented through a 35-ml linear 15 to 30% sucrose gradient in 0.5% SDS buffer at 22 C for 17 h at 95,000 × g in the SW27 rotor. HeLa 18S ribosomal RNA served as a marker. Samples from each fraction were counted in a xylene based scintillant and the 20S dsRNA (indicated by brackets) was pooled.*

SDS buffer, was chromatographed through 2% agarose (Fig. 3A). The excluded peak, containing the partially double-stranded RI RNA, was pooled, ethanol precipitated, and reapplied to the agarose column. The excluded fractions of RI RNA (Fig. 3B) were pooled, ethanol precipitated, resuspended in 0.1% SDS buffer plus 20 mM EDTA, and frozen at −70 C. The 35S RNA from the initial agarose column was further purified by centrifugation through a linear 35-ml 15 to 30% sucrose gradient in 0.5% SDS buffer at 95,000 × g in the SW27 rotor (Fig. 3C). The fractions containing 35S RNA were pooled, ethanol precipitated, resuspended in 0.1% SDS buffer, and frozen at −70 C.

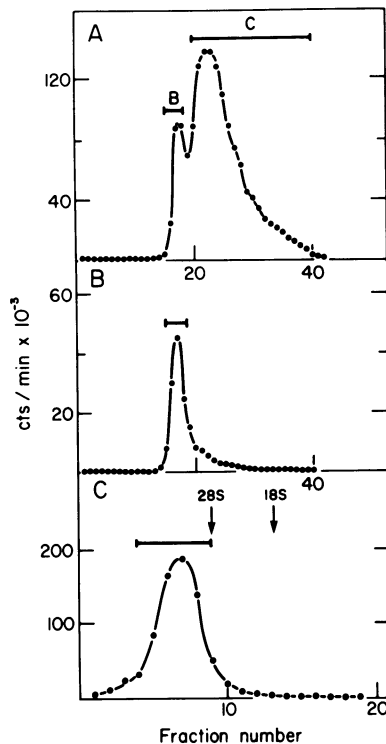**Isolation of poliovirus polyribosomal RNA.** Two

FIG. 3. *Fractionation of 2 M LiCl precipitate from a 3-h infection continuously labeled with [³H]adenosine. (A) The 2 M LiCl precipitate containing the 35S and RI RNA was extracted with phenol-chloroform-isoamyl alcohol and chromatographed through 2% agarose. Fractions of 1.5 ml were collected and samples from each fraction were counted in a xylene-based scintillant. The excluded fractions of RI RNA (B) and the partially included fraction of the 35S single-stranded RNA (C) were pooled. (B) The RI RNA from pool B of (A) was rechromatographed through 2% agarose. The excluded fractions of RI RNA were pooled. (C) The 35S RNA from pool C of (A) was sedimented through 15 to 30% sucrose in 0.5% SDS buffer as in Fig. 2B.*

methods were used to isolate poliovirus polyribosomal RNA from a labeled cytoplasmic extract of cells infected for 3 h to which 50 µg of dextran sulfate per ml had been added.

(i) The cytoplasmic extract, adjusted to a final concentration of 1% DOC and 1% Brij, was applied to a linear 35-ml 15 to 30% sucrose gradient in RSB buffer (10 mM NaCl, 10 mM Tris, pH 7.5, 1.5 mM magnesium acetate) and centrifuged at 4 C in the SW27 rotor at 95,000 × g for 80 min. The gradients were collected into 1-ml fractions through a recording Gilford spectrophotometer. The virus-specific polyribosomes as determined by A_{260} (20) were pooled, diluted threefold with ice cold RSB buffer, and collected by centrifugation at 4 C in the type 30 Spinco rotor for 90 min at 95,000 × g. The pellet was resuspended in 1% SDS buffer and 20 mM EDTA, adjusted to 0.4 M sodium acetate, and precipitated with 2.5 volumes of 95% ethanol. The ethanol precipitate was resuspended in 0.5% SDS buffer, and centrifuged through a linear 35-ml 15 to 30% sucrose gradient in 0.5% SDS buffer at 22 C in the SW27 rotor at 95,000 × g for 10.5 h. The 35S polio polyribosomal RNA was pooled, ethanol precipitated, resuspended in 0.1% SDS buffer plus 20 mM EDTA, and frozen at −70 C.

(ii) The cytoplasmic extract was centrifuged through a 31-ml 15 to 30% sucrose gradient with a 4-ml 60% sucrose cushion in RSB buffer for 2.5 h at 95,000 × g in the SW27 rotor at 4 C. The 60% sucrose cushion containing the polyribosomes and membrane-bound replication complex was diluted fivefold with RSB buffer, made 20 mM EDTA, 1% SDS, and 2 M LiCl, and stored at −20 C. The LiCl precipitate containing the poliovirus polyribosomal RNA was resuspended in 0.5% SDS buffer and chromatographed through 2% agarose (Fig. 4A). In this way the small amount of RI in the replication complex which would also pellet into the 60% sucrose cushion could be separated from the poliovirus polyribosomal RNA. The 35S polyribosomal RNA which was partially included in the agarose gel matrix was further purified by sedimentation through a linear 15 to 30% sucrose gradient in 0.5% SDS buffer at 95,000 × g in the SW27 rotor (Fig. 4B). The fractions containing 35S RNA were pooled, ethanol precipitated, resuspended in 0.1% SDS buffer, and frozen at −70 C.

Determination of nucleotide composition and chain length. To determine the nucleotide composition of the material resistant to RNase, ³²P-labeled RNA was eluted from gels with 0.5 M sodium acetate, pH 7.0, 1 mM EDTA, and 0.2% SDS. The eluate was precipitated with ethanol, resuspended in 20 µl of 1 M KOH, and incubated at 37 C for 24 h. The alkaline hydrolysate was subjected to electrophoresis along with nucleotide markers on Whatman 540 paper in 0.5% pyridine, and 5% acetic acid (pH 3.5) for 90 min at 3,000 V. The paper was dried and the radioactivity in each nucleotide was determined by counting in a toluene-based scintillant.

To determine the chain length of [³H]poly(A), an alkaline hydrolysate prepared as above was subjected to electrophoresis for 60 min. The paper was dried and
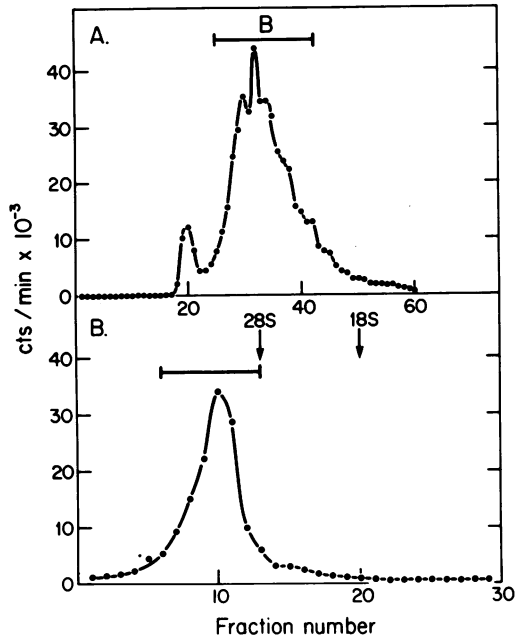

FIG. 4. *Isolation of poliovirus polyribosomal RNA.* (A) *The polyribosomal RNA from a 3-h infection continuously labeled with [³H]adenosine isolated from the 60% sucrose cushion (method II) was chromatographed through 2% agarose. Fractions of 1.5 ml were collected and samples from each fraction were counted in a xylene-based scintillant. The fractions containing the partially included 35S polyribosomal RNA (B) were pooled. (B) The RNA from pool B of (A) was sedimented through 15 to 30% sucrose in 0.5% SDS buffer as in Fig. 2B.*

the radioactivity in the adenosine and adenosine 2′,3′-monophosphate spots determined by solubilizing the radioactivity with 0.1 M NaOH (37 C for 2 h) followed by neutralization with acetic acid.

Preparation of [³H]adenine and [³H]uracil f2 bacteriophage RNA. The procedure was a modification of that used to prepare Qβ phage (25). Briefly, 25 ml of *Escherichia coli* grown logarithmetically to an A_{260} of 2 were infected with 5 PFU per cell of f2 phage. Twenty minutes later, 1 mCi of [³H]adenine or [³H]uracil were added. After 3 h, the cells were lysed with chloroform and lysozyme (100 µg/ml) and 115 mg of polyethylene glycol plus 35 mg of NaCl were added per ml of lysate. After 18 h at 4 C, the precipitate was collected by centrifugation and the pellet was dissolved in 1/30 volume of 0.1 M Tris-hydrochloride, pH 7.8. The cell debris was removed from the phage by centrifugation and 0.625 g of CsCl was added per ml of supernatant. The phage particles were banded in the CsCl by centrifugation in a Spinco SW50.1 rotor at 40,000 rpm for 22 h. The banded phage was extracted with phenol:chloroform:isoamyl alcohol (50:48:2) three times and the aqueous layer containing the phage RNA was precipitated with ethanol. The

ethanol-precipitated RNA was resuspended in 0.1% SDS buffer plus 20 mM EDTA and frozen at −70 C.

Preparation of [³H]adenosine HeLa cytoplasmic RNA. Five hundred milliliters of HeLa cells at 4×10^5 cells/ml were concentrated 10-fold and treated with 0.05 μg of actinomycin D per ml. Sixty minutes later, 50 μCi of [³H]adenosine per ml was added. Two hours after the addition of label a cytoplasmic extract was made by breaking the cells in the presence of 50 μg of dextran sulfate per ml with a Dounce homogenizer. The cytoplasmic extract was adjusted to 0.5% SDS, 50 mM EDTA (pH 7.2), and 0.4 M sodium acetate and extracted two times with phenol:chloroform:isoamyl alcohol (50:48:2) and two times with chloroform:isoamyl alcohol (96:4). The aqueous phase was precipitated with ethanol, resuspended in 0.1% SDS buffer and 20 mM EDTA, and frozen at −70 C.

Poly(U) binding of viral RNA, T_1 plus pancreatic RNase digestion of RNA, and isolation and polyacrylamide gel electrophoresis of the poly(A). These methods have been described in detail previously (23).

Materials. Actinomycin D was a generous gift from Merck, Sharp, and Dohme. ³²PO₄ in HCl-free solution, [2,8-³H]adenosine (34.2 Ci/mmol), [5,6-³H]uridine (45.0 Ci/mmol), [¹⁴C]uridine, [³H]adenine, and [³H]uracil were purchased from New England Nuclear; pancreatic RNase (RNase A) and RNase T_1 were obtained from Worthington Biochemical Corp.; poly(A) and poly(U) were from Miles Laboratories; SDS and formamide from Matheson Coleman and Bell; diethylpyrocarbonate, acrylamide, and N,N-methylene bisacrylamide from Eastman, proteinase K from EM Laboratories; and 2% agarose (Sepharose 2B) from Pharmacia Fine Chemicals.

RESULTS

To investigate the content and size of poly(A) on the various species of poliovirus-related RNAs, a standard procedure was developed utilizing mainly established methods (see reference 4 for review of methodology and Materials and Methods for details). The basic strategy (Fig. 1) was to make a cytoplasmic extract and treat it with 1% SDS and 2 M LiCl to separate the high salt-soluble materials (dsRNA and virions) from the insoluble materials (RNAs containing single-stranded regions). Further fractionation by agarose gel chromatography and sucrose gradients allowed the isolation of pure species of RNA. For isolation of polyribosomal RNA, most methods gave degraded RNA. Method (i), as described in Materials and Methods, gave only a 20% yield of intact 35S RNA. Method (ii), which involved centrifuging the polyribosomes onto a cushion, gave a much better yield but did not control as well contamination with non-polyribosomal RNA.

Poly(U) binding and RNase-resistance. The different species of poliovirus-related RNAs harvested at 3 or 6 h postinfection were assayed

for their ability to bind to poly(U) filters (Table 1). All species of RNA bound to the filters, although with differing efficiencies, indicating that all species contained poly(A) regions. Binding efficiency was independent of labeling time or the particular radioactive label used ([³H]adenosine or [³H]uridine). [³H]poly(A) itself bound with 100% efficiency whereas less than 1% of f2 phage RNA, which contains no poly(A) (7, 27), was bound. The dsRNA bound with lowest efficiency of the various virus-specific RNA species probably because of the poly(U) in the complementary RNA strand; poly(A)·poly(U) binds poorly to poly(U) filters (unpublished data).

A second method for assaying poly(A) content of RNAs is determination of the fraction of RNA resistant to pancreatic plus T_1 RNases. To avoid scoring double-stranded RNA regions, the various tested species were first denatured by brief alkali treatment before nuclease digestion. All of the viral RNAs contained about 4% RNase-resistant material if [³H]adenosine-labeled species were investigated (Table 1). {Poliovirus is 7,500 nucleotides in length and has 29% AMP (4); for such an RNA, 4% RNase resistance of [³H]adenosine label corresponds to 87 nucleotides of AMP in poly(A) per molecule.} Virion RNA labeled with [³H]uridine had only 0.78% resistance; [³H]adenosine-labeled HeLa cell cytoplasmic RNA had 30% resistance assayed in this fashion which agrees with previous results (9, 16); and [³H]poly(A) was completely nuclease resistant. The differences in poly(A) content implied by the different percentages of RNase resistance in Table 1 are corroborated by later data on the size of poly(A) stretches (see below). However, the high percentages of poly(A) in both 3 and 6 h RI were unexpected and will be discussed later.

None of the viral RNA species bound to poly(U) with 100% efficiency (Table 1). To determine if this implied that a fraction of the molecules lacked poly(A), virion RNA which passed through a poly(U) filter was recovered and separated from any poly(U), which might have been eluted from the filter, by denaturation in the presence of excess poly(A) followed by sucrose gradient centrifugation. This RNA rebound to poly(U) with an efficiency identical to its original efficiency of binding and contained an equal percentage of RNase-resistant [³H]adenosine as the RNA which initially bound to the filter (Table 2). Thus, no evidence for a fraction of RNA lacking poly(A) could be found. If the RNA which initially passed through the poly(U) filters was assayed immediately for binding to a fresh poly(U) filter, no

TABLE 1. *Poly(U) binding and RNase resistance of RNA species[a]*

RNA species	Time of harvest (h)	Label	% Bound to poly(U) filters	% Resistance to T_1 plus pancreatic RNases
Virion RNA	3	[^{3}H]adenosine	63	3.8 (2.8 to 5.1)
Virion RNA	6	[^{3}H]adenosine	55	3.8 (3.5 to 4)
Virion RNA	6	[^{3}H]uridine	56	0.78
Cytoplasmic	3	[^{3}H]adenosine	45	4.1 (3.5 to 4.5)
Cytoplasmic	6	[^{3}H]adenosine	46	4.1 (3.7 to 4.5)
Replicative intermediate ..	3	[^{3}H]adenosine	58	4.1 (3.6 to 4.8)
Replicative intermediate ..	6	[^{3}H]adenosine		9.0 (8.1 to 10)
Replicative intermediate ..	3	[^{3}H]uridine		1.25 (1.03 to 1.48)
Polyribosomal	3 [method (i)]	[^{3}H]adenosine		4.3
Polyribosomal	3 [method (ii)]	[^{3}H]adenosine	56	4.1
dsRNA	6	[^{3}H]adenosine	30	3.5
HeLa cytoplasmic	3	[^{3}H]adenosine		30
Poly(A)			100	100
f$_2$ Bacteriophage		[^{3}H]adenine	0.58	
f$_2$ Bacteriophage		[^{3}H]uracil	0.35	

[a] Poliovirion, cytoplasmic, RI, polyribosomal and dsRNA, f$_2$ phage RNA, and HeLa cytoplasmic RNA were prepared as described in the Materials and Methods. To determine the percentage of resistance to T_1 plus pancreatic RNases, 0.1 ml of each sample in 0.1% SDS buffer plus 20 mM EDTA was placed into 0.4 ml of 0.1 M NaOH and incubated at room temperature for 1.5 min. The solution was then adjusted to 0.25 M Tris-hydrochloride, pH 7.5, 0.3 M NaCl, and 0.03 M sodium acetate in a final volume of 4 ml and treated with 10 μg of pancreatic RNase per ml and 10 U of T_1 RNase per ml for 60 min at 37 C. The samples were then precipitated with 12.5% trichloroacetic acid. Values of percentage of binding to poly(U) filters and of percentage of resistance to T_1 plus pancreatic RNases represent the average of at least two independent determinations. Numbers in parentheses indicate the range of values obtained.

binding was observed (unpublished data). Apparently sufficient poly(U) is eluted from the filters to coat the poly(A) on the unbound poliovirus RNA and this must be removed to demonstrate that the unbound molecules can be bound by a second filtration through poly(U) filters.

Size of the poly(A) sequences on continuously labeled molecules. To determine whether there was any difference in the size of the poly(A) sequences on the different species of poliovirus RNA, the poly(A) which resisted digestion by RNase was bound to poly(U) filters, eluted, and subjected to electrophoresis in 10% polyacrylamide gels (see Fig. 6-9). For comparison, the electrophoretic pattern of the poly(A) on HeLa cytoplasmic RNA labeled for 3 h with [^{3}H]adenosine in the presence of 0.05 μg of actinomycin D per ml is shown in Fig. 5. The major peak of radioactivity in the HeLa RNA (fractions 10 to 25) represents poly(A) of approximately 180 to 220 nucleotides in length. The minor peak of radioactivity (fractions 45 to 55) probably represents mitochondrial poly(A) (approximately 50 nucleotides in length [21]).

The poly(A) of virion RNA was heterogeneous (Fig. 6). The size distribution of the poly(A) was the same whether the virions were harvested after 3 or 6 h (Fig. 6A and B), whether the virion RNA was preselected on poly(U) filters (Fig. 6C), or whether the virion RNA did not bind to poly(U) filters (Fig. 6D). As expected, no oligonucleotides containing uridine were detected by gel electrophoresis after RNase digestion and poly(U) selection (Fig. 6E). The small peak of [^{3}H]adenosine radioactivity which sometimes appeared in fractions 70 to 80 probably represents a labeled oligonucleotide contaminant. From the positions of the 4S and 5S RNA markers, it was estimated that the virion poly(A) spans approximately 50 to 125 nucleotides. This estimate agrees both with percentage of [^{3}H]adenosine resistant to RNase (indicating 87 nucleotides) and with an estimate of 71 nucleotides which was obtained by determining the ratios of AMP to adenosine in the poly(A) (Table 3).

The size distributions of the poly(A) on cytoplasmic RNA at 3 h after infection (Fig. 7C) and on polyribosomal RNA at 3 h after infection obtained by either method (i) or (ii) (Fig. 7A and B) are identical to that on virion RNA. Of the single-stranded RNA species, only the cytoplasmic RNA isolated 6 h after infection (Fig. 7D) contained a population of poly(A) molecules which was larger than that seen on virion RNA.

In contrast to the size distribution of virion,

TABLE 2. *Rebinding of virion RNA to poly(U) filters*[a]

Sample	% Rebound to poly(U) filters	% Resistance to T₁ plus pancreatic RNases
Virion RNA bound to poly(U) filters	46	3.7
Virion RNA not bound to poly(U) filters	55	4.2

[a] [³H]adenosine 6-h virion RNA was bound to poly(U) filters in the presence of 0.2 ml of 0.3 M NaCl, 0.5% SDS, and 10 mM Tris-hydrochloride (pH 7.6), and the filter was washed with 10 ml of this buffer (wash containing virion RNA not bound to poly(U) filter). The filter was then washed with two 5-ml aliquots of a buffer containing 0.3 M ammonium acetate in 50% ethanol. RNA bound to the filters was eluted with four 1-ml aliquots of a 65 C solution of 90% formamide, 2 mM Tris-hydrochloride (pH 7.3), and 100 μg of poly(A) (containing virion RNA bound to poly(U) filters) per ml. The solutions containing the RNA both bound and not bound to the poly(U) filters were made 0.4 M in sodium acetate and precipitated with 2.5 volumes of ethanol and carrier yeast tRNA. The ethanol-precipitated RNA not bound to poly(U) filters was dissolved in 4 ml of a 65 C solution of 90% formamide, 2 mM Tris-hydro-chloride (pH 7.3), and 100 μg of poly(A) per ml to complex the poly(U) which may have come off the filter. After 5 min at room temperature the solution was made 0.4 M in sodium acetate and precipitated with 2.5 volumes of ethanol. The ethanol-precipitated 35S virion RNA both bound and not bound to the poly(U) filters was purified from the excess poly(A) and poly(A)·poly(U) complexes by sedimentation through sucrose. The 35S RNA was pooled, ethanol precipitated, and tested for its capacity to rebind to poly(U) filters and for its percentage of resistance to T₁ plus pancreatic RNases. The values represent the average of three independent determinations.

cytoplasmic, and polyribosomal poly(A), the poly(A) on the dsRNA was much larger (30, Fig. 8). Its migration on 10% polyacrylamide gels was consistent with a length of from 50 to 250 nucleotides. The average size of the poly(A) (140 nucleotides) and the percentage of the [³H]adenosine label resistant to RNase (3.5%) was consistent with each dsRNA molecule containing only one sequence of poly(A). The dsRNA isolated 4 h after infection contained a higher percentage of long sequences than that isolated 6 h after infection. The dsRNA isolated after 6 h, however, contained some poly(A) longer than any found at 4 h. It is possible that the poly(A) sequences on the dsRNA may become shorter as the RNA ages in the cell cytoplasm as is the case with HeLa cell mRNA (22).

Electrophoretic analysis on 10 or 5% poly-acrylamide gels of the poly(A) on the RI showed that the size of the poly(A) on the RI isolated 3 h postinfection (Fig. 9D) was similar to that on the virion, cytoplasmic, and polyribosomal RNA. However, the RI isolated 4 h or later in the infection (Fig. 9B, C and E) contained very large sequences of poly(A) of 200 nucleotides and longer. Although the 3-h RI containing an average of four to six growing strands (4) displays a heterogenous distribution on a su-crose gradient extending from 20S to 70S, the 6-h RI containing fewer nascent strands (average = 2.4; calculated from RNase resistance of the native RI as described in reference 4) sediments as a more homogenous species from 20S to 28S. The percentage of [³H]adenosine label in the denatured 6-h RI (9.0%) and the average size of this poly(A) (400 to 500 nucleotides) is consistent with a structure which is essentially double stranded with a few nascent strands and which contains a very long sequence of poly(A).
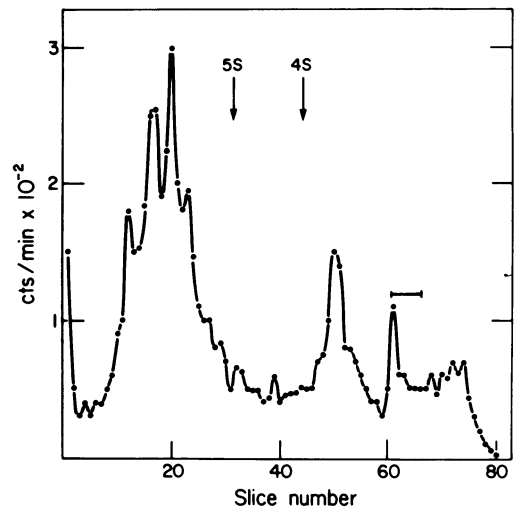

FIG. 5. *Polyacrylamide gel electrophoresis of poly(A) sequences in HeLa cytoplasmic RNA.* [³H]adenosine-labeled HeLa cytoplasmic RNA was treated with 0.1 M NaOH at room temperature for 1.5 min, neutralized with 0.5 M Tris (pH 7.5), digested with T₁ plus pancreatic RNases, bound to poly(U) filters, and eluted. 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 electro-phoresed in 10% polyacrylamide gels at 7.5 mA/gel for 5.5 h. Slices (2 mm) of the gel were directly counted in a toluene-based scintillation fluid containing 3.5% Nuclear Chicago solubilizer. The horizontal bar indi-cates the position of the bromophenol blue dye marker.
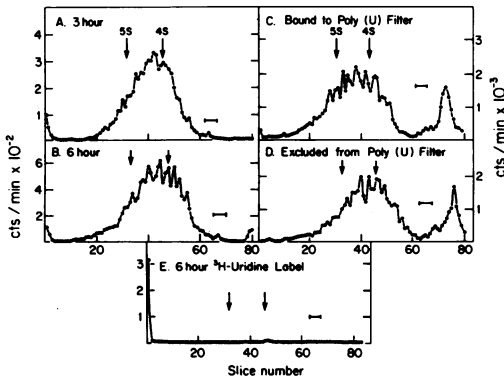
FIG. 6. Polyacrylamide gel electrophoresis of poly(A) sequences in continuously-labeled virion RNA. The RNA was treated as in Fig. 5. (A) [³H]-adenosine virion RNA isolated from a 3-h infection. (B) [³H]adenosine virion RNA isolated from a 6-h infection. (C) [³H]adenosine 6-h virion RNA which was bound to a poly(U) filter as described in Table 2. (D) [³H]adenosine 6-h virion RNA which did not bind to a poly(U) filter as described in Table 2. (E) [³H]uridine 6-h virion RNA.

TABLE 3. Determination of length of poly(A) in viral RNA[a]

Trial	Counts/min in adenosine (A)	Counts/min in AMP	A/AMP ratios
1	170	12,065	1/71
2	173	14,304	1/82
3	395	23,923	1/61

[a] Poly(A) was isolated from gels similar to those seen in Fig. 6B. Gel slices from the major region of radioactivity were broken by passage through a syringe needle in a solution of 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. The solution was centrifuged and passed through a glass fiber filter to remove pieces of gel. The eluate was precipitated with ethanol, hydrolyzed with 1 M KOH, and subjected to electrophoresis as described in the Materials and Methods.

To determine the nucleotide composition of the RNase-resistant sequences, infected cells were labeled with ³²PO₄ and harvested 4 h after infection. Poly(A) was isolated from RI RNA, dsRNA, and 35S cytoplasmic RNA 4 h after infection and subjected to polyacrylamide gel electrophoresis. Radioactivity in alternate gel slices was determined and patterns similar to those in Fig. 7C, 8A, and 9B were found. The remaining fractions from the peak of radioactivity were pooled and separated from the polymerized acrylamide by centrifugation and filtration through glass fiber filters. The recovered oligonucleotides were hydrolyzed and analyzed

for base composition. The 35S cytoplasmic poly(A) was 98% AMP, whereas the RI and dsRNA poly(A) were 88 and 90% AMP, respectively. The only contaminating nucleotide in the dsRNA and RI poly(A) preparations was UMP, which probably represents attachment to the poly(A) of a small amount of the poly(U) found in complementary RNA in both dsRNA (28, 30) and the RI (31; D. H. Spector, manuscript in preparation).
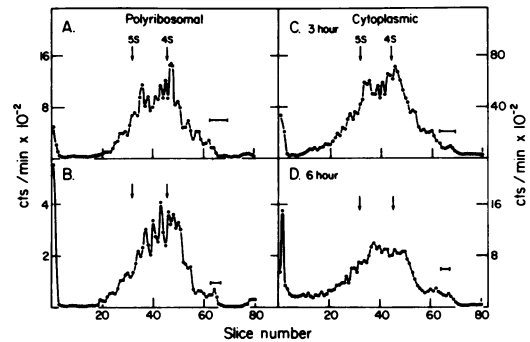

FIG. 7. Polyacrylamide gel electrophoresis of poly(A) sequences in continuously labeled [³H]adenosine-labeled polyribosomal and cytoplasmic 35S RNA. The RNA was treated as in Fig. 5. (A) Polyribosomal RNA isolated by method I from a 3-h infection. (B) Polyribosomal RNA isolated by method II from a 3-h infection. (C) Cytoplasmic 35S RNA isolated from a 3-h infection. (D) Cytoplasmic 35S RNA isolated from a 6-h infection.
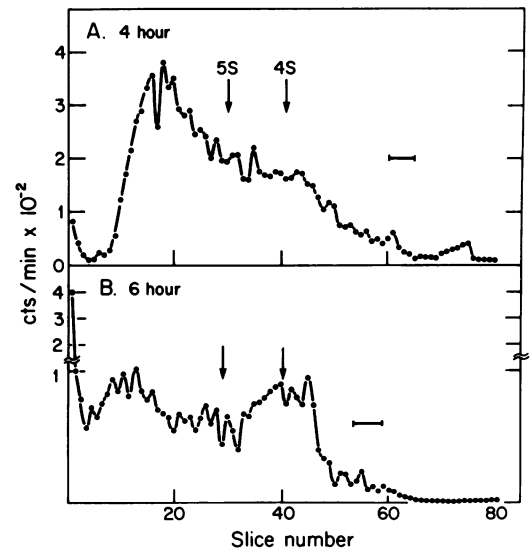

FIG. 8. Polyacrylamide gel electrophoresis of poly(A) sequences in continuously labeled [³H]adenosine-labeled dsRNA. The RNA was treated as in Fig. 5. (A) dsRNA isolated from a 4-h infection. (B) dsRNA isolated from a 6-h infection.
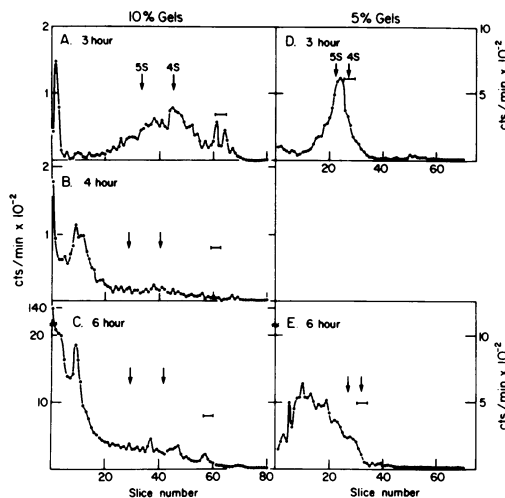
FIG. 9. *Polyacrylamide gel electrophoresis of poly(A) sequences in continuously labeled [³H]adenosine replicative intermediate RNA isolated from 3- (A and D), 4- (B), and 6-h (C and E) infections. The RNA was treated as in Fig. 5. (A, B, C) 10% polyacrylamide gels, 7.5 mA/gel, 5.5 h. (D, E) 5% polyacrylamide gels, 7.5 mA/gel, 3 h.*

Size of the poly(A) on pulse-labeled RNA molecules.

To study the origin of the large sequences of poly(A) appearing on the continuously labeled RI 4 h after infection, and on a small population of the 6 h 35S cytoplasmic RNA, we pulse-labeled the viral RNAs with [³H]adenosine. The RI, ds-, viral, and 35S cytoplasmic RNA labeled from 2.25 to 3.25 h (early) or from 4 to 5 h (late) after infection were purified from cells harvested at the end of the labeling period. Poly(A) was isolated from these pulse-labeled molecules and analyzed by electrophoresis on 10% polyacrylamide gels (Fig. 10). Comparing the two times of labeling, there were no differences in the size of the poly(A) on the virion RNA (Fig. 10A and E) and on the dsRNA (Fig. 10D and H) synthesized early or late after infection. However, the size of the poly(A) on the RI (Fig. 10C and G) synthesized late in infection is at least two to three times the size of that early in infection. Analysis of the poly(A) on 35S RNA molecules synthesized late in infection (Fig. 10F) demonstrated that approximately 50% of the poly(A) sequences were larger than those synthesized early in infection (Fig. 10B). The heterogeneity present in the size distribution of this late 4-h cytoplasmic poly(A) was not due to degradation during the 1-h labeling period as labeling for as short a period as 5 min at 4 h post-infection produced the same pattern (data not shown). From these results, it

appears that late in infection large sequences of poly(A) are being added to 35S RNA molecules but that only molecules with poly(A) of smaller size (50 to 150 nucleotides) are being encapsidated into virions.

Kinetics of the addition of poly(A) to poliovirus RNA. In an attempt to determine whether the poly(A) in the 35S RNA molecules was added in the RI or added post-transcriptionally in the cytoplasm, we studied the kinetics of the synthesis of poly(A) on the RI and 35S RNA molecules. Three hours after infection, [³H]adenosine was added to a culture of infected cells. At 2, 4, 6, 8, and 30 min later samples were taken and the RI and 35S were purified as described in Table 4. ³²P-labeled RI and 35S RNA were added at the beginning of
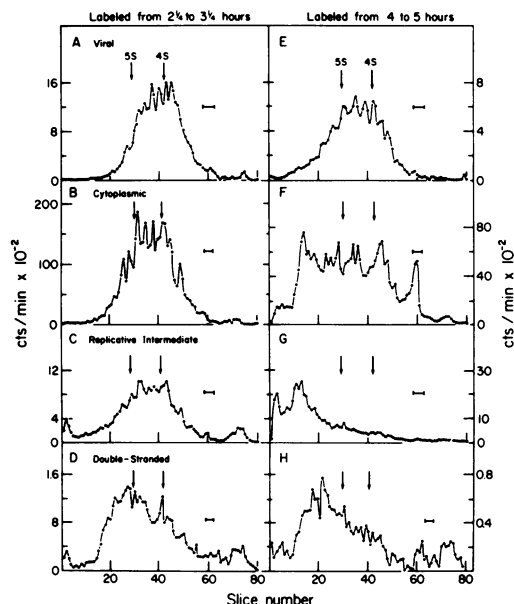

FIG. 10. *Polyacrylamide gel electrophoresis of poly(A) sequences on viral RNA labeled with [³H]adenosine from 2.25 to 3.25 h (A to D) and from 4 to 5 h (E to H) postinfection. A culture of 8 × 10⁸ HeLa cells was infected with 50 PFU per cell of poliovirus in the presence of 10 μg of actinomycin D per ml. At 2.25 h, the culture was divided into two portions containing 2 × 10⁸ cells and 6 × 10⁸ cells. The culture containing 2 × 10⁸ cells was labeled with 50 μCi of [³H]adenosine per ml from 2.25 to 3.25 h postinfection and the culture containing 6 × 10⁸ cells was labeled from 4 to 5 h postinfection. Immediately after the labeling period a cytoplasmic extract was made by breaking the cells with a Dounce homogenizer and the various species of viral RNA were separated. The RNA was treated as in Fig. 5. (A, E) Viral RNA. (B, F) Cytoplasmic 35S RNA. (C, G) Replicative intermediate RNA. (D, H) dsRNA.*

TABLE 4. *Kinetics of poly(A) addition*[a]

Time of labeling (min)	Total counts/min × 10⁻⁵		Poly(A) counts/min × 10⁻³		% Poly(A)		RI poly(A)/35S poly(A)
	RI	35S	RI	35S	RI	35S	
2	2.5 (2.45)	1.4	3.8 (2.55)	25	1.52 (1.04)	22.6	1/6.6 (1/9.8)
4	4.8 (4.59)	4.3	8.1 (5.85)	45	1.69 (1.27)	10.5	1/5.6 (1/7.7)
6	6.8 (6.36)	8.8	9.4 (6.2)	64	1.39 (0.98)	7.3	1/6.7 (1/10.4)
8	7.9 (7.1)	16	11 (6)	100	1.4 (0.85)	6.25	1/9.1 (1/16.6)
30	12 (8.65)	67	26 (11.7)	286	2.16 (1.32)	4.3	1/11.1 (1/24.5)

[a] A culture of 4×10^8 cells were infected with 50 PFU per cell of poliovirus in the presence of 10 μg of actinomycin D per ml. Three hours later, 100 μCi of [³H]adenosine per ml was added. Later (2, 4, 6, 8, and 30 min), samples were taken and placed onto frozen crushed Earle saline and, after washing the infected cells with Earle saline, a cytoplasmic extract was made by breaking the cells with a Dounce homogenizer. ³²P-labeled 35S RNA and RI RNA were added to each homogenate to monitor losses and the homogenates were adjusted to 10 mM EDTA, 1% SDS, and 2 M LiCl and were placed at −20 C overnight. Single-stranded cytoplasmic 35S RNA and RI RNA were removed by centrifugation, resuspended in 0.5% SDS buffer, and chromatographed once through 2% agarose. The excluded fractions of RI RNA and the partially included fractions of 35S RNA were pooled, ethanol precipitated, and resuspended in 0.1% SDS buffer plus 20 mM EDTA. Duplicate samples of RI and 35S RNA from each time point were either precipitated with trichloroacetic acid (total counts per minute) or digested with T₁ plus pancreatic RNases after denaturation with 0.1 M NaOH for 1.5 min at room temperature and bound to poly(U) filters [poly(A) counts per minute]. The values listed are for 10 ml of the original infection and reflect corrections for losses during the purification procedure. Since previous experiments had demonstrated that about 3 to 5% of 35S RNA elutes with the RI during a single agarose purification, the numbers in parentheses reflect a correction assuming 5% contamination.

the purification process to correct for losses. Table 4 shows the total and poly(A)-associated [³H]adenosine radioactivity of RI and 35S RNA after different times of pulse. In an attempt to limit loss of RNA during purification, 35S RNA and RI were purified from the 2 M LiCl precipitate by a single cycle of agarose chromatography. Previous experiments had demonstrated that about 3 to 5% of 35S RNA elutes with the RI during a single agarose purification. The numbers in parentheses in Table 4 reflect a correction assuming 5% contamination and are portrayed in Fig. 11.

There was a lag in the incorporation of total [³H]adenosine label into completed 35S RNA molecules whereas no lag was evident in the labeling of RI (Fig. 11A). Such a result is consistent with the RI being the site of synthesis of 35S RNA molecules. In contrast to the total incorporation of label, there was no lag evident in the appearance of poly(A) in 35S RNA molecules (Fig. 11B). Even at the earliest point of the pulse there was more labeled poly(A) in 35S RNA molecules than in the RI. The high percentage of radioactivity in the poly(A) of 35S RNA at the earliest point of the pulse is consistent with replication proceeding in a 5' to
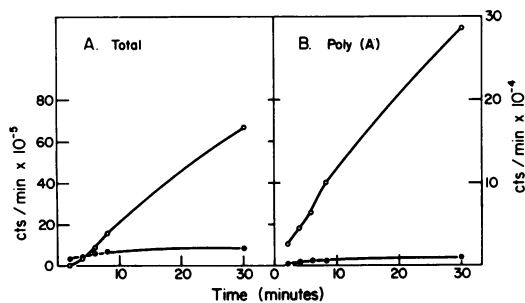

FIG. 11. *Kinetics of the addition of poly(A) to poliovirus RNA (see Table 4 for details). The values in parentheses in Table 4 are plotted for total counts per minute (A) and for poly(A) counts per minute (B). Symbols:* ●, *replicative intermediate RNA;* ○, *cytoplasmic 35S RNA.*

3' direction and with the poly(A) segment being the last nucleotides added to newly synthesized molecules. However, we cannot say from these data whether or not poly(A) is added to 35S RNA in the RI or just after release of a molecule of RNA from the RI. If, as seems likely, poly(A) is added by copying the poly(U) in the RI, 35S RNA molecules with their attached poly(A) do not remain in association with the

RI long enough after their synthesis to observe kinetically this poly(A) addition.

From the kinetics of poly(A) appearance in the RI and 35S RNA (Fig. 11B), it would be expected that no nascent poly(A) could be identified in the 3-h RI. In fact, if the RI consists exclusively of a complete complementary RNA molecule plus growing viral RNA molecules, no poly(A) should ever be present in the RI. Should a single chain of viral RNA with 75 nucleotides of AMP in poly(A) be in the RI, then at most 1% of the RI should be poly(A) (calculated assuming four to six growing viral RNA molecules per RI molecule). In spite of this logic, poly(A) does appear in the 3-h RI (Fig. 11B) and in the steady-state there is much more than 1% (Table 1). Furthermore, even after 2 min of labeling with [³H]adenosine, the poly(A) on RI molecules has the same size distribution as the poly(A) on 35S RNA.

To determine whether the high percentage of RNase resistance on the RI was due to the presence of poly(A) on growing strands, we heated both RI and viral RNA to 75 C for 5 min and then quickly cooled the samples. This procedure will release growing strands from the RI and leave a partially double-stranded backbone (2). If the growing strands do not contain poly(A) then dissociation of the RI should reduce the percent of binding to poly(U) filters. Table 5 shows that heat treatment had very little effect on the poly(U) binding of virion RNA whereas the efficiency of binding of heat-treated RI was 50% that of the unheated RI. To further investigate whether growing plus strands had poly(A), we heat dissociated a mixture of ³²P-labeled virion RNA and [³H]adenosine-labeled RI and then subjected the mixture to agarose chromatography (Fig. 12). The double-stranded RI backbone was excluded (fractions 14 to 20) whereas the dissociated plus strands were retained on the column and eluted in a broad peak. All fractions

TABLE 5. Effect of heating RI on poly(U) binding[a]

Sample	% Poly(U) bound	
	Unheated	Heated
RI	65	33
Virion	63	58

[a] [³H]adenosine-labeled 6-h virion RNA and 3-h RI were heated separately in 0.3 ml of 0.1% SDS buffer plus 20 mM EDTA to 75 C for 5 min and quickly cooled in an ice bath. Values of percentage of binding to poly(U) filters represent the average of four independent determinations.
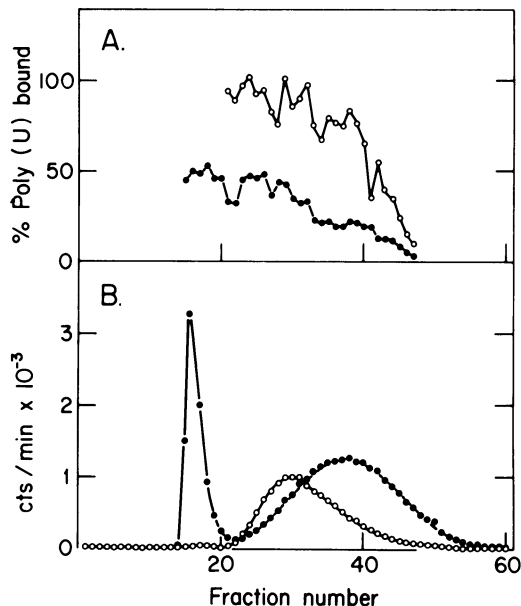

FIG. 12. Agarose chromatography of heat-denatured replicative intermediate RNA. A mixture of [³²P]-labeled virion RNA and 3-h [³H]adenosine-labeled replicative intermediate RNA heated in 1 ml of 0.1% SDS buffer plus 20 mM EDTA to 75 C for 5 min and quickly cooled was chromatographed through 2% agarose in 0.5% SDS buffer. Samples from each fraction were both counted in a xylene-based scintillant and bound to poly(U) filters. (A) Percentage of each sample which bound to a poly(U) filter. Values have been normalized so that the highest percentage of binding of ³²P-labeled virion RNA equals 100%. The acutal highest value for binding of ³²P-labeled virion RNA was 68%. Symbols: O, ³²P-labeled virion RNA; ●, [³H]adenosine replicative intermediate RNA. (B) Total counts per minute in a sample from each fraction. Symbols: O, ³²P-labeled virion RNA; ●, [³H]adenosine replicative intermediate RNA.

were then tested for the capacity to bind to poly(U) filters. Of the ³H label, the double-stranded RI backbone and the largest dissociated molecules bound with the highest efficiency. As the size of the dissociated strands decreased so did the ability to bind to poly(U) decrease. The curve of binding efficiency of the ³²P-labeled virion RNA followed that of the largest released chains suggesting that in the RI only completed 35S plus strands have poly(A). Part of the poly(A) in the RI must therefore be due to completed 35S RNA molecules associating with the RI and able to be released by temperatures below the melting point of dsRNA. There is also poly(A) left in the undissociated backbone of the RI.

Effect of guanidine on poly(A) synthesis.

To further study the site of synthesis of poliovirus poly(A), we utilized the drug guanidine. Earlier results have shown that the majority of the RNA made in the presence of guanidine does not enter polyribosomes (14) or become encapsidated into virions (3) but rather is held up in a complex larger than the replication complex called the guanidon (3).

At 2.25 h after infection, the infected cells were split into two cultures, one of which was made 2 mM in guanidine. Fifteen minutes later both cultures received 75 µCi of [³H]adenosine per ml, and 30 min after the addition of label 35S cytoplasmic, viral, ds-, and RI RNA was purified. Poly(A) was purified from these various RNA molecules and analyzed on 10% polyacrylamide gels (Fig. 13). Since a negligible amount of virions were made in the presence of guanidine, they could not be studied. The amount of poly(A) in the 35S cytoplasmic, RI, and dsRNA made both in the absence and presence of guanidine was comparable (Table 6). The values for the amount of poly(A) in these species made in the absence of guanidine

TABLE 6. Poly(A) on RNA molecules synthesized in the presence or absence of guanidine[a]

Sample	% RNase resistant and poly(U) bound	
	Plus guanidine	Minus guanidine
Cytoplasmic	5.4	2.6
RI	2.1	2.1
RF	1.2	1.6

[a] A culture of 8 × 10⁸ HeLa cells were infected with 50 PFU per cell of poliovirus in the presence of 10 µg of actinomycin D per ml. After 135 min the culture was separated into two portions, one of which contained 2 × 10⁸ cells/ml and the other 6 × 10⁸ cells/ml. The culture containing 6 × 10⁸ cells/ml was made 2 mM in guanidine-hydrochloride. Fifteen minutes after the addition of guanidine both cultures received 75 µCi of [³H]adenosine per ml and after 30 min of labeling cytoplasmic extracts were made by breaking the cells with a Dounce homogenizer. The cytoplasmic 35S, RI, and dsRNA were purified as described in the Materials and Methods.
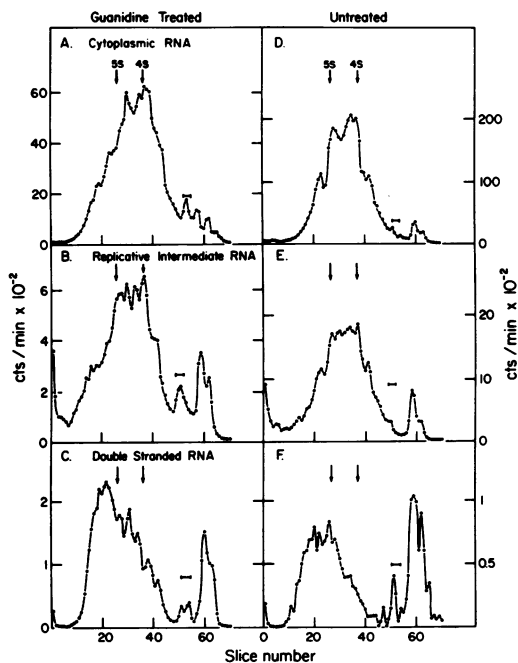

FIG. 13. Polyacrylamide gel electrophoresis of poly(A) sequences on polio RNA labeled in the presence (panels A, B, C) and absence (panels D, E, F) of 2 mM guanidine HCl. The RNA was prepared as in Table 6 and treated as in Fig. 5 except the gels were run at 7.5 mA/gel for 5 h. (A, D) Cytoplasmic 35S RNA. (B, E) Replicative intermediate RNA. (C, F) dsRNA.

were about 60% of those seen in Table 1. This discrepancy reflects the different methods used to measure the poly(A). In this experiment the poly(A) was measured as those sequences both resistant to T₁ plus pancreatic RNases and poly(U) bound. The poly(U) binding step results in a loss of about 40% of the poliovirus poly(A) sequences. The slightly higher percentage (5.4%) of poly(A) in the cytoplasmic single-stranded RNA made in the presence of guanidine is probably a reflection of the greater degree of inhibition of single-stranded RNA synthesis (6, 17) and thus longer period of time needed to reach steady-state values. As shown in Fig. 13 the poly(A) sequences on these species of RNA as determined by their migration on polyacrylamide gels were identical. These results suggest that poly(A) is added to 35S RNA molecules in the replication complex and that the guanidine-sensitive step is not involved with poly(A) addition.

DISCUSSION

From these studies we conclude that all species of poliovirus RNA (virion, RI, ds-, polyribosomal and 35S cytoplasmic RNA) contain poly(A). At the time when RNA synthesis is maximal (3 h after infection), the poly(A) on the virion, RI, polyribosomal, and 35S cytoplasmic RNA was found to be approximately 75 nucleotides in length. Thus, at this time in the infection, the size of the poly(A) does not seem to serve a regulatory role in determining whether a 35S RNA molecule will be translated,

encapsidated into a virion, or used as a template for the replication of minus strand.

In agreement with the studies of Yogo and Wimmer (30) we find that the size of the poly(A) on the dsRNA is at least twice the size of that found on virion RNA. Furthermore, after 4 h of infection, sequences of poly(A) at least as large as those found on dsRNA appear on RI RNA. These late RI molecules which are excluded from an agarose column are partially single stranded as indicated by their insolubility in 2 M LiCl. However, in contrast to 3-h RI molecules which display a heterogenous distribution from 20S to 70S, the late RI molecules sediment as a relatively homogenous species between 28S and 20S. It is likely that these late RI molecules represent dsRNA with a very long poly(A) tail and 1 to 2 small strands of nascent RNA attached. The presence of these very large poly(A) sequences on the RI and dsRNA later in the infection provide additional support that the dsRNA is a by-product of the RI and not just a random association of plus and minus strands (18).

At the same time that we find sequences of poly(A) as large as those on the dsRNA appearing on the RI, we also find that many 35S cytoplasmic RNA molecules have large poly(A) sequences. However, only molecules with smaller size poly(A) are encapsidated into virions. Whether encapsidation involves selection of molecules with shorter sequences of poly(A) or cleavage of longer sequences is unknown. Coincidentally, at this time of infection (4 h postinfection), the poliovirus polyribosomes are disintegrating and RNA synthesis is decreasing. It is unclear whether these long sequences of poly(A) are the cause, the result, or unrelated to the decrease in the translation and replication of poliovirus RNA.

The kinetic studies presented here are consistent with a model in which replication of viral RNA proceeds in a 5' to 3' direction with poly(A) being the last thing added at the 3' end of the molecule (29). This addition of poly(A) to completed 35S RNA molecules occurs without a lag. Although these kinetic studies do not indicate a simple precursor-product relationship between the RI and 35S poly(A), it is likely that the poly(A) sequences on the plus strand of poliovirus RNA are transcribed from the poly(U) sequences found in the dsRNA (28, 30) and the RI (31; D. H. Spector, manuscript in preparation). However, our results cannot rule out the possibility of some posttranscriptional polyadenylation.

The kinetics of the appearance of poly(A) in the RI and 35S RNA suggest that little poly(A) should be found in the RI. Yet at steady state, much more poly(A) is found on the RI than can be accounted for by considering the RI as a structure consisting of a minus strand hydrogen bonded to four to six growing plus strands (4). The experiments presented here on the poly(U) binding capacity of the heat-dissociated RI indicate that there is little if any poly(A) on nascent strands. It is more likely that released 35S RNA molecules reassociate with the RI either in vivo or during the purification procedure (18). If poly(A) is being added to completed 35S RNA molecules in the RI, then the presence of these 35S molecules associated with the RI is obscuring this.

Although we cannot show directly that poly(A) is added to 35S RNA molecules in the RI, several experiments suggest that the poliovirus poly(A)-adding activity is probably associated with the replication complex. The replication complex is a membrane-bound structure with an average sedimentation rate of 250S which is the intracellular site for viral RNA synthesis (12). In the accompanying paper (24) we demonstrate that poly(A) is added to poliovirus RNA newly synthesized in an in vitro extract containing the replication complex and other membrane-bound structures. The experiments in this paper which show that all species of poliovirus RNA synthesized in the presence of the inhibitor guanidine contain the same amount and size of poly(A) as those synthesized in its absence provide additional support for the replication complex as the site of poly(A) addition. In the presence of guanidine, RNA is synthesized and accumulates in a structure called the "guanidon" which sediments faster than the replication complex (3). This structure, which functions as the site of RNA synthesis, differs from the normal replication complex in that newly made RNA is unable to leave the guanidon to form polyribosomes or virus particles (3, 14).

The biological function of the 3'-terminal poly(A) is unknown. It has been suggested that it may play some role either in the processing and transport of mRNA from the nucleus to the cytoplasm or in the translation of the mRNA (1, 8). In a previous publication (23) we demonstrated that the 3'-terminal poliovirus poly(A) does serve a biological function since severe reduction of the size of the poly(A) by RNase H markedly decreased the specific infectivity of the poliovirus RNA molecule. Furthermore the virions in the few plaques deriving from infection with RNase H-treated RNA had normal amounts and size of poly(A) indicating that mechanisms exist in infected cells to regen-

erate normal-sized poly(A) from truncated poly(A). The experiments presented here also suggest that the size of the poliovirus poly(A) is important since poliovirus RNA molecules actively engaged in replication, translation, or virion formation all have normal size poly(A). It is only later in infection when viral functions are coming to a halt that we find the presence of large size poly(A) on the RI, ds- and 35S cytoplasmic RNA, none of which, however, is encapsidated into virions.

ACKNOWLEDGMENTS

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