JOURNAL OF VIROLOGY, Dec. 1975, p. 1512-1527
Vol. 16, No. 6

Printed in U.S.A.

Replication of Picornaviruses

I. Evidence from In Vitro RNA Synthesis that Poly(A) of the Poliovirus Genome is Genetically Coded

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Received for publication 3 July 1975

A crude replication complex has been isolated from poliovirus-infected HeLa cells and used for synthesis of poliovirus replicative intermediate (RI) RNA, replicative form (RF) RNA, and single-stranded (SS) RNA in vitro. All three classes of virus-specific RNA synthesized in vitro are shown to contain poly(A). Poly(A) of RF and of SS RNA [RF-poly(A) and SS-poly(A)] has a chain length (50 to 70 nucleotides) that is shorter than that of poly(A) of in vivo-synthesized RNAs. Poly(A) of RI [RI-poly(A)], however, is at least 200 nucleotides long and, therefore, larger than poly(A) of RI isolated from HeLa cells 4 h after infection. The crude membrane-bound replication complex contains a terminal adenylate transferase activity that is stimulated by Mn^{2+} and the addition of an $(Ap)_5A_{OH}$ primer. This transferase activity is found also in extracts of mock-infected cells. Partial purification of the replication complex in a stepwise sucrose gradient, in which the viral replicase is associated with the smooth cytoplasmic membrane fraction, does not remove the terminal transferase. However, when the partially purified replication complex is treated with deoxycholate and sedimented through a sucrose gradient, a soluble replication complex can be isolated that is free from terminal adenylate transferase. This soluble replication complex was found to synthesize viral RNA-linked poly(A) longer in chain length than that synthesized by the crude replication complex. Taking into account the 5'-terminal poly(U) in poliovirus minus strands, our data suggest that polyadenylation of poliovirus RNA occurs by transcription and not by end addition. When compared to other viral systems, poliovirus and, probably, all picornaviruses appear to be unique in that the poly(A) of their genome is genetically coded.

Poly(A) covalently linked to mRNAs of eukaryotic cells has been a conundrum ever since its discovery some years ago (for a review, see reference 25). Its mode of biosynthesis is poorly understood and its biological function remains obscure. Poly(A) was originally thought to play a crucial role in the synthesis of cellular mRNA's and their transport to the cytoplasm. It was, furthermore, suggested that poly(A) might be a requirement for an mRNA to function in protein synthesis. However, the lack of poly(A) in a large proportion of mRNA's of HeLa cells (1, 29) and of sea urchin embryos (31) and the absence of poly(A) in all reovirus mRNA's (46) indicate that transcription, processing, transport, and translation of mRNA's can occur in eukaryotic cells without the involvement of poly(A).

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Mounting evidence suggests that poly(A) of cellular mRNA is synthesized by post-transcriptional addition. Terminal adenylate transferases, which are presumably involved in this process, have been isolated from mammalian cells and characterized (for a review, see reference 11). Terminal adenylate transferase has also been found in vaccinia virions (12, 30, 32, 42). This enzyme is likely to polyadenylate viral transcripts by end addition because vaccinia virus-specific mRNA contains poly(A) (22), whereas the viral genome does not contain tracts of poly(dT) (32, 42). However, no evidence has been presented that directly links any of the characterized terminal adenylate transferases with the poly(A) synthesis of mRNA.

Single-stranded (SS) RNA viruses, which replicate in the cytoplasm, have been classified into plus-strand and minus-strand viruses (5). The genome RNAs of minus-strand viruses,

such as rhabdoviruses, serve as templates in primary transcription that precedes viral protein synthesis early in infection. Transcription can be carried out in vitro with detergent-disrupted virions because of a virion-associated RNA polymerase (7). Viral mRNA's isolated from infected cells or synthesized in vitro are poly(A)-linked (40). The genome (template) RNA does not contain segments of poly(U) (18, 28), suggesting again post-transcriptional end addition as the mechanism of poly(A) synthesis (48). In contrast to vaccinia virus, however, no poly(A) polymerase activity has been found so far to be associated with minus-strand virions (8).

Picorna and togaviruses are plus-strand RNA viruses, the single-stranded genome of which is polyadenylated and serves as mRNA during initiation of infection (40). Poly(A) in poliovirus RNA was discovered by Armstrong et al. (2) and subsequently was shown by us to be 3′ terminal and heterogeneous in size with an average chain length of 89 adenylate residues (50).

We have studied the biosynthesis of the homopolymeric tail of the poliovirus genome. Our strategy for solving the problem was the following: first, we have analyzed the structure of polio minus strands (that is, RNA complementary to the genome) for a sequence complementary to poly(A). This led to the discovery of poly(U) in minus strands of polio double-stranded RNA (referred to as replicative form [RF]; 51) and of the polio replicative intermediate (RI; 52) in the 5′-terminal position (49). Second, we have studied the synthesis of poliovirus RNA in a cell-free system with special emphasis on the possible involvement of terminal adenylate transferase. As we shall outline in this report, we have used a poliovirus-specific replication complex, first isolated from infected HeLa cells by Baltimore (3), which is membrane bound (16, 21) and does not respond to exogenous polio RNA template (19). Partial purification of the replication complex was achieved by solubilizing it with deoxycholate followed by sucrose gradient centrifugation (15). We have found that this complex is free of detectable poly(A) polymerases or terminal adenylate transferases, yet it synthesizes poly(A)-linked polio RNA in vitro. The structure of polio minus-strand RNA, together with the data presented here, strongly suggests that the genome-linked poly(A) of poliovirus is genetically coded.

We propose that the biosynthesis of poly(A) in poliovirus replication occurs by means of transcription. Such a mechanism is unique when compared with the other systems that have been analyzed so far.

(This work was presented in part at the 73rd Annual Meeting of the American Society for Microbiology, 6 to 11 May 1973, Miami Beach, Fla.)

MATERIALS AND METHODS

Cells and virus. The growth of strain S3 HeLa cells in suspension culture and the propagation of poliovirus type 1 (Mahoney) have been described previously (50).

Preparation of the crude replication complex. HeLa cells were washed once in Earle saline, suspended at a concentration of 4×10^7 cells/ml in Eagle minimal essential medium without serum, and infected with 40 to 50 PFU of virus per cell. After adsorption at room temperature for 30 min, the cells were diluted to 4×10^6/ml with warm medium containing horse serum or heat-inactivated calf serum (5%) and incubated at 37 C. We have found that the growth of HeLa cells and the virus yield were independent of the serum used. The crude polio replication complex was isolated as follows (27). Cells were harvested 4 h after infection, washed once with Earle saline, and suspended in reticulocyte standard buffer (RSB; 0.01 M Tris-hydrochloride, pH 7.35; 0.01 M KCl) to which $MgCl_2$ had been added (to 1.5 mM) at a concentration of 2×10^7 cells/ml. After swelling for 5 min at 0 C, the cells were disrupted with a stainless-steel Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at $600 \times g$ for 5 min, and the replication complex was sedimented from the supernatant by centrifugation at $30,000 \times g$ for 20 min. The resulting pellet was then suspended at approximately 10 mg of protein/ml in suspending buffer (0.12 M Tris-hydrochloride, pH 8.0; 0.06 M KCl). Such preparations can be stored for several months at −70 C without loss of activity.

Fractionation of the crude replication complex. The crude replication complex was fractionated by equilibrium sedimentation in a discontinuous sucrose gradient (16). The discontinuous gradient was formed by layering the following sucrose-RSB solutions in a 36-ml tube: 3 ml of 60% sucrose in RSB, 7 ml of 45% sucrose, 7 ml of 40% sucrose, 10 ml of the crude replication complex containing 30% sucrose (2 to 3 mg of protein per ml), 7 ml of 25% sucrose in RSB and, lastly, 3 ml of RSB. The gradients were centrifuged at $86,000 \times g$ and 4 C for 12 to 17 h in a Spinco SW27 rotor. Caliguiri and Tamm (16) observed seven fractions when the total cytoplasmic extract was separated by isopycnic banding, the top fraction (fraction 1) being a lipid layer. In our case, most of the lipids had been removed during the preparation of the crude replication complex. The first visible band in our gradients contains smooth membranes. This fraction has been designated by Caliguiri and Tamm (16) as fraction 2 and was found to contain poliovirus RNA polymerase at the highest specific activity when compared with the other fractions. It was collected with a long tip pipette as follows. A 2-ml volume from the top of the gradient

was discarded. The next 5 ml was collected, diluted with RSB, and sedimented at 40,000 rpm and 4 C for 90 min in a Spinco type 40 rotor. The pellet was dissolved in 0.2 to 0.4 ml of suspending buffer and assayed.

Solubilization of the replication complex. Dissociation of the replication complex from membranes was achieved as follows (15). The pellet of the smooth microsomal fraction (fraction 2) was suspended in 0.1 ml of RSB containing 1.5 mM $MgCl_2$, and sodium deoxycholate was added to a final concentration of 0.5%. The mixture was incubated at 0 C for 10 min and sedimented through a 15 to 30% sucrose-RSB gradient at 58,000 rpm and 4 C for 40 min in a Spinco SW65 rotor. ^{32}P-labeled poliovirus was run in a parallel gradient as a 150S marker. Fractions (32 drops each), collected by puncturing the bottom of the centrifuge tube, were assayed for polymerase activity after the addition of one-fifth volume of RSB. Fractions from the gradient containing the ^{32}P-labeled poliovirus were collected in small glass scintillation vials, and the ^{32}P was located by determining Cerenkov radiation. Peak fractions of polymerase activity and poliovirus usually coincided. The three fractions of the gradient containing the replication complex corresponding to peak fractions in the marker gradient were, therefore, routinely combined and used as the solubilized replication complex.

Assay of polymerase activity. The standard reaction mixture in a final volume of 200 μl contained the following constituents (unless otherwise noted in the text): enzyme solution, 100 μl; Tris-hydrochloride (pH 8.0), 12 μmol; magnesium acetate, 2 μmol; phosphoenolpyruvate, 2 μmol; pyruvate kinase, 8 μg; actinomycin D, 4 μg; DNase, 4 μg; GTP, CTP, and UTP, 0.06 μmol each; and [^3H]ATP (specific activity, 0.415 Ci/mmol), 0.012 μmol. In some experiments, [^3H]GTP was used as the labeled substrate instead of [^3H]ATP. In this case, 0.06 μmol of ATP and 0.012 μmol of [^3H]GTP (specific activity, 0.416 Ci/mmol) were added to the reaction mixture. The polymerization was carried out at 37 C. It was stopped by placing duplicate 20-μl samples into 1 ml of cold 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate. After 30 min at 0 C, the precipitate was collected on membrane filters and washed five times with 2 ml of 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate. The dried filters were immersed in a toluene-based scintillation fluid and counted in a liquid scintillation counter. In some experiments, the reaction was terminated by spotting 20- to 50-μl samples onto squares (2 by 2 cm) of Whatman DEAE filter paper. Unincorporated ATP was removed by washing the filters with 0.5 M Na_2HPO_4 (37). The filters were counted as above. The two methods gave very similar results. The DEAE filter paper method, however, was found to be easier when the processing of large numbers of samples was necessary, and it has the additional advantage of greater reproducibility in counting the tritium isotope. All results are given after subtraction of the zero time values (sample taken immediately after mixing the reaction mixture with the enzyme).

Isolation of poliovirus-specific RNAs. For preparative synthesis of RNA, [^3H]ATP at a specific activity of 1.66 Ci/mmol was used as the labeled substrate. The reaction was stopped by adding EDTA to a final concentration of 20 mM and sodium dodecyl sulfate (SDS) to 0.5%. RNA was deproteinized by shaking the mixture twice with 0.5 volume of RSB-saturated phenol and 0.5 volume of chloroform-isopentanol (24:1, vol/vol) for 10 min at room temperature (51). The phenol phases were then washed serially with NTE buffer (0.1 M NaCl; 0.01 M Tris-hydrochloride, pH 7.5; 0.001 M EDTA). Both aqueous phases were combined and the RNA was precipitated twice with 2 volumes of ethanol. The RNA was fractionated by precipitation in high-salt and gel filtration (4, 52) as follows. The ethanol precipitate was dissolved in NTE buffer at a concentration of 50 to 100 absorbancy units at 260 nm (A_{260}) per ml. LiCl (4 M) was added to a final concentration of 2 M, and the mixture was kept at 0 C overnight. SS RNA and partially double-stranded RI, which precipitate under these conditions, were collected by centrifugation. Double-stranded RNA (RF) was recovered from the supernatant by ethanol precipitation. Both RNA fractions were dissolved in NTE buffer containing 0.5% SDS and fractionated in the same buffer in a column (1.5 by 70 cm) of Sepharose 2B. Polio RI and RF elute under these conditions in the void volume, whereas SS RNA is included and elutes as a broad peak. Fractions containing SS RNA, RI, and RF were collected and ethanol precipitated. The identities of the RNA species were verified by their sensitivity to RNase A, their migration in 1.8% polyacrylamide-0.5% agarose gels, and their sedimentation in sucrose density gradients (52).

Isolation of poly(A) fragments. SS RNA was digested with 2 μg of RNase A and 50 U of RNase T1 per 1 A_{260} unit of RNA for 30 min at 37 C in a 100-μl solution of 0.2 M NaCl, 0.1 M Tris-hydrochloride (pH 7.5), and 5 mM EDTA. The reaction was interrupted by adding a 2-ml solution of 0.3 M NaCl, 5 mM Tris-hydrochloride (pH 7.5), and 1 mM EDTA in 7 M urea. The mixture was applied to the DEAE-Sephadex column with a bed volume of 1 ml (equilibrated with the same buffer; 50). The column was washed with 2 ml of buffer, and 8 A_{260} units of tRNA were applied to the column. Unbound material was eluted with 30 ml of this buffer, and polynucleotides bound to the column were eluted with a linear NaCl gradient (total volume, 50 ml) from 0.3 to 0.7 M in a solution of 5 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 7 M urea. Fractions (1.5 ml) were collected and monitored by absorbance at 260 nm and for radioactivity. Peak fractions were pooled and precipitated with 2 volumes ethanol in the presence of tRNA (0.2 A_{260} unit/ml) and sedimented at 15,000 rpm and 4 C for 6 h in a Spinco SW27 rotor.

Polio-specific RF and RI were denatured in 1 ml of 1 mM EDTA in the presnce of 0.2 μg of poly(A)/ml at 100 C for 2 min. The solution was rapidly cooled, mixed with a 0.1-ml solution of 0.2 M Tris-hydrochloride (pH 7.5), 0.01 M EDTA, and 50 U of RNase T1, and incubated for 30 min at 37 C. Poly(A)-containing fragments were isolated by binding to poly(U) fiberglass filters as previously described (41, 51). The

mixture was passed slowly through a poly(U) filter in binding buffer (0.12 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5; reference 41). Unbound polynucleotides were washed from the filter with binding buffer. Bound polynucleotides were eluted with a solution of 2 ml of 0.5% SDS, 0.02 M Tris-hydrochloride (pH 7.4), and 1 mM EDTA containing 10 μg of poly(A)/ml for 5 min at 65 C. The extraction was repeated twice. Under these conditions of binding to poly(U) filters, no loss of poly(A) that originated from viral RNA was observed. The material in the combined extracts was precipitated in the presence of carrier tRNA and further analyzed by ion-exchange chromatography after digestion with RNase A and T1 as described above.

End-group determination. Purified poly(A) was digested with 20 U of RNase T2 for 3 h at 37 C in a 20-μl solution of 0.05 M ammonium acetate (pH 4.5) and 1 mM EDTA. The digest was analyzed on thin-layer plates, precoated with silica gel, by using isopropanol-concentrated ammonia-water (7:2:1, vol/vol/vol) as solvent in the presence of authentic markers. Appropriate areas were scraped from the plate. Nucleosides and nucleotides were extracted from the silica gel with a 0.5-ml solution of 0.025 N HCl, 0.5 mM 2',3'-AMP, and 0.5 mM adenosine for 5 min at 65 C. The extraction was repeated twice; the extracts were combined, neutralized with Tris-hydrochloride, and counted in a liquid scintillation counter.

Polyacrylamide gel electrophoresis. High-molecular-weight RNAs were analyzed with 1.8% polyacrylamide-0.5% agarose gels by the method of Yogo and Wimmer (52). Three milliliters of 30% acrylamide, 55 μl of ethylene diacrylate, 10 ml of 5× E buffer (0.18 M Tris-hydrochloride, 0.15 M NaH_2PO_4, 5 mM EDTA, pH 7.6), 5 ml of glycerol, and 6.7 ml of water were combined and degassed, and 40 μl of *N,N,N',N'*-tetramethyl ethylene diamine was added. This solution was mixed with 25 ml of 1% agarose ("Seakem," Marine Colloid, Inc.) in water that had been boiled and cooled to 56 C. A 0.25-ml portion of 10% ammonium persulfate was added, and the mixture was poured into Plexiglas electrophoresis tubes. After 1 h, the gels were topped with E buffer containing 10% glycerol.

For the analysis of poly(A) fragments, 10% polyacrylamide gels were prepared as follows: 10 ml of 30% acrylamide, 70 μl of ethylene diacrylate, 6 ml of 5× E buffer, 3 ml of glycerol, and 11 ml of water were mixed and degassed. A 25-μl portion of *N,N,N',N'*-tetramethyl ethylene diamine and 0.1 ml of 10% ammonium persulfate were added, and the mixture was poured into electrophoresis tubes. The gels were topped with E buffer containing 10% glycerol, left at room temperature for 1 h to polymerize and then stored at 4 C. For electrophoresis, the gels were prerun for 30 min at 5 mA/gel with E buffer containing 0.2% SDS and 10% glycerol (1.8% gels were inverted for electrophoresis). Gels were scanned for absorbance at 280 nm (50) or cut into 1-mm slices, incubated overnight with 0.2 ml of concentrated ammonia, and counted in a scintillation fluid.

Protein assay. Protein concentrations were determined by the method of Lowry et al. (26), with bovine serum albumin as a standard.

Materials. [^{3}H]ATP (30 Ci/mmol) and [^{3}H]GTP (5 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass.; RNase A (fraction XII), pyruvate kinase (type III), and actinomycin D were from Sigma Chemical Co., St. Louis, Mo.; RNase T1, RNase T2, and phosphoenolpyruvate were obtained from Calbiochem, Los Angeles, Calif.; DNase was purchased from Worthington Biochemicals Corp., Freehold, N. J.; ATP, CTP, GTP, and UTP were supplied by Boehringer Mannheim Corp., New York; silica gel plates (E. Merck AG., Darmstadt, Germany) were obtained from Brinkmann Instruments Inc., Westbury, N. Y.; Sepharose 2B and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; poly(A), poly(U), [^{3}H]poly(A), and $(Ap)_5A_{OH}$ were from Miles Laboratories, Inc., Elkhart, Ind.

RESULTS

Kinetics of [^{3}H]ATP and [^{3}H]GTP incorporation. The properties of the crude replication complex as isolated from infected HeLa cells have been analyzed previously in several laboratories (for a review, see reference 24). The complex is associated with cellular membranes and contains virus-specific RNAs. RNA functioning as template is tightly bound to the polio-specific polymerase (21). Under appropriate conditions, all three species of viral RNA, SS, RI, and RF, are labeled in vitro (20, 27). Removal of RNA from protein components results in the loss of polymerase activity that cannot be restored through the addition of exogenous RNA template (K. Dorsch-Häsler and E. Wimmer, unpublished data). In all published studies, the incorporation of labeled UMP or GMP, but not of AMP, into viral RNA in the presence of the other unlabeled precursors has been analyzed. ATP was presumably not used as the labeled precursor because of the high background polymerization that occurs when labeled ATP alone is added to the complex (see below). In our study, the method of isolating the crude replication complex by McDonnell and Levintow (27) proved to be the most suitable and was employed throughout the course of this work.

A comparison of the incorporation of [^{3}H]-AMP and [^{3}H]GMP into RNA at a concentration of 0.033 mM is presented in Fig. 1. Incorporation increases linearly with time for [^{3}H]AMP up to 30 min and for [^{3}H]GMP up to 60 min. The rate of [^{3}H]AMP and [^{3}H]GMP incorporation is linear for approximately the same length of time, even when low concentrations (0.0018 mM) of the labeled substrates are used (data not shown). The concentration of labeled ATP, although below the apparent K_m (Fig. 2),

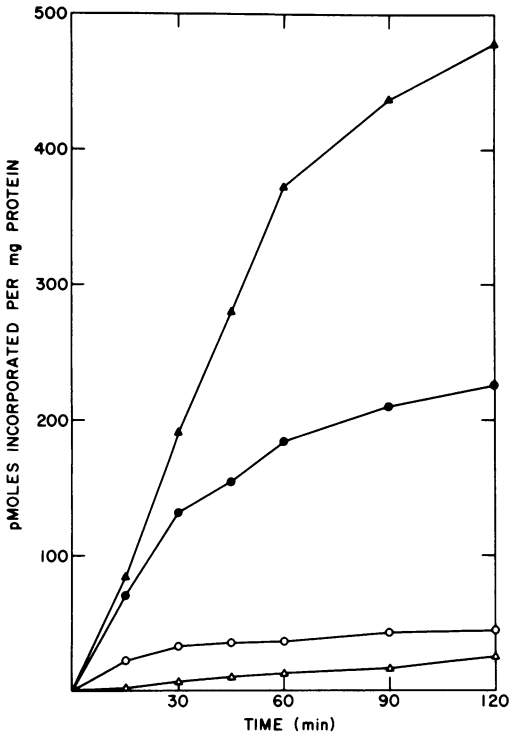

FIG. 1. *Kinetics of [³H]AMP and [³H]GMP incorporation. The crude replication complex was incubated in an assay mixture containing either all four nucleoside triphosphates, one of them being labeled (▲, ●), or only one nucleoside triphosphate (△, ○). The concentration of the unlabeled nucleoside triphosphates was 0.3 mM; the ³H-labeled nucleoside triphosphates were added at a concentration of 0.033 mM. Duplicate 20-μl samples were assayed for acid-precipitable radioactivity after incubation at 37 C for the indicated length of time as described in the text. Symbols: ▲, incorporation of [³H]GMP; ●, incorporation of [³H]AMP.*

was sufficient to insure that the rate of RNA synthesis did not change during the course of our assays. It should be pointed out that only a very small proportion of the substrate is incorporated into RNA; that is, the concentration of nucleoside triphosphates does not appreciably change during the incubation. For this reason, the extent of the polymerization was approximately proportional to the amount of crude polymerase extract, when monitored during the linear phase of RNA synthesis (Table 1). We used the crude polymerase extract at a protein concentration of 5 mg/ml in all of the experiments described below.

As can be seen in Fig. 1, more [³H]GMP compared with [³H]AMP is incorporated under the given conditions, presumably because of a higher affinity of the enzyme for GTP. This is supported by Fig. 2, which shows Lineweaver-Burk plots indicating that the apparent K_m for GTP is about 4×10^{-6} (a similar result has been found by Ehrenfeld et al. [19]) and that the apparent K_m for ATP is about 2.2×10^{-4}. One must consider, however, that the replication complex used in these experiments is crude and might contain more than one enzyme capable of incorporating AMP into acid-insoluble material. Indication for such an activity comes from control reactions in which labeled substrate only was added to the incubation mixture (Fig. 1). Whereas the incorporation of [³H]GMP is reduced to 3 to 5%, incorporation of [³H]AMP is reduced to only 25 to 35% when compared with the complete reaction mixture after 15 min of incubation. A very similar reaction can be observed in extracts from mock-infected cells, which will be discussed in a later section.

In vitro product of the crude replication complex. For analyses of viral RNAs synthesized by the crude replication complex, the reaction mixture was incubated for 1 h at 37 C in

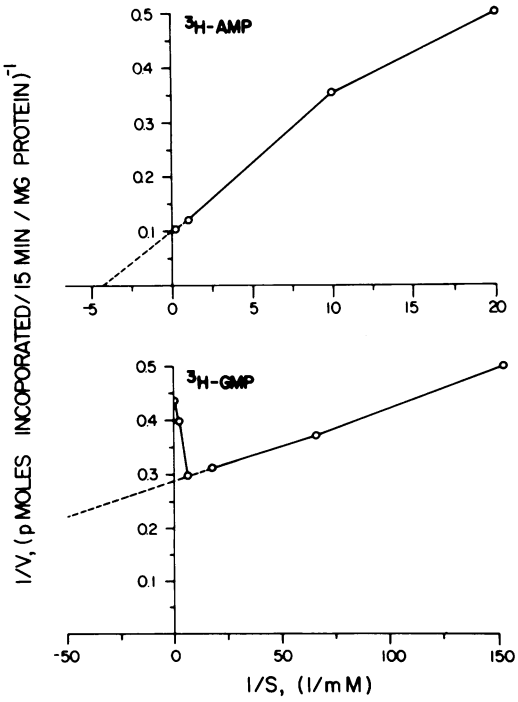

FIG. 2. *Lineweaver-Burk plots of incorporation of [³H]AMP and [³H]GMP into RNA in vitro by the crude replication complex. The reaction mixtures contained varying amounts of [³H]ATP or [³H]GTP of constant specific radioactivities. Duplicate 20-μl samples were assayed for radioactivity binding to DEAE paper after 15 min incubation at 37 C.*

TABLE 1. *Dependence of [³H]AMP incorporation on the protein concentration of the crude replication complex*[a]

Protein concn (mg/ml)	Counts/min incorporated/15 min per 20 μl-reaction mixture
7.5	2,736
5.0	2,132
2.5	1,178
0.5	267

[a] A standard polymerase assay was performed in the presence of various concentrations of the crude replication complex preparation. The samples were incubated for 15 min at 37 C and DEAE-paper binding radioactivity was determined of duplicate 20-μl samples.

the presence of [³H]ATP and unlabeled nucleoside triphosphates and subsequently deproteinized with phenol-chloroform (see above). RNA was separated into RI, RF, and SS RNA by precipitation in 2 M LiCl and gel filtration on Sepharose 2B. Of the total radioactivity recovered in polio-specific RNAs, 47.6% was SS, 38.9% was RI, and 13.5% was RF. These yields differ considerably from yields obtained in vivo: viral RNA isolated from a cytoplasmic extract 4 h after infection is more than 90% SS RNA (6, 14).

An analysis of the in vitro-synthesized RNAs on 1.8% acrylamide-0.5% agarose gels shows that SS RNA and RF are rather homogeneous in size. They migrate to the same position as the corresponding in vivo species (Fig. 3A and B). In vitro RI migrates as a more heterogeneous peak, slightly slower than the RF, while some material does not enter the gel (Fig. 3C). When compared to in vivo RI isolated from a cytoplasmic extract, however, the in vitro RI migrates faster, which indicates that it might contain fewer nascent RNA strands (52). Analyses of sedimentation velocities in sucrose density gradients verified the nature of the RNA species (data not shown). The data are in agreement with previous studies that have shown that all virus-specific RNA species (RI, RF, SS RNA) are labeled by the crude replication complex in vitro (20, 27).

The resistance of in vitro-synthesized RNA (labeled with [³H]adenosine) to RNase A (20 μg/ml) in 0.3 M NaCl is shown in Table 2. As expected, RF is highly resistant to degradation. In vitro RI is resistant to degradation to a larger extent than RI isolated from infected cells (4, 10, 52). The reason is undoubtedly its content of long poly(A) fragments (see below). Furthermore, RI isolated from a crude replication complex after incubation for 1 h is probably the product of a runoff synthesis of preinitiated strands (see below) and is, therefore, to a higher proportion double stranded. Single-stranded poliovirus RNA synthesized in vitro

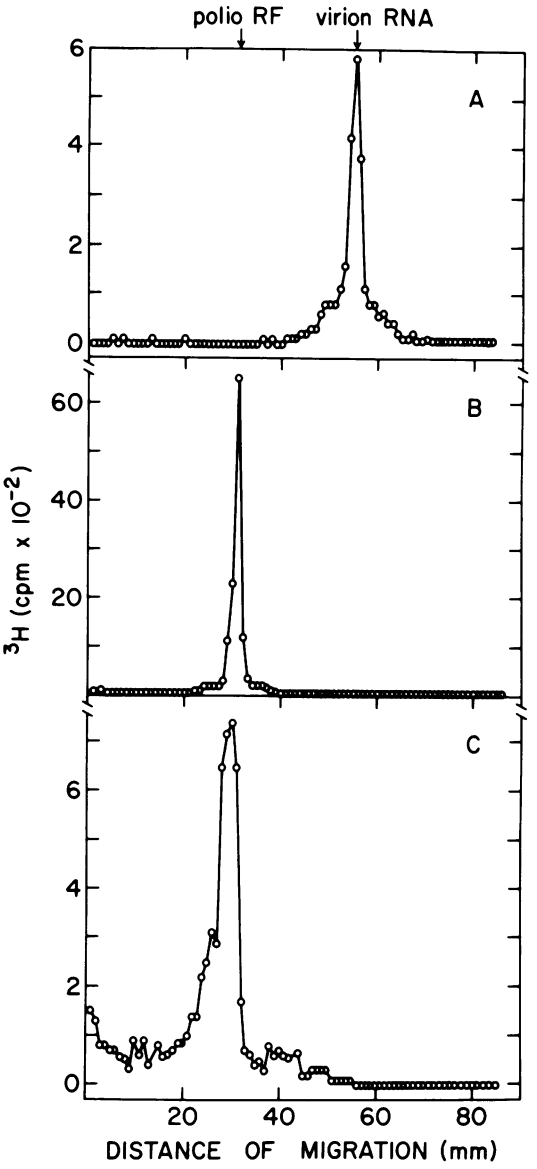

FIG. 3. *Electrophoretic analysis of poliovirus-specific RNA synthesized in vitro by the crude replication complex. [³H]AMP-labeled RNA was phenol extracted from a standard reaction mixture after 60 min incubation and separated by LiCl fractionation and chromatography on Sepharose 2B. Electrophoresis of the RNA samples in 1.8% polyacrylamide-0.5% agarose gels was carried out for 3.5 h at 5 mA/gel. The gels were analyzed for radioactivity as described in the text. (A) SS RNA, (B) RF, (C) RI.*

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

Sample	Resistance (%)
In vitro 35S RNA	8.6
In vitro RI	58.4
In vitro RF	98.25
[^{3}H]poly(A)	100
Virion RNA	6.8
HeLa 28S rRNA	5.3

[a] The in vitro RNAs were prepared as described in the legend to Fig. 3. Samples were incubated for 30 min at 24 C in 0.3 M NaCl and 0.03 M sodium citrate containing 10 μg of RNase A/ml. The RNase-resistant fraction was determined by assaying the remaining acid-precipitable radioactivity. All RNAs were labeled with [^{3}H]AMP, except for virion RNA and HeLa 28S rRNA, which were labeled in vivo with [^{3}H]uridine.

shows considerable resistance to endonucleolytic degradation, indicating the presence of poly(A) (Table 2).

Poly(A) is not degraded by RNase A in 0.3 M NaCl (9). Resistance to only RNase A under these conditions, however, is a poor measure of the poly(A) content of an SS RNA since 6.8% of polio [U-^{3}H]RNA and 5.3% of ribosomal 28S [U-^{3}H]RNA remain acid precipitable (Table 2), presumably because of the high degree of secondary structure of the RNAs.

Viral RNA-linked poly(A) synthesized in vitro. Poly(A) in viral RNA synthesized in vitro by the crude replication complex was then analyzed by isolating it from endonuclease digests of purified SS RNA, RI, and RF. SS RNA was digested with RNase A-RNase T1 at 0.2 M NaCl, followed by chromatography on DEAE-Sephadex in 7 M urea (see above). RI and RF were first denatured in 1 mM EDTA at 100 C for 2 min in the presence of unlabeled poly(A) and then digested with RNase T1 in 0.02 M Tris-hydrochloride, pH 7.5. Digestion at a low salt concentration reduces the possibility of partial reassociation of complementary sequences. It necessitates the omission of RNase A from the digest (9), which in turn has the advantage that filtration of digests through poly(U) filters yields 100% binding, even of short poly(A) (30 to 50 adenylate residues). When RNase A is used, poly(U) on the filter is undoubtedly degraded during the binding step which, in our hands, interferes with the efficient binding of shorter poly(A).

Poly(A) recovered from either ion-exchange columns or poly(U) filters (see above) was then subjected to electrophoresis on 10% polyacrylamide gels. As can be seen, poly(A) from SS RNA [SS-poly(A)] migrates faster than poly(A) from virion RNA [virion-poly(A)] or tRNA (Fig. 4A). Similarly, poly(A) from RF [RF-poly(A)] is shorter in chain length than virion-poly(A) or tRNA and only slightly larger than SS-poly(A) (Fig. 4A). In contrast, poly(A) from in vitro RI [RI-poly(A)] is extremely long and heterogeneous, with an estimated chain length >200 nucleotides (Fig. 4B).

The lengths of in vitro-synthesized poly(A)'s differ considerably from in vivo-synthesized poly(A)'s of corresponding RNA species (50, 51, 52). SS RNA and RI isolated 3 to 4 h after the infection of HeLa cells with poliovirus contain poly(A) that is, on the average, 90 adenylate residues long (52) whereas poly(A) from in vivo RF is approximately 200 nucleotides long (51). These differences cannot be due to nucleolytic degradation of poly(A) in vitro because (i) the long RI-poly(A) was isolated from the same incubation mixture that contained the short SS-poly(A) and RF-poly(A), and (ii) all products labeled by the crude replication complex during a pulse of 30 min remained acid precipitable during a chase of 60 min (20, 27, and our own unpublished data).

Poly(A) synthesized in vitro was shown to be covalently linked to polio RNA by the following procedure. ^{3}H-labeled SS RNA was incubated with an excess of unlabeled poly(A) in 85% dimethyl sulfoxide at 37 C for 30 min (23). Intact poliovirus-specific RNA was recovered by sucrose density centrifugation. This RNA contained the same amount of labeled poly(A) as did untreated RNA after RNase A and RNase T1 digestion. The length of the poly(A) fragment of both untreated and dimethyl sulfoxide-treated RNA was the same, as demonstrated by DEAE-Sephadex chromatography (data not shown).

The content of poly(A) of in vitro RNAs is similar to that observed in in vivo RNAs (Table 3). However, if the sizes of SS-poly(A) and RF-poly(A) of the in vitro RNAs are taken into consideration (Fig. 4A), the poly(A) content in these RNAs is too high.

We interpret this discrepancy as an indication that in vitro poly(A) synthesis occurs primarily as a runoff addition to nascent RNA strands and that little, if any, chain initiation occurs.

End group analysis by the digestion of purified SS-poly(A) with RNase T2 followed by thin-layer chromatography yielded AMP and adenosine in a ratio of 73 to 1 (two experiments). This indicates that poly(A) in in vitro SS RNA is 3'-terminal and shorter than virion-poly(A).

Incorporation of ATP in the absence of

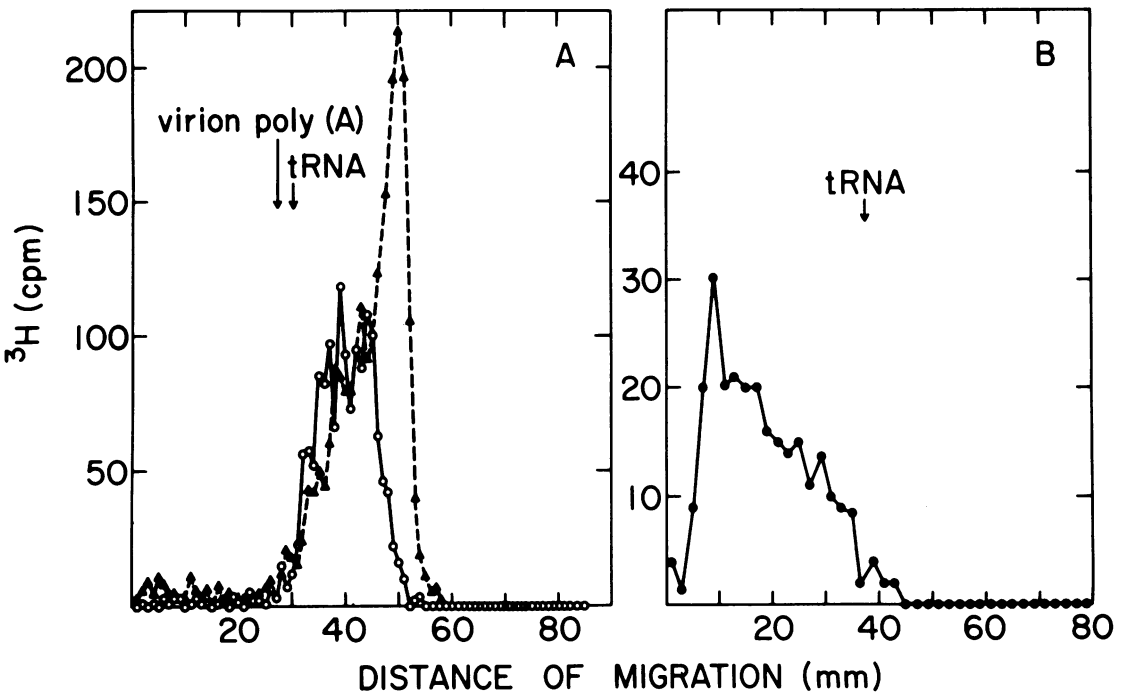

FIG. 4. *Polyacrylamide gel electrophoresis of poly(A) sequences of polio-specific RNAs labeled in vitro with [^{3}H]AMP. The RNAs were isolated as described in the legend to Fig. 3. Poly(A) from in vitro-labeled single-stranded RNA and from ^{32}P-labeled virion RNA was obtained by digestion with RNase A and T1, followed by ion-exchange chromatography on a DEAE-Sephadex column in 7 M urea. In vitro-synthesized RI and RF were first heat denatured in the presence of an excess of unlabeled poly(A) (0.2 μg/ml), digested with RNase T1, and bound to poly(U) filters. The bound material was eluted, redigested with RNase A and T1 after ethanol precipitation, and further purified by ion-exchange chromatography. Electrophoresis and analysis for radioactivity were performed as described in the text. Yeast tRNA was run on a parallel gel and scanned for absorbance at 280 nm in a Gilford spectrophotometer. Symbols: (A) △, SS-poly(A); ○, RF-poly(A); (B) RI-poly(A).*

TABLE 3. *Poly(A) content of in vivo- and in vitro-synthesized polio-specific RNAs*

Form of RNA	% Poly(A) of RNA synthesized		
	In vivo	In vitro	
		By crude replication complex	By replication complex in smooth microsomal fraction
RI	0.4[a]	2.52 ± 0.031[b]	2.61[b]
RF	0.72 ± 0.06[c]	2.05 ± 0.37[b]	2.18[b]
SS RNA	1.14 ± 0.02[d]	2.49 ± 0.23[e]	2.50[e]

[a] Reference 52.
[b] Determined by digestion with RNase T1 followed by poly(U)-filter binding.
[c] Reference 51.
[d] Reference 50.
[e] Determined by digestion with RNase A and T1 followed by DEAE-Sephadex chromatography (52).

viral RNA synthesis. When the crude replication complex is incubated with [^{3}H]ATP as the only substrate, acid-precipitable material is synthesized (Fig. 1). This background reaction could be a result of the end addition of adenylate residues to incomplete viral RNA chains and/or to short primer molecules yielding poly(A). It might also be the result of a turnover of the 3′ end of the tRNA. We have, therefore, studied RNA synthesis in extracts from mock-infected cells and have compared the products with those of the crude replication complex. In the presence of all four precursors, no [^{3}H]GMP is incorporated into acid-precipitable material by extracts of the mock-infected cells (Fig. 5B), confirming that the replication complex is present only in poliovirus-infected cells. In contrast, [^{3}H]ATP is incorporated by an extract from uninfected cells in the presence of all four precursors (Fig. 5A) approximately to the same extent as by an extract from infected cells when the three other precursors are omitted (Fig. 1).

RNA synthesized by extracts from infected and mock-infected cells labeled in vitro with [^{3}H]ATP in the presence or absence of the other nucleoside triphosphates was analyzed by zone

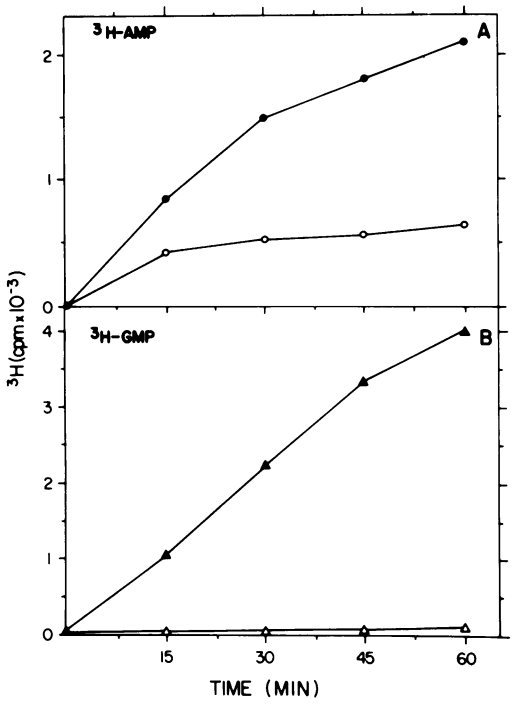

FIG. 5. *Incorporation of [³H]AMP (A) and [³H]GMP (B) into RNA by extracts from polio-infected (closed symbols) and mock-infected (open symbols) HeLa cells. Extracts from mock-infected cells were prepared in the same way as the crude replication complex, except that medium was added instead of virus suspension at the onset of the infection. A standard polymerase assay was performed with a reaction mixture containing all four nucleoside triphosphates.*

sedimentation through sucrose density gradients (Fig. 6). The product of the polymerization of the crude replication complex in the complete reaction mixture resolves into three peaks with sedimentation values of 35S, 18S and 4S, corresponding to the sedimentation values of authentic poliovirion RNA, polio RF, and tRNA, respectively (Fig. 6A). As indicated, a considerable amount of each fraction is RNase resistant. This is due to the poly(A) content of the high-molecular-weight RNAs and the double strandedness of the RI, which sediments over a wide range, and of the RF. Most of the small 4S peak is RNase sensitive. RNase-resistant material in the 4S region (approximately 10% of the total counts) binds to poly(U) filters and is, therefore, considered to be adenosine-rich RNA (see below). The sedimentation profile changes when the crude replication complex is incubated with [³H]ATP as the only substrate (Fig. 6B). The small amount of material sedimenting between 18S and 50S is presumably viral RNA that has been adenylated at the 3′ end by end addition. The 4S peak is prominent relative to high-molecular-weight RNA. Because of its senstivity to RNase, we consider it to be terminally labeled tRNA. The presence of tRNA in the crude replication complex can be expected since a fraction of poliovirus-specific polyribosomes is membrane bound (16, 35, 39) and will cosediment at 30,000 × *g* together with the complex. Very little RNA larger than 4S is labeled in extracts from mock-infected cells, regardless of whether the incubation was carried out in the presence of all nucleoside triphosphates (Fig. 6C) or in the presence of [³H]ATP alone (Fig. 6D). Labeled 4S

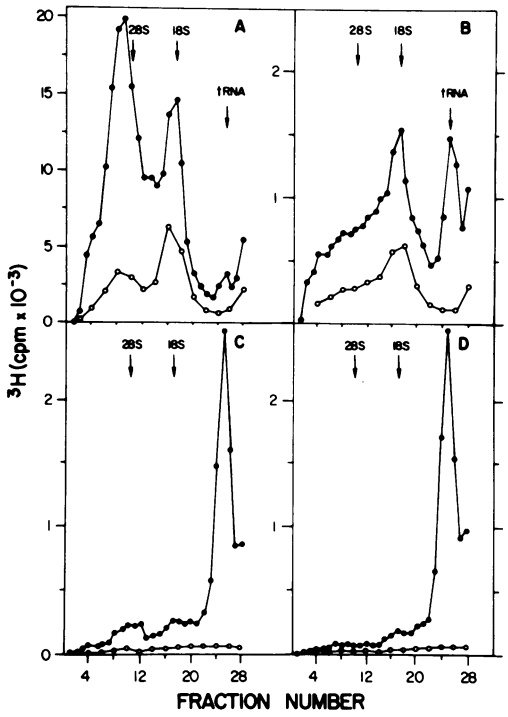

FIG. 6. *Sedimentation of RNA synthesized in vitro by extracts from infected and from mock-infected cells. The crude replication complex preparation from infected cells (A, B) and mock-infected cells (C, D) was incubated for 60 min with [³H]ATP in the presence (A, C) and in the absence (B, D) of the three other nucleoside triphosphates. Total RNA was isolated by extraction with phenol-chloroform-SDS and precipitated with 2 volumes of ethanol. The samples were sedimented through a 15 to 30% sucrose gradient in NTE buffer for 10 h at 40,000 rpm and 4 C in an SW41 rotor. Portions from each fraction were precipitated with trichloroacetic acid (●), and portions from alternate fractions were tested for resistance to RNase A (○) by incubation for 30 min at 24 C in 0.3 M NaCl and 0.03 M sodium citrate in the presence of 10 μg of RNase A/ml.*

RNA, on the other hand, is found in both gradients (Figs. 6C and D) to approximately the same extent as in extracts from infected cells. The 4S RNA in Fig. 6A to D is probably tRNA originating from membrane-bound cellular polyribosomes (see above). This is supported by the finding that the 4S material, which is sensitive to RNase A, comigrates with marker tRNA on 10% polyacrylamide gels (data not shown). As indicated, very little RNase-resistant material is synthesized by the uninfected cell extract under standard conditions of incubation.

The presence of some poly(A) with an S value of 4 or smaller, which was synthesized by the crude replication complex (Fig. 6A), prompted us to test cell extracts for the presence of terminal adenylate transferase. Such an enzyme has been characterized recently by Tsiapalis et al. (47); it is primer dependent and five times more active with Mn^{2+} than with Mg^{2+}. Extracts from mock-infected and infected cells were incubated in the presence of 0.9 mM Mn^{2+} or 10 mM Mg^{2+} with or without $(Ap)_5A_{OH}$ as primer (47). RNA was synthesized with [^{3}H]ATP in the presence and absence of the other three precursors. The RNA was then deproteinized with phenol-chloroform, heat denatured, and digested with RNase T1. The presence of poly(A) was determined by passing the digests through poly(U) filters. The results are shown in Table 4 and can be summarized as follows. First, the crude RNA polymerase is less active with Mn^{2+} than it is with Mg^{2+} (Table 4). The addition of $(Ap)_5A_{OH}$ to the Mn^{2+}-containing reaction stimulates total RNA synthesis and, in particular, poly(A) synthesis, indicating the presence of a terminal adenylate transferase. This is supported by the finding that, with [^{3}H]ATP as the sole substrate and Mn^{2+}, only poly(A) is synthesized. The nature of the primer in this reaction has not been determined, but it might be preexisting, short RNA molecules in the crude replication complex. Second (Table 4), a terminal adenylate transferase activity can also be detected in extracts from uninfected cells. This activity is prominent in the presence of manganese ions and is stimulated greatly by $(Ap)_5A_{OH}$. However, not all acid-precipitable material synthesized with Mn^{2+} in the absence of the primer was bound to poly(U) filters after RNase treatment, the reason for which is unknown. When the divalent cation is magnesium, little of the total counts incorporated are resistant to RNase T1 and bind to poly(U) filters. The bulk of the acid-insoluble material synthesized with Mg^{2+} represents, presumably, 4S RNA (Fig. 6C and D).

The terminal adenylate transferase activity, which we have found in the crude replication complex, is likely to be of cellular origin. Consequently, this activity could be considered simply a contamination of the crude replication complex. Alternatively, it could be involved in the poly(A) synthesis of poliovirus-specific

TABLE 4. *Effect of manganese ions and a primer on RNA synthesis*[a]

Substrate mixture	Extract from polio-infected cells				Extract from uninfected cells			
	Mg^{2+}		Mn^{2+}		Mg^{2+}		Mn^{2+}	
	Total counts/min	% Bound to poly(U) filters[b]	Total counts/min	% Bound to poly(U) filters[b]	Total counts/min	% Bound to poly(U) filters[b]	Total counts/min	% Bound to poly(U) filters[b]
[^{3}H]ATP, GTP, CTP, UTP	17,015	21.6	8,088	34.6	4,197	7.3	4,311	13.1
[^{3}H]ATP, GTP, CTP, UTP, $(AP)_5A_{OH}$	ND[c]	ND	9,807	65.9	ND	ND	5,293	84.2
[^{3}H]ATP	3,557	67.5	7,079	100	3,079	9.11	6,600	48.8
[^{3}H]ATP, $(Ap)_5A_{OH}$	ND	ND	7,838	100	ND	ND	8,259	100

[a] The crude replication complex preparation from poliovirus-infected and mock-infected cells was incubated in the presence of 0.9 mM Mn^{2+} or 10 mM Mg^{2+} with or without $(Ap)_5A_{OH}$ (12 μM). RNA was labeled in vitro with [^{3}H]AMP in the presence and absence of the other three nucleoside triphosphates. Total counts were determined by measuring the DEAE-paper binding radioactivity from duplicate 20-μl samples after 60 min at 37 C. RNA was then isolated from the reaction mixtures (240 μl) by deproteinization with phenol-chloroform followed by ethanol precipitation. The ethanol precipitate was dissolved in water. Acid-precipitable counts were determined from a portion of each RNA sample. Another portion was heat denatured in the presence of cold poly(A), digested with RNase T1, and filtered through poly(U) filters, and the dried filters were counted in a liquid scintillation counter. Acid-precipitable counts were taken as 100% for the determination of poly(U)-filter binding.

[b] After heat denaturation and digestion with RNase T1.

[c] ND, Not determined.

RNAs. To answer this question we have attempted to separate the poly(A) polymerase from the replication complex.

Fractionation of the crude replication complex on a sucrose gradient. The crude replication complex was subjected to density equilibrium centrifugation in a discontinuous sucrose gradient (16). The smooth cytoplasmic fraction, which was visible as a slightly turbid band (for details, see above), was isolated, and, in agreement with the findings of Caliguiri and Tamm (16), it was found to contain polio RNA polymerase activity. The partially purified polio RNA polymerase can be characterized as follows. The rate of incorporation of [^{3}H]AMP is constant for 20 min and thereafter decreases rapidly (data not shown). The specific activity of the complex is 5- to 15-fold higher than that of the crude enzyme, depending on the enzyme preparation. ^{3}H-labeled RNA synthesized for 30 min at 37 C by the partially purified replication complex was deproteinized and analyzed by zone sedimentation in a 15 to 30% sucrose gradient. Figure 7 shows that, relative to double-stranded RNA species, less 35*S* RNA was synthesized. SS RNA, RI, and RF were purified by LiCl precipitation and gel filtration, and their properties were verified by electrophoresis on 1.8% polyacrylamide-0.5% agarose gels (data not shown). Poly(A) was then isolated from each RNA species by the procedures described in the previous sections. As determined by poly(U)-filter binding, the content of poly(A) in RNAs synthesized by the partially purified replication complex is slightly higher than that of RNAs synthesized by the crude replication complex (Table 3). Unexpectedly, we have found, however, that the in vitro poly(A) of RNA from the partially purified replication complex is longer and more heterogeneous than previously observed (Fig. 8). We estimate that SS-poly(A) has an average chain length of 200 nucleotides (Fig. 8A) whereas a proportion of the RI-poly(A) barely enters the gel (Fig. 8B). Considering the poly(A) content (Table 3), these data might indicate that the RNAs were labeled internally to a larger extent than those synthesized by the crude complex. Alternatively, not all RNA strands may be terminated with poly(A).

Omission of GTP, CTP, and UTP from the incubation mixture of the partially purified replication complex results in a 95% decrease in [^{3}H]AMP incorporation. Material synthesized under these conditions can be considered to be

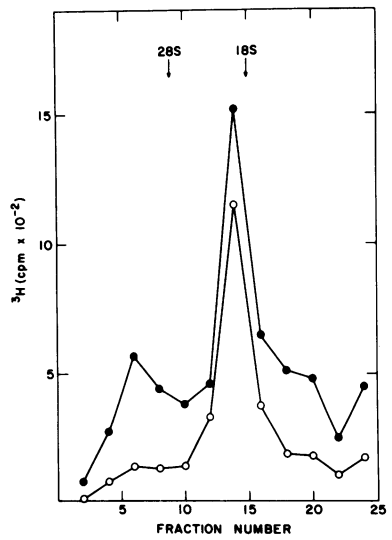

FIG. 7. *Velocity sedimentation of RNA synthesized by the partially purified replication complex. The membrane pellet from fraction 2 was dissolved in buffer and incubated for 30 min at 37 C with a complete incubation mixture. RNA was extracted with phenol-chloroform-SDS and centrifuged through a 15 to 30% sucrose gradient in NTE buffer. Fractions were analyzed for acid-insoluble radioactivity* (●) *and RNase resistance* (○).

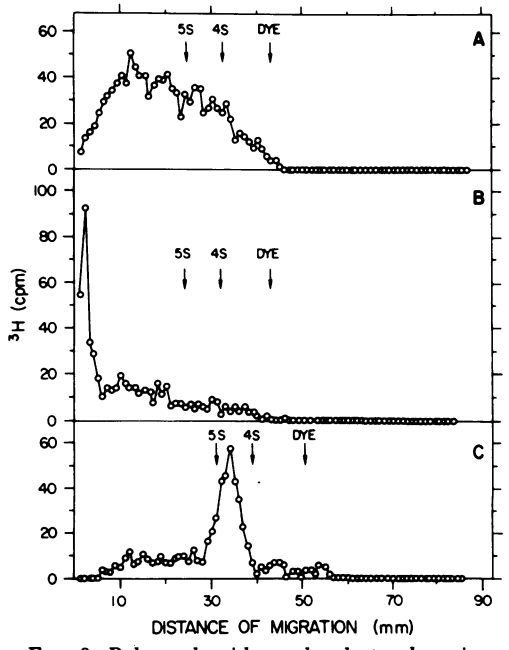

FIG. 8. *Polyacrylamide gel electrophoresis of poly(A) fragments from SS RNA (A), RI (B), and RF (C) synthesized in vitro by the partially purified replication complex. RI-poly(A) and RF-poly(A) were isolated by digestion with RNase T1 after heat denaturation and binding to poly(U) filters. SS-poly(A) was isolated by digestion with RNase A/T1 in 0.2 M NaCl and ethanol precipitation.*

poly(A) since it is resistant to RNase T1 and binds 100% to poly(U) filters (data not shown). We, therefore, tested the partially purified enzyme preparation for the presence of terminal adenylate transferase. The experiment was performed in the same way as described for the crude enzyme preparation, except that uninfected cells were not tested. The results are shown in Fig. 9. The partially purified polio polymerase is inhibited by Mn^{2+} in the same way as the crude enzyme. Oligo(A) also has an inhibitory effect on the polio polymerase. On the other hand, the poly(A) polymerizing activity seen in the presence of Mg^{2+} and ATP as the only substrate is stimulated approximately threefold when Mg^{2+} is replaced by Mn^{2+}. Addition of $(Ap)_5A$ to the Mn^{2+}-dependent reaction results in a sevenfold stimulation. All of the material synthesized in reaction mixtures with ATP as the only substrate binds to poly(U) filters (data not shown). Repeated suspension of the smooth microsomal fraction in suspension buffer and sedimentation by centrifugation did not lower the ATP polymerizing activity. These data indicate that terminal adenylate transferase activity in poliovirus-infected HeLa cells might be membrane bound. However, these experiments did not answer the question of whether terminal adenylate transferase is involved in viral RNA replication. In an attempt to solve this problem, we treated the replication complex with detergent, subjected it to density gradient centrifugation, and analyzed the products synthesized by it.

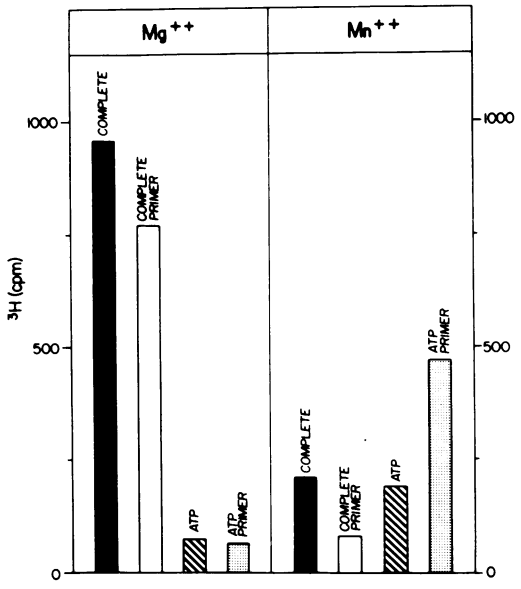

FIG. 9. *Effect of Mn^{2+} and a primer on RNA synthesis by the partially purified replication complex. [^{3}H]AMP was incorporated into RNA by the partially purified replication complex in the presence or absence of the other three nucleoside triphosphates and/or the primer $(Ap)_5A_{OH}$ (final concentration 12 μM). The divalent cations $Mg(Ac)_2$ or $MnCl_2$ were added to the reaction mixtures at concentrations of 10 mM and 1 mM, respectively. DEAE-paper binding radioactivity was determined after 15 min at 37 C.*

Poly(A) synthesis by the solubilized replication complex. To dissociate the poliovirus-specific replication complex from membranous material, we treated the smooth microsomal fraction (see above) with deoxycholate (final concentration 0.5%) for 10 min at 0 C, and we subjected the mixture subsequently to sucrose density gradient centrifugation. As has been shown by Caliguiri (13), polio RNA polymerizing activity separates under these conditions into two major fractions: (i) a smaller complex sedimenting at 70S (and synthesizing predominantly RF) and (ii) a larger complex sedimenting between 75 and 260S with a peak at approximately 150S. The larger, solubilized replication complex synthesizes RI, RF, and SS RNA in vitro (13). We have only tested the solubilized 150S replication complex for poliovirus-specific RNA synthesis and terminal riboadenylate transferase activity. The results shown in Fig. 10 indicate distinct differences from the partially purified replication complex (Fig. 9). First, RNA synthesis by the 150S replication complex requires Mg^{2+} whereas Mn^{2+} does not activate polymerization. It is interesting to note that $(Ap)_5A_{OH}$ has no inhibitory effect on polio-specific RNA synthesis. Second, no AMP is incorporated into acid-insoluble material when the complex is incubated with [^{3}H]ATP alone in the presence of Mg^{2+} or Mn^{2+}, with or without the $(Ap)_5A_{OH}$ primer. This clearly shows that the 150S replication complex is free from terminal riboadenylate transferase activity. A search for this activity in other fractions of the sucrose gradient has not been carried out in this study.

RNA synthesized by the 150S replication complex was analyzed by zone sedimentation through sucrose density gradients after deproteinization with phenol-chloroform (Fig. 11). The sedimentation profile of in vitro-synthesized RNA is similar to that obtained from RNA labeled by the partially purified, membrane-bound replication complex (Fig. 7). Because of a lack of sufficient material, all fractions containing viral RNA (fractions 4 to 22) were combined, and the RNA was precipitated with ethanol. The RNA was then heat denatured and digested with RNase T1, and the digest was filtered through poly(U) filters.

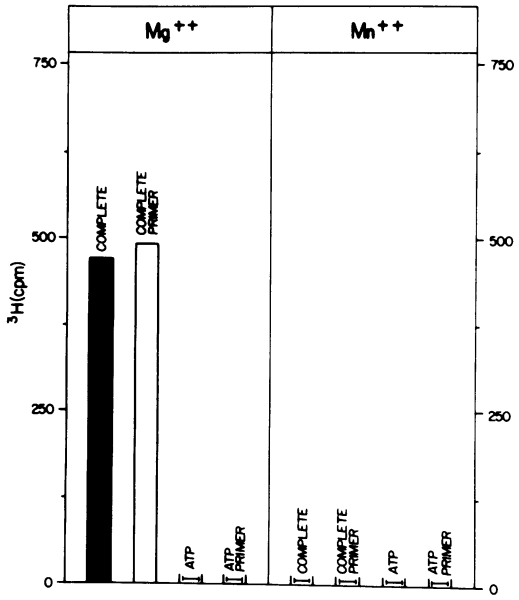

FIG. 10. *Effect of Mn^{2+} and a primer on RNA synthesis by the solubilized replication complex. The pelleted, smooth microsomal fraction was suspended in RSB and divided. One-half was made 1 mM in $MgCl_2$ (sample 1); the other half was made 1 mM in $MnCl_2$ (sample 2). Deoxycholate was added to a concentration of 0.5%. After incubation for 10 min at 0 C, both samples were centrifuged through a 15 to 30% sucrose gradient in RSB containing 1 mM $MgCl_2$ (sample 1) or 1 mM $MnCl_2$ (sample 2). ^{32}P-labeled poliovirus was run on a third gradient as marker. After centrifugation for 40 min at 58,000 rpm in a Spinco SW65 rotor, the solubilized replication complexes were isolated as described in the text. The complexes were assayed for poliovirus-specific RNA synthesis and terminal riboadenylate transferase activity as in Fig. 9.*

Of the [^{3}H]AMP labeled digest 10.9% was found to bind to poly(U) filters. The bound material was confirmed to be poly(A) by the following procedure: it was eluted from the poly(U) filter, digested again with RNase T1 and RNase A in 0.2 M NaCl, and subsequently chromatographed on a column of DEAE-Sephadex in 7 M urea in the presence of a tRNA marker. Figure 12 shows that heterogeneous poly(A) elutes after tRNA. We estimate that the average chain length of the poly(A), by comparison with other elution profiles (50, 51), ranges from below 100 to above 200 adenylate residues with very long poly(A) molecules at the trailing edge of the peak. Gel electrophoresis of this material was not carried out.

Our data show that poly(A)-linked poliovirus RNA is being synthesized in the absence of detectable terminal adenylate transferase activity.

DISCUSSION

The complex that we have used to study viral RNA replication in vitro had previously been shown to reflect replication in vivo in that it synthesizes all three classes of viral RNA, the RI being the precursor for SS RNA (20, 27). All three classes of viral RNA, which are labeled in vitro (Fig. 3), contain poly(A) covalently linked

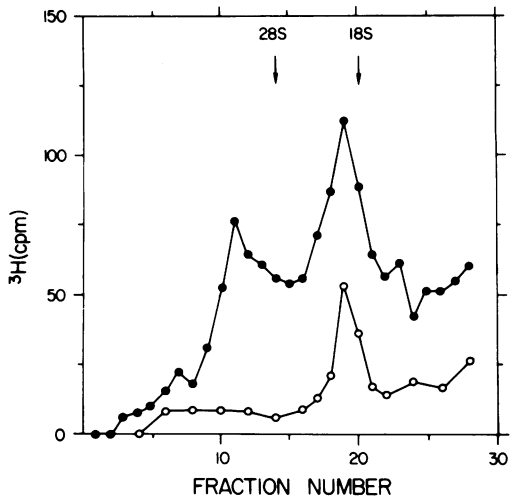

FIG. 11. *Velocity sedimentation of RNA labeled in vitro with [^{3}H]AMP by the solubilized replication complex. The complete reaction mixture was incubated for 30 min at 36 C. RNA was isolated by extraction with phenol-chloroform and precipitated with ethanol. The ethanol precipitate was sedimented through a 15 to 30% sucrose gradient in NTE buffer and assayed for acid-precipitable (●) and RNase A-resistant (○) radioactivity.*

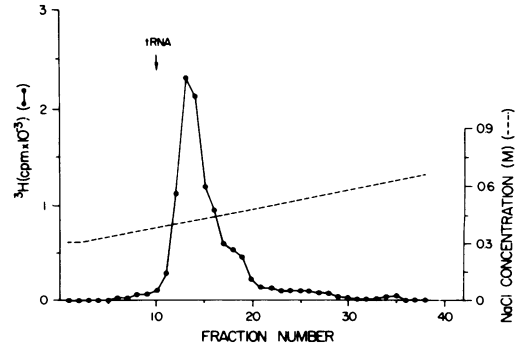

FIG. 12. *Ion-exchange chromatography of poly(A) of polio-specific RNA synthesized by the detergent-treated replication complex. Fractions from the sucrose gradient in Fig. 11 containing virus-specific RNA (fractions 4 to 22) were collected and precipitated with ethanol. Poly(A) was isolated as described in the text and analyzed on a DEAE-Sephadex column in 7 M urea. The arrow indicates the position where tRNA elutes.*

to the nucleotide chain (Fig. 4). Its position was determined to be 3'-terminal in SS RNA, as is the case for virion RNA (50). The size of the RNA-linked poly(A) increases after the replication complex has been partially purified (Fig. 8). RI-poly(A) and SS-poly(A) are, in fact, much larger than that of in vivo-synthesized RI or SS RNA isolated 4 h after infection (52). It is surprising that poly(A) can be recovered at all from RI because the structure of RI during RNA synthesis has been proposed to be essentially single stranded (33), in which case the content of poly(A) should not exceed 0.4% (52). The extremely long poly(A) recovered from in vitro RI argues against an artifact of the isolation procedure, namely that poly(A) containing plus strands aggregate with minus strands during deproteinization. A similar argument has been presented previously for in vivo RF (51).

Both the crude replication complex and the partially purified replication complex contain an activity that is capable of synthesizing poly(A) in the presence of only ATP. This activity was found also in extracts of mock-infected cells. It is stimulated when Mn^{2+} is substituted for Mg^{2+} and an oligo(A) primer is added (Table 4 and Fig. 9) and is, therefore, similar or identical to the terminal adenylate transferase purified by Tsiapalis et al. (47). In our experiments, the enzyme was found in the smooth membrane fraction from which it could not be removed by repeated sedimentation. The terminal adenylate transferase present in our crude or partially purified replication complex might, therefore, be membrane bound. The enzyme, however, is with great likelihood not involved in viral RNA replication because no free poly(A) is synthesized by the solubilized replication complex (Fig. 10) although the complex synthesizes RNA-linked poly(A) (Fig. 12). These data exclude a mechanism of poly(A) synthesis in poliovirus RNA by which preformed poly(A) is linked to the viral RNA. The absence of terminal adenylate transferase in the replication complex, together with the presence of poly(U) in minus strands, strongly suggests that poly(A) in poliovirus RNA is genetically coded.

Therefore, we propose that, after its translation, the infecting viral RNA is transcribed yielding a minus strand with 5'-terminal poly(U) (49). Subsequently, progeny plus-strand RNA is synthesized, the 3'-terminal poly(A) of which being transcribed from poly(U) prior to the release from the RI. Heterogeneity in size and variation of the average chain length of poly(A) in viral RNA synthesized in vivo (50, 51, 52) or in vitro might be explained by premature chain termination yielding short poly(A) (Fig. 4) or elongation by a mechanism of repeated slippage (translational motion of the transcript along the template; see references 17, 34, 38) yielding very long poly(A) segments (Fig. 8 and 12).

It has recently been shown that the partial removal of poly(A) from poliovirus RNA results in a decrease of the specific infectivity of the viral RNA (44). A possible reason for this finding might be that the poliovirus-specific replicase may require a sequence of adenylic acid residues longer than 25 nucleotides to initiate the transcription of poly(U) in minus strands.

Transcription of poly(A) is consistent with the finding that cordycepin does not inhibit polio RNA synthesis in HeLa cells at a concentration (25 to 30 μg/ml) at which it interferes with cellular mRNA synthesis (36). We have found, however, that cordycepin at higher concentrations (60 μg/ml) decreases the rate of RNA synthesis and virion formation although the viral RNA synthesized contains poly(A) and the virions produced are infectious (K. Dorsch-Häsler, Y. Yogo, R. A. Miller, and E. Wimmer, manuscript in preparation). Interestingly, cordycepin 5'-triphosphate completely and irreversibly inhibits polio RNA synthesis in vitro. This is not due to interferences with RNA-linked poly(A) synthesis but to chain termination (Dorsch-Häsler et al., in preparation).

mRNA's of DNA viruses, whether synthesized in the nucleus or cytoplasm of the infected cell, are polyadenylated after transcription, presumably by enzymes distinct from the transcriptase (11). Similarly, all mRNA's of minus-strand RNA viruses are polyadenylated after transcription (40) although evidence obtained with vesicular stomatitis virus indicates that end addition of poly(A) may be carried out by the virion-associated transcriptase (8). It is noteworthy that genome size plus strands of vesicular stomatitis virus, which can be isolated from infected cells in the form of a ribonucleoprotein, also contain poly(A) (43). These 40S plus strands, which are not translated, function as template in viral genome synthesis, yet their poly(A) is not transcribed into poly(U) during the process. Thus, the replicase of vesicular stomatitis virus appears to ignore the poly(A) of its template RNA, in contrast to the replicase of poliovirus.

It is likely that the unique mechanism of poly(A) synthesis by transcription observed in poliovirus replication applies to all picornaviruses.

After this manuscript was completed, a publication by Spector and Baltimore (45) appeared in which the addition of poly(A) to poliovirus

RNAs in vitro is described. Spector and Baltimore (45) carried out their experiments exclusively with a crude replication complex; in this regard, their results are very similar to ours.

ACKNOWLEDGMENTS

We thank Ruth Ann Miller and Maureen V. McLeod for excellent technical assistance and Barbara Morgan Detjen for suggestions in writing the manuscript. We are grateful to Robert G. Roeder, Sanford R. Simon, and Akio Nomoto for discussions. The supply of stocks of S3 HeLa cells by Elvera Ehrenfeld and David Baltimore is appreciated.

This work was supported by Public Health Service grant CA-14151 from the National Cancer Institute.

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