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Poliovirus Infection and Poly(A) Sequences of Cytoplasmic Cellular RNA

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The influence of polio infection on Poly(A) sequences of cellular cytoplasmic RNA was investigated. In the presence of guanidine, cellular protein synthesis was still shut off after poliovirus infection, although there was no viral RNA synthesis. The Poly(A) of these cells was unchanged with respect to quantity, size, and linkage to cellular cytoplasmic RNA. This finding strongly suggests that the shut off of cellular protein synthesis is not caused by a change of the Poly(A) sequences of cellular mRNA.

The infection of HeLa S3 cells with poliovirus causes a rapid disappearance of cellular protein synthesis (16) with concurrent loss of cellular polyribosomes (19). This also occurs in the presence of guanidine, which inhibits viral RNA synthesis (11). These conditions are therefore well suited for the study of a possible influence of viral infection on already present cellular RNA, in particular mRNA. Most mRNA species in animal cells carry a polyadenylic acid-rich sequence [Poly(A)] on their 3' end (2, 4, 5, 7, 9), various functions of which are being discussed (6, 14, 17). Recent experiments with sea urchin eggs have indicated that Poly(A) may play an important role in translation (20). It has also been shown that in HeLa S3 cells these Poly(A) sequences are successively degraded with increasing age of mRNA molecules (17). The present work examines whether infection of HeLa S3 cells with poliovirus leads to a more rapid degradation of Poly(A) sequences which in turn might explain the observed effect on cellular protein synthesis.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells (Flow Lab., Irvine, Scotland) were grown in suspension culture as described (1). Poliovirus type 1 Mahoney was isolated and purified from lysates of infected HeLa cells (1). The virus titer was determined by the plaque test (3).

Labeling of cellular RNA. HeLa cells (2×10^8) were stirred for 30 min at 37 C in 30 ml of spinner medium supplemented with 5% calf serum (CS) and actinomycin D (0.05 μg/ml; a gift from Bayer AG., Wuppertal-Elberfeld). They were then labeled with either [^{3}H]adenosine (Amersham-Buchler, specific activity 11.8 Ci/mM, 200 μCi) or [^{3}H]uridine (Amersham-Buchler, specific activity 2 Ci/mM, 200 μCi). After 2 h of labeling, the cells were harvested by centrifugation and unincorporated label was removed by resuspension of the cells in 50 ml of warm (37 C) medium and centrifugation ($400 \times g$, 37 C). Each pellet was resuspended in 20 ml of spinner solution without CS. As indicated in Fig. 1, one-half of each culture (10 ml) was infected with 500 PFU/cell in the presence of 2 mM guanidine and actinomycin D (5 μg/ml). The uninfected half of the two cultures was used as a control. After 45 min, 40 ml of spinner medium with CS, guanidine (2 mM), and actinomycin D (5 μg/ml) was added to each culture. This concentration of actinomycin D prevents incorporation into RNA of nucleoside and nucleotide precursors from the cellular pool. Cultures were then incubated for 4 h. Under these conditions, cellular protein synthesis came to a halt after 2.5 to 3 h (Fig. 2).

Cell fractionation. Cells were harvested for 4 h postinoculation, washed four times with ice-cold phosphate-buffered saline, allowed to swell in 2 ml of hypotonic buffer A (50 mM Tris-hydrochloride pH 7.6, 50 mM KCl, 1 mM $MgCl_2$) (9) for 10 min, and broken with 10 strokes in a Dounce homogenizer. Nuclei and large cellular debris were removed by centrifugation (10 min, $1,000 \times g$, 4 C).

Extraction of RNA-containing Poly(A) sequences. The cytoplasmic fraction was extracted at pH 9 with sodium dodecyl sulfate (SDS)-phenol as described by Lee et al. (9). Traces of phenol were removed with ether. RNA was precipitated with ethanol at −20 C, the precipitate was collected by centrifugation (30 min at $2,000 \times g$, 4 C), and the RNA pellet was dissolved in 0.6 ml of distilled water. All solutions used for the isolation and further characterization of the RNA had been freed of RNase activity by the addition of diethylpyrocarbonate (Baycovin, Bayer AG., Leverkusen) and were subsequently sterilized.

Kinetics of RNA degradation. The mixture contained 10 μliters of RNA extract (38 μg of RNA), 20 μg of pancreatic RNase (20 μliters), 120 Kunitz units of T1 RNase (10 μliters), and 0.5 ml of buffer B (0.01 M Tris-hydrochloride, 7.5; 0.2 M KCl) at 37 C. After various incubation periods, 100 μg of bovine serum albumin was added, and the reaction was stopped with 0.5 ml of trichloroacetic acid (10% solution). The

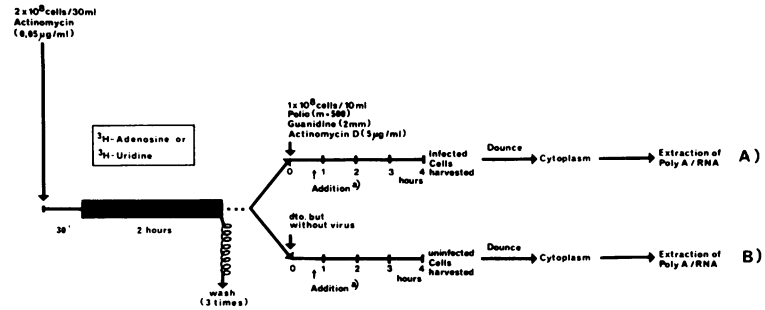

FIG. 1. *Scheme of basic protocol followed in the experiments. Addition (a): 40 ml of medium with serum, actinomycin D (5 µg/ml), and guanidine (2 mM).*

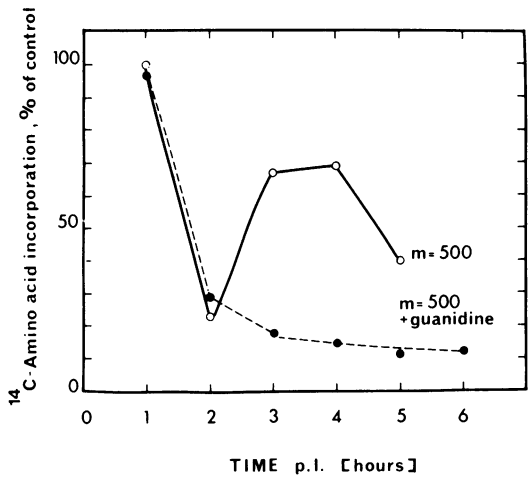

FIG. 2. *The shut off of the cellular protein synthesis by poliovirus infection. Symbols: O——O, without guanidine multiplicity of infection 500 PFU; O-----O, with guanidine (2 mM), multiplicity of infection 500 PFU. Each set of the experiment contained 6 × 10⁷ cells in 50 ml of spinner medium with actinomycin D (5 µg/ml). Samples of 2.4 × 10⁶ cells were withdrawn and incubated for 10 min with 1 ml of spinner medium containing 2 µCi of [¹⁴C]amino acid mixture (54 mCi/matom C). The pulse was terminated by the addition of cold PBS and immediate centrifugation in the cold. The cells were resuspended and washed three times in PBS. The cell pellet was precipitated with 3 ml of 5% trichloroacetic acid and collected on membrane filters. After drying, the radioactivity was determined. 100% = 9.900 counts/min.*

precipitated radioactivity was collected on a membrane filter (Sartorius, Göttingen) and washed with 5% trichloroacetic acid solution. Dried filters were placed in a counting vial containing a mixture of 1,4-bis - (5 - phenyloxazolyl)benzene - 2,5 - diphenyloxazole-toluene and the samples were counted in a Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Nitrocellulose filter binding test. This test was performed by the method of Lee et al. (9), with 10 µliters of RNA solution in 1 ml of binding buffer. Samples to be RNase treated were incubated for 15 min at 37 C in the following incubation mixture: 10 µliters of RNA (19 µg), 10 µliters of double-concentrated buffer B, 1 µg of pancreatic RNase, and 3 Kunitz units of T1 RNase. After 15 min of incubation at 37 C, 1 ml of binding buffer was added and the samples were treated as described by Lee et al. (9). Sedimentation and electrophoresis on polyacrylamide gels were performed as described in the figure legends.

RESULTS

(i) Degradation with T1 RNase and pancreatic RNase. At high ionic strength (0.2 M KCl), the [³H]adenosine-labeled Poly(A) piece of the mRNA has been shown to be RNase resistant (8), whereas the [³H]uridine label is degraded by the enzyme and becomes acid soluble. The kinetics of the degradation are depicted in Fig. 3. [³H]uridine-labeled RNA was completely degraded within five min, whereas only 85% of the [³H]adenosine-labeled RNA was

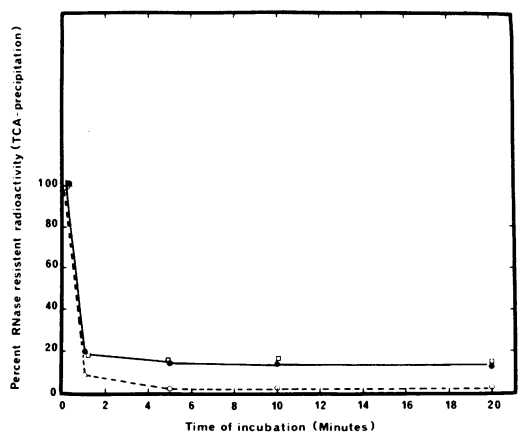

FIG. 3. *Kinetics of RNase degradation of extracted RNA. [³H]adenosine labeled, from infected cells (□), 100% = 6,911 counts/min. [³H]adenosine labeled, from uninfected cells (●), 100% = 5,978 counts/min. [³H]uridine labeled, from infected cells (○), 100% = 3,644 counts/min.*

degraded, the remaining 15% being resistant to further degradation by both pancreatic and T1 RNase. Uninfected and infected cells were found to contain the same amount of RNase-resistant Poly(A) sequences. Identical results were obtained after sucrose density centrifugation (Fig. 6) of such RNA preparations, when individual fractions were analyzed for their Poly(A) content based on RNase resistance (Table 1).

TABLE 1. *Resistance to RNase of cytoplasmic RNA from infected and uninfected HeLa S3 cells*

Sample of RNA, label	Trichloroacetic acid-precipitable radioactivity counts/min		Resistance (%)
	Without RNase	With RNase	
[^{3}H]adenosine, infected cells[a]	18,791	2,645	14
[^{3}H]adenosine, uninfected cells	24,325	2,679	11
[^{3}H]uridine, infected cells[b]	1,715	35	2
[^{3}H]uridine, uninfected cells	1,444	39	3

[a] Gradients, see Fig. 5.
[b] Gradients not shown; for test see Material and Methods.

(ii) Binding to nitrocellulose filters. As previously demonstrated by Lee et al. (9), Poly(A) containing RNA characteristically binds to nitrocellulose filters at high salt concentrations. This binding test system was used to analyze the total RNA before and after RNase treatment. With untreated RNA, no difference was observed for binding of [^{3}H]adenosine-labeled and [^{3}H]uridine-labeled material from both infected and uninfected cells. After RNase treatment, however, only the [^{3}H]adenosine label was bound, and here again, no difference was observed in infected and uninfected cells (Table 2). Again, this experiment clearly demonstrated that RNA from infected and uninfected cells contained the same amounts of Poly(A).

TABLE 2. *Binding to nitrocellulose filters of cytoplasmic RNA from infected and uninfected cells*

Sample of RNA, label	Binding to membrane filter (counts/min)	
	Without RNase	With RNase
[^{3}H]adenosine, infected cells	3,580	1,271
[^{3}H]adenosine, uninfected cells	3,650	1,021
[^{3}H]uridine, infected cells	209	12
[^{3}H]uridine, uninfected cells	178	1

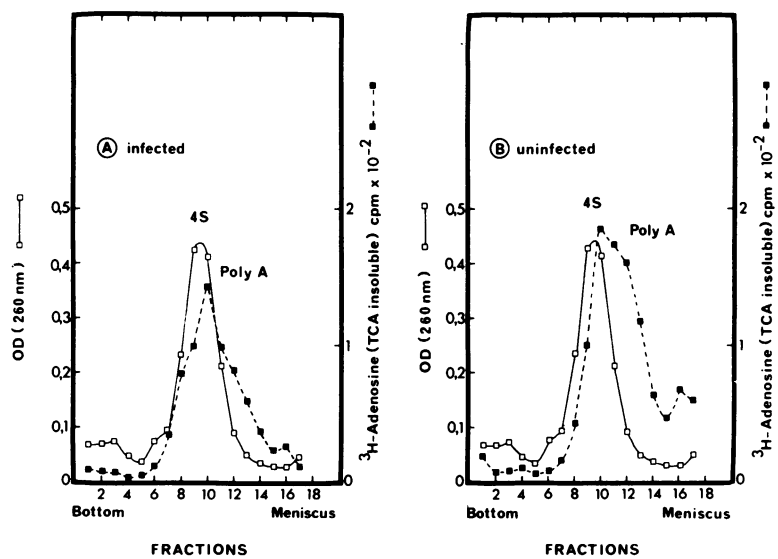

FIG. 4. *Sedimentation of RNase-resistent Poly(A) segments on a sucrose gradient. After RNase treatment, samples were extracted again with phenol-SDS at pH 9.0 (9) and the extract was layered on a sucrose gradient (5 to 20% [wt/vol] in 0.01 M Tris-hydrochloride pH 7.5, 0.2 M KCl). tRNA from E. coli (25 μg) (Boehringer & Söhne, Germany) was added as a marker. Ultracentrifugation was performed in an SW50.1 rotor (Beckman) at 45,000 rpm, 15 C, for 9 h. Fractions (0.25 ml) were monitored at 260 nm and precipitated with trichloroacetic acid, and the radioactivity was counted.*

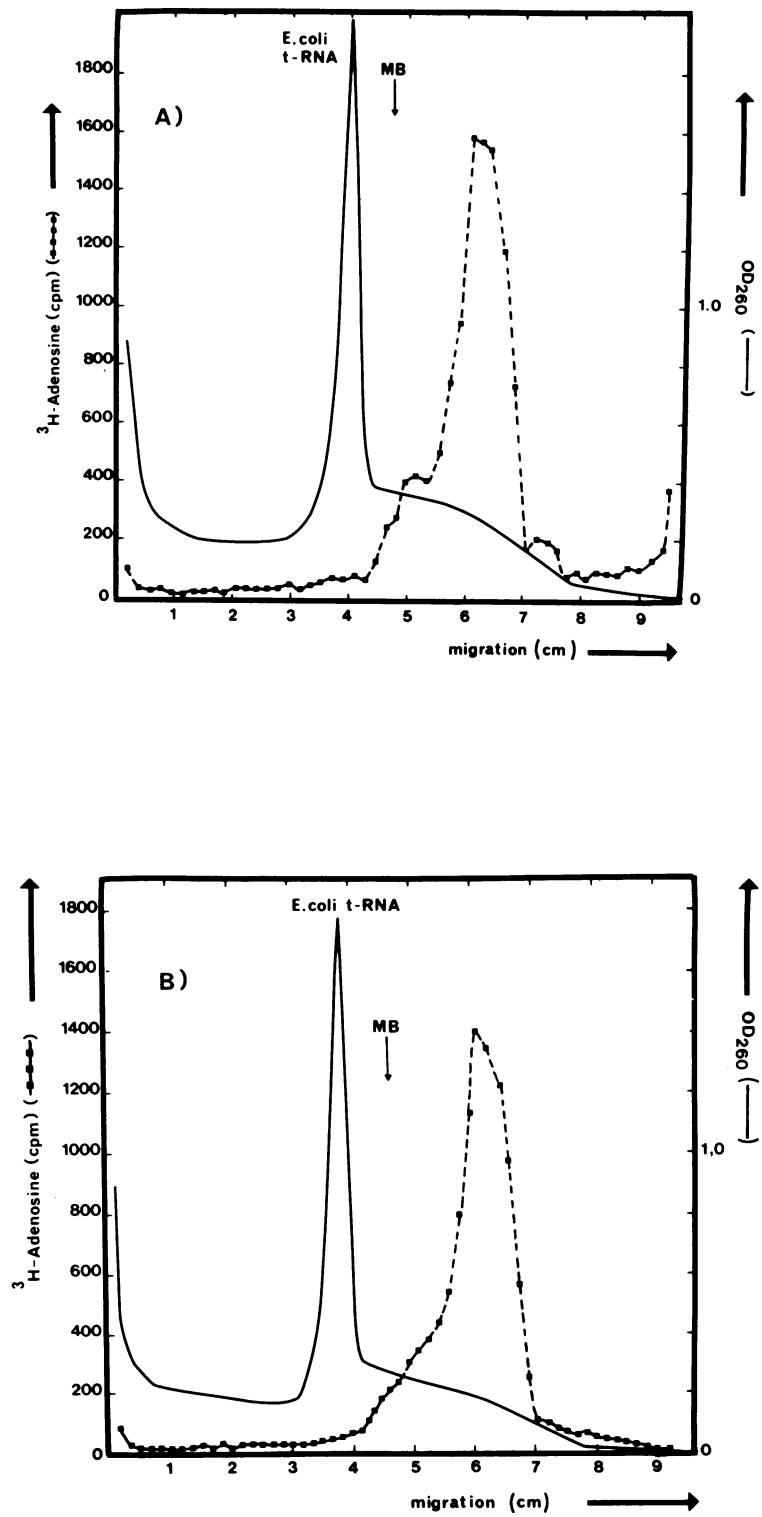

FIG. 5. *SDS-polyacrylamide gel electrophoresis of the RNase-resistent Poly(A) segments from (A) infected and (B) uninfected cells. Gels (9-cm length and 10% with respect to acrylamide concentration) were prepared by the method of Loening (10). Pre-electrophoresis was carried out at 7 mA/gel for 1.5 h. The buffer (pH 7.6) used contained per liter: 0.036 M Tris, 0.03 M $NaH_2PO_4 \cdot H_2O$, 0.01 M EDTA disodium salt, and 0.2% SDS. RNase-treated RNA samples were applied, with tRNA from E. coli (5 μg) and methylene blue was added as marker. Electrophoresis was performed at 5 mA/gel for 2 h. Gels were subsequently scanned at 260 nm in a disc gel scanning device in combination with a PMQ-II spectrophotometer (Zeiss, Oberkochen, Germany), and then sliced into disks of 1.6-mm thickness. After solubilization in 0.5 ml of a 10% aqueous solution of piperidine for 2 h at 65 C, Bray solution was added and ^3H radioactivity was counted in a liquid scintillation counter.*

(iii) Sedimentation behavior of Poly(A) segments. Studies by numerous authors have shown that the RNase-resistant Poly(A) piece from cellular and viral mRNA sediments as a 4*S* molecule in sucrose gradient centrifugation (2, 5, 7-9, 14). When the sedimentation behavior of RNase-resistant Poly(A) sequences was compared to that of *Escherichia coli* tRNA, no differences could be detected between Poly(A) sequences from infected and uninfected cells (Fig. 4). In both cases the Poly(A)-rich segment had a sedimentation coefficient slightly less than 4*S*.

(iv) SDS-polyacrylamide gel electrophoresis of Poly(A) segments. When Poly(A) segments were subjected to co-electrophoresis with tRNA from *E. coli* and methylene blue as a marker through 10% acrylamide gels as described by Loening (10), we obtained the results shown in Fig. 5. [^{3}H]adenosine-labeled Poly(A) migrated slightly faster than *E. coli* tRNA and methylene blue. Again, no difference was seen between preparations from infected and uninfected cells. This finding further strengthens the notion that Poly(A) sequences from the two sources are identical in size.

(v) Zonal centrifugation of the cytoplasmic RNA and Poly(A) distribution. Although the integral length of Poly(A) has been shown to be conserved in infected cells, the possibility remains that Poly(A) is no longer bound to mRNA. Considering the results obtained from the filter binding test performed with total RNA of the cytoplasm, this notion seems to be unlikely.

Direct evidence comes from the distribution

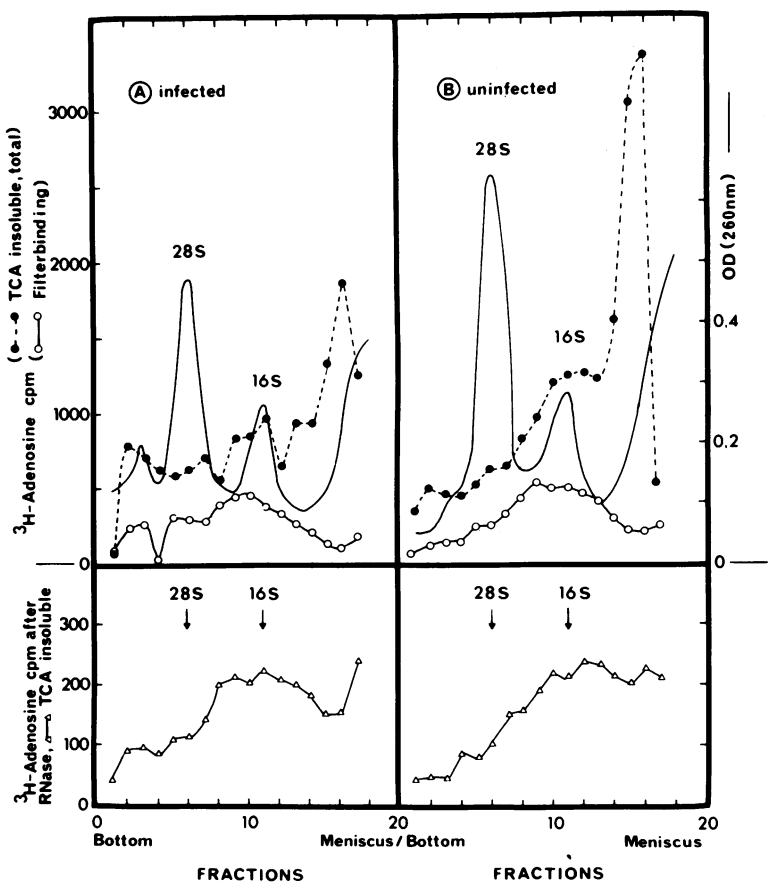

FIG. 6. *Sedimentation of [^{3}H]adenosine-labeled cellular RNA extracts on a sucrose gradient. The RNA extract was layered on a sucrose gradient (5 to 20% [wt/vol]) in buffer A with 0.5 ml of 2 M sucrose cushion. Centrifugation was carried out in an SW50.1 rotor at 45,000 rpm for 150 min at 4 C. Absorbance of fractions was measured at 260 nm. Each fraction (20 μliters) was precipitated with trichloroacetic acid after addition of 100 μg of bovine serum albumin (●-----●). In another 0.1-ml amount, RNase resistance was examined (△——△). A third sample of each fraction (0.1 ml) was subjected to the filter binding test (○——○). For convenience, values obtained with trichloroacetic acid precipitation were corrected to 0.1-ml volumes.*

of [^{3}H]adenosine-labeled Poly(A) when extracted RNA material from infected and uninfected cells is analyzed on a sucrose gradient (Fig. 6). Again no difference between the two preparations can be detected. This clearly demonstrates that the (Poly(A) segment is still bound to RNA after infection of HeLa S3 cells with poliovirus.

DISCUSSION

In the experiments described in this report, no detectable differences were observed between the Poly(A) sequences in cellular cytoplasmic RNA from uninfected and poliovirus-infected cells.

Both amount and size of these sequences are identical. Furthermore, the binding of Poly(A) to mRNA is the same in uninfected and infected cells. Thus, the 3′ end of cellular mRNA seems to be unimpaired after infection with poliovirus, although cellular protein synthesis is drastically inhibited. Similar results were shown by Soria and Huang (18) on mRNA of vesicular stomatitis virus after superinfection by poliovirus. In this situation, the vesicular stomatitis virus protein synthesis is inhibited without change in the Poly(A) sequences of the mRNA. The "shut-off" phenomenon therefore does not seem to be the result of rapid alteration of the Poly(A) piece after infection.

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