The stability of purified poliovirus RNA in cell-free translation systems prepared from HeLa cells or rabbit reticulocytes has been examined. Degradation of the RNA occurs with a $t_{1/2}$ of approximately 35 min at 30°C under conditions used for in vitro translation. Degradation is due in part to activity of the cell lysate, and in part to contaminants in the commercial preparations of creatine phosphokinase used in the energy-regenerating system. Addition of crude preparations of initiation factors significantly slows degradation, presumably as a result of protein-RNA interactions which confer resistance to nuclease action. Prior treatment of RNA with methylmercury hydroxide has no effect on degradation rates. On the other hand, endogenous mRNA, present as a messenger ribonucleoprotein particle in extracts from poliovirus-infected HeLa cells, remains completely intact during in vitro translation. These infected cell extracts synthesize the normal complement of viral proteins and utilize two different initiation sites for translation. Treatment of the infected cell extract with micrococal nuclease destroys the endogenous mRNA. Subsequent addition of exogenous RNA to the same extract results in the formation of a protein-associated RNA particle with sedimentation properties slightly different from the endogenous messenger ribonucleoprotein, and the added RNA is unstable. We conclude that two initiation sites can be utilized on intact poliovirus mRNA, and fragmentation of the RNA is not a prerequisite for generation of a second site in this RNA.

Numerous studies have been conducted in an effort to elucidate the mechanism by which eukaryotic ribosomes select appropriate sites on messenger RNA for initiation of protein synthesis. Several considerations have led to proposals such as the "scanning model" presented by Kozak (1), in which a 40 S ribosomal subunit (with associated initiation factors and initiator tRNA) binds initially at or near the 5' terminus of a mRNA and subsequently migrates until the first AUG codon is encountered. This type of mechanism is consistent with the observed monocistronic character of most eukaryotic mRNAs, and with the facilitating effect of the 5'-terminal m'G cap group, regardless of the distance between that cap and the initiator codon.

Several years ago, we reported the utilization of two initiation sites during translation of poliovirus RNA in vitro (2), and subsequent studies have extended these observations (3-5). Initiation at more than one site on a single species of mRNA has also been reported for tobacco rattle virus RNA-2 (6), tobacco necrosis virus (7), flavivirus 42 S RNA (8), carnation mottle virus (9), alfalfa mosaic virus RNA-3 (10), cowpea mosaic virus genome RNA (11), Semliki Forest virus genome RNA (12), and Southern bean mosaic virus (13).

A major difficulty in evaluating all of these reports is the possibility of cleavage or fragmentation of the mRNA in the cell extract during translation. It has been well documented that fragmentation of mRNA permits ribosome binding and opens new initiation sites at sequences which are apparently not available in the intact RNA (14-18) even after efforts to denature the RNA (19). In addition, incubation of mRNA in cell-free extracts has been reported to result in degradation (18). Thus, RNA fragmentation might conceivably precede and be required for initiation at multiple sites.

The above considerations prompted us to examine the stability of poliovirus RNA in cell extracts under conditions where translation is occurring at two initiation sites. We show here that exogenous polio RNA is indeed degraded in both HeLa cell and rabbit reticulocyte extracts. However, endogenous mRNA present in extracts from virus-infected cells remains intact and stable throughout an incubation in which translation is occurring from two initiation sites. The endogenous mRNA persists as a ribonucleoprotein particle which has different properties from the protein-associated particle formed by exogenous RNA in the same extract.

**EXPERIMENTAL PROCEDURES**

**Cells and Virus**—The growth of suspension cultures of HeLa S3 cells and the purification of the Mahoney strain of poliovirus type 1 have been described previously (2).

**Preparation of RNAs for Translation**—Poliovirus RNA was extracted from purified virions as described previously (22). Radiolabeled preparations were obtained by incubation of poliovirus-infected cells (5 x 10⁶/ml) with 10 μCi/ml of [³H]uridine from 1½ to 6 h post-infection in the presence of 2 μg/ml of actinomycin D (gift of Merck, Sharp and Dohme). After purification and RNA extraction, yields of 100,000 cpm/μg of RNA were obtained. Vascular stomatitis virus mRNA was extracted from infected cells 4½ h post-infection, and was purified by chromatography on oligo(dT) cellulose (20). HeLa cell poly(A)-containing mRNA was prepared similarly from uninfected cells. All RNA samples were stored frozen at 1 mg/ml in sterile H₂O.

**Cell-free Translation**—Conditions for preparation of and translation in S10 extracts from HeLa cells and rabbit reticulocyte lysates have been described (21). When indicated, extracts were treated with 5 μg/ml of micrococcal nuclease for 10 min at 18°C prior to supplementation with mRNA (22). Crude initiation factors were prepared as described (21), and used to stimulate translation in most experiments. Translation products were labeled either with [³⁵S]methionine or with N-formyl [³⁵S]Met-RNA (23), prepared and utilized as described previously (4, 23). The labeled polypeptides were analyzed by
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Degradation of Polio RNA in Protein-synthesizing Systems—Translation of exogenous mRNAs in a variety of extracts prepared from rabbit reticulocytes, ascites tumor cells, or cultured mammalian cells has become a standard procedure for numerous types of studies in many laboratories. Micrococcal nuclease treatment of cell extracts is often utilized to eliminate endogenous protein synthesis prior to supplementation with the mRNA of interest (22). We have previously used such extracts, prepared from cultured HeLa cells or from rabbit reticulocytes, to study translation of poliovirus RNA in vitro, and we have shown accurate and efficient production of poliovirus proteins (21; see Fig. 3, below). Despite successful translation, analysis of the RNA during the translation reaction generally showed it to be fragmented and degraded to significant extents. Fig. 1 shows a velocity sedimentation analysis of radiolabeled poliovirus RNA added to a micrococcal nuclease-treated HeLa cell S10 extract under conditions used for translation. Prior to incubation in the translation reaction, the RNA was shown to be intact by analytical agarose gel electrophoresis in methylmercury hydroxide (not shown). After 15 min of translation, approximately 65% of the RNA remained as material sedimenting at 35 S, while increas-

![Image](https://example.com/fig1.png)

**Fig. 1.** Sedimentation analysis of polio RNA incubated in a HeLa cell-free translation reaction. Uninfected HeLa cell extracts were prepared and treated with micrococcal nuclease as described under "Experimental Procedures" for translation of polio RNA. Four μg of purified [3H]RNA were added to each 50 μl of translation reaction, and samples were incubated at 30°C for 0, 15, or 60 min. Reactions were stopped by dilution with SDS-containing buffer and analyzed by velocity sedimentation in sucrose gradients as described. Sedimentation was from right to left. Ribosomal RNA present in the cell extract served as absorbance markers at 28 and 18 S, and identified the peak of radioactivity as 35 S. Total radioactivity recovered from each gradient was 468,000 cpm-0 min incubation; 434,000 cpm-15 min incubation; 400,000 cpm-60 min incubation.

SDS-polyacrylamide gel electrophoresis, according to the method of Laemmlil (24), in gels containing either 10% acrylamide or linear gradients of 10-24% acrylamide. When necessary, gels were fluorographed in sodium salicylate (25) before exposure to x-ray film.

Analysis of RNA in Translation Reactions—Radiolabeled poliovirus RNA in cell-free translation reactions was analyzed after different times of incubation by dilution with the reaction mixture with 1% SDS, 0.1 M NaCl, 0.02 M EDTA, 0.01 M Tris-HCl, pH 7.4, and sedimentation through linear 15–30% (w/w) sucrose gradients in the Spinco SW 27 rotor. Fractions were collected through a Gilford recording spectrophotometer, and precipitated with 7% trichloroacetic acid. Precipitates were collected onto Whatman GF/F filters, washed with ethanol, dried, and analyzed for radioactivity by scintillation spectrometry.

Alternatively, samples were electrophoresed in composite agarose-polyacrylamide gels prepared by a method originally developed by M. Stewart, National Institutes of Health. Vertical slab gels (14 x 16 cm) consisted of 2% polyacrylamide, 0.05% bisacrylamide, 0.32% agarose (Seakem), and 1% sodium dodecyl sulfate in TSE buffer (0.01 M Tris-HCl, pH 8.3, 0.1 M NaCl, 0.002 EDTA). After addition of 0.025 ml of N,N,N',N'-tetramethylethylenediamine/50 μl of gel solution and 0.06% ammonium persulfate, the gel solution was poured on top of a prepolymerized plug consisting of 20% acrylamide, 1% SDS in TSE buffer. Electrophoresis was for about 8 h at 90 mA. The gel running buffer (TSE buffer, 0.3% SDS) was recirculated to maintain equal pH in the anode and cathode buffer chambers. After electrophoresis, the gels were fixed for 10 min in 10% acetic acid, transferred to 30% paper, and dehydrated with two changes of methanol. The gel thickness was reduced by evacuation for about 15 min. The gel was then soaked for 3 h in 20%, 2,5-diphenyloxazole in methanol and rinsed with water to precipitate the fluor. Gels were wrapped in plastic wrap and exposed to Kodak X Omat film at -70°C.

Electrophoresis under denaturing conditions was performed in agarose gels containing 5 mM methylmercury hydroxide as described (26).

The abbreviations used are: SDS, sodium dodecyl sulfate; mRNP, messenger ribonucleoprotein.

![Image](https://example.com/fig2.png)

**Fig. 2.** Degradation of polio RNA incubated with different components of the translation reaction. A, 2 μg of [3H]-labeled polio RNA were incubated for 0 or 60 min in 50 μl of sterile 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, containing 6.5 μg of creatine phosphokinase (CPKase). Samples were analyzed as described in the legend to Fig. 1. Total radioactivity recovered from each gradient was 251,000 cpm-plus initiation factor preparations; 197,000 cpm-without initiation factor preparations.
were analyzed by SDS-polyacrylamide gel electrophoresis as described. Initiation factor preparations have been reported previously, and these were utilized for translation conditions utilized for translation. The possibility was therefore considered that endogenous mRNA present in an infected cell extract might be a more stable template for protein synthesis by virtue of protein associations or other mechanisms occurring in vivo. Previous work with extracts from infected cells translating endogenous mRNA, had already shown that two initiation sites were utilized for viral protein synthesis (2–5).

Poly(A)-containing RNA (lanes a–d), vesicular stomatitis virus mRNA (lanes e–h) and poliovirus RNA (lanes i–l), treated with several concentrations of mercuric hydroxide. No significant effects on translation rates or products were observed. Translation in Extracts from Polio-infected Cells—A major rationale for these studies was to determine whether the two initiation sites utilized for translation of polio RNA were both available on intact RNA molecules, or whether utilization of a second site was secondary to fragmentation of the RNA. The above results stymied our efforts to answer this question, since RNA degradation was occurring under the conditions utilized for translation. The possibility was therefore considered that endogenous mRNA present in an infected cell extract might be a more stable template for protein synthesis by virtue of protein associations or other mechanisms occurring in vivo. Previous work with extracts from infected cells translating endogenous mRNA, had already shown that two initiation sites were utilized for viral protein synthesis (2–5). Accordingly, virus-infected cells were labeled with [3H]uridine for 3 h prior to harvesting and utilization for preparation of an S10. Fig. 4A shows the polypeptide products

**Fig. 3.** Effect of methylmercury hydroxide treatment of mRNA on translation in HeLa cell extracts. Polyacontaining HeLa cell mRNA, vesicular stomatitis virus (VSV) mRNA, and poliovirus RNA were prepared as described. Samples of each were incubated for 3 min at room temperature with 0, 2, 3, or 5 mM methylmercury hydroxide (MMH) prior to addition to translation reactions containing micrococcal nuclelease-treated HeLa cell extracts. After 60 min of incubation at 30°C, each sample was diluted with gel sample buffer and 20-μl samples were analyzed by SDS-polyacrylamide gel electrophoresis as described.

The degradation of exogenous RNA is due in part to activity in the lysate itself, and in part to nuclease activity apparently contaminating the commercial preparations of creatine phosphokinase used in the energy-regenerating system needed for translation. Fig. 2A shows a sucrose gradient analysis of polio RNA incubated for 60 min in the presence of 130 μg/ml of creatine phosphokinase alone, in the absence of cell lysate or other components of the translation reaction. Degradation is even greater than when the RNA is incubated in the complete reaction mixture (compare Fig. 2A with the 60-min time point in Fig. 1 or 2B). In addition, RNA degradation is significantly slowed by the addition to a standard reaction mixture of crude preparations of initiation factors. We routinely supplement translation reactions with initiation factors to stimulate translation (21). Fig. 2B shows a sucrose gradient profile of RNA incubated for 60 min in the same reaction mixture in the presence and absence of initiation factors; little detectable 35S RNA remained in the reaction without initiation factors after 60 min. Associations between mRNA and proteins in initiation factor preparations have been reported previously, and we have demonstrated an increase in the sedimentation coefficient of other mRNAs after incubation with initiation factors in a translation reaction (20).

Treatment of conalbumin mRNA with 2.5 mM methylmercury hydroxide has been reported to stimulate translation, presumably by denaturation of the RNA so as to promote more efficient ribosome binding (27). Since preliminary experiments had suggested that methylmercury hydroxide also inhibited RNase A-catalyzed hydrolysis of poly RNA under some conditions, we examined the effect of prior methylmercury treatment on the stability of poly RNA under translation conditions. No difference in the rate of RNA degradation was observed (not shown). Fig. 3 shows the translation products of three different mRNA populations, total HeLa cell poly(A)-containing RNA (lanes a–d), vesicular stomatitis virus mRNA (lanes e–h) and poliovirus RNA (lanes i–l), treated with several concentrations of methylmercury hydroxide. No significant effects on translation rates or products were observed.
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FIG. 5. Sedimentation of polio messenger ribonucleoprotein particles from infected HeLa cell extracts. Poliovirus-infected HeLa cells were labeled with 10 μCi/ml [3H]uridine for 3 h prior to harvest at 4 h post-infection and preparation of a cell-free protein-synthesizing extract. A sample (extract recovered from 1.6 X 10^6 cells) was adjusted to 0.02 M EDTA, diluted with 1 ml of 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 0.002 M EDTA, and sedimented through 15-30% (w/w) sucrose gradients prepared in the same buffer. Centrifugation was for 17 h at 19,000 rpm in the Beckman SW 27 rotor at 4°C. Gradients were collected through a Gilford recording spectrophotometer and fractions were analyzed for radioactivity after precipitation with 7% trichloroacetic acid. The arrows indicate the positions in the gradient of ribosomal monosomes and subunits, measured by absorbance at 260 nm.

FIG. 6. Stability of endogenous polio mRNA during in vitro translation in an infected cell extract. [3H]uridine-labeled infected cell extracts were prepared as described and incubated under translation conditions as shown in Fig. 4. After 0, 15, 30, and 60 min, samples were adjusted to 0.02 M EDTA and sedimented through EDTA-containing sucrose gradients as described in the legend to Fig. 5. Samples of each gradient fraction were analyzed for radioactivity in order to locate the position of the mRNP, and the remainder of these fractions (14-18, Fig. 5) were precipitated overnight at -10°C with 2.5 volumes of ethanol. The precipitates were collected by sedimentation, dissolved in 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.002 M EDTA, 0.2% SDS, and sedimented through sucrose gradients as in Fig. 1. Each fraction was analyzed for radioactivity as described.
peak has been previously described and characterized (28) and identified as viral mRNA bound to proteins.

**Stability of mRNA in Infected Cell Extracts during Translation—**Radiolabeled, infected cell S10 extracts were translated in vitro for 60 min, and aliquots were removed at several times during the incubation for the subsequent isolation of viral mRNA. Each sample was sedimented through EDTA-containing sucrose gradients similar to that shown in Fig. 5, and the mRNP peak was pooled and precipitated with ethanol. The gradient profiles of the mRNP-containing extracts were identical at all time points examined. Fig. 6 shows a sucrose gradient sedimentation analysis of the viral mRNA isolated after 0, 15, 30, and 60 min of translation. The mRNA remained intact, and no loss of any material during the incubation was observed. Fig. 7 shows similar samples from another experiment in which the recovered RNA was analyzed by agarose-polyacrylamide gel electrophoresis. Again, the endogenous mRNA was stable throughout the incubation time, while protein synthesis was occurring.

**Addition of Exogenous mRNA to an Infected Cell Extract—**The above results suggested that endogenous viral mRNA, present as mRNP particles, in an infected cell extract was stable during translation, whereas exogenous viral RNA added to translation extracts was not. In order to confirm this conclusion, the endogenous mRNA in an infected cell extract was hydrolyzed with micrococcal nuclease, and that same extract was then supplemented with exogenous [3H]RNA for protein synthesis. Fig. 8A shows that micrococcal nuclease treatment of the labeled, infected cell S10 abolished the endogenous mRNA peak. Exogenous viral RNA added to this extract does appear to associate with proteins to form an mRNP structure which sediments in EDTA-containing sucrose gradients at approximately 65 S (Fig. 8B). This complex sediments slightly slower than the endogenous mRNP complex, but sediments faster than protein-free 35 S RNA. However, with time of incubation under translation conditions, the exogenous mRNP peak can be observed to degrade (Fig. 8B).

Analysis of the exogenous RNA as a function of time of incubation again showed degradation to occur with a $t_{1/2}$ of approximately 35 min.

**DISCUSSION**

Although translation of purified mRNAs in vitro has become a common procedure for identifying specific gene products as well as for analyzing translation mechanisms, few studies have evaluated the stability of the mRNA under translation conditions. Lawrence (18) has documented the formation of an 18 S messenger activity of adenovirus 2 polypeptide pVIII during translation of purified 27 S mRNA for 100,000 protein. The generation of the smaller mRNA appeared to result from a nonspecific degradation of the larger. Several other investigators have reported a preferential degradation of experimentally uncapped mRNAs, compared with their capped counterparts (29-31), thus leading to the suggestion that modification of the 5' end increases the stability of mRNA by virtue of conferring protection against nuclease action. For a naturally uncapped mRNA such as poliovirus RNA, the 5'-terminal protein could be considered as serving a similar protective role, although the apparent ubiquity in cell extracts of a 5'-terminal protein-unlinking activity (32) makes this unlikely.

In this report, we have shown that degradation of exogenous poliovirus RNA does indeed occur during translation, in both HeLa cell and rabbit reticulocyte extracts, despite relatively efficient synthesis of all of the known gene products (21, 33). Crude preparations of initiation factors significantly slow the degradation process, probably as a result of protein-RNA interactions which protect the RNA from nuclease. The resulting increased stability of the mRNA may in fact account for the apparent stimulatory activity of initiation factors in some translation systems, and interpretations of initiation factor activity in vitro should be made with some caution.

As reported previously (28, 34), incubation of polio RNA with cell extracts results in the association of proteins to yield a ribonucleoprotein particle of approximately 65 S in EDTA-containing sucrose gradients. Unlike the endogenous mRNP particle isolated from infected cells, however, the exogenous RNA with associated proteins, is not stable. The mRNP particle found in vitro is completely stable, and it must be assumed that the associated protein complement and/or the chemical nature of the association renders the RNA stable. The description of mRNP particles and their associated proteins isolated from a variety of cells and tissues has been reviewed (35). The composition of the polio mRNP particles which we have isolated in this study has not been examined.

The finding that endogenous mRNA in an infected HeLa cell extract remains intact during translation under conditions when two initiation sites are utilized, eliminates the possibility that the second site is generated secondary to fragmentation of the RNA. How ribosomes ultimately select an AUG codon for initiation of translation on an uncapped mRNA such as polio, remains an open and intriguing question.
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