Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 4157–4161, October 1975
Microbiology

Complete translation of poliovirus RNA in a eukaryotic cell-free system

(protein synthesis/picornaviruses/polyproteins/proteolysis)

LYDIA VILLA-KOMAROFF*, NAOMI GUTTMAN, DAVID BALTIMORE, AND HARVEY F. LODISH

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139

Contributed by David Baltimore, July 7, 1975

ABSTRACT **Poliovirus RNA stimulates incorporation of ^{35}S from both [^{35}S]methionine and formyl-[^{35}S]methionyl-$tRNA_f^{Met}$ in cell-free systems derived from HeLa cells or from poliovirus-infected HeLa cells. The largest product formed under the direction of the viral RNA is the same size as the polyprotein thought to represent translation of the entire RNA. Synthesis of this polyprotein and other large products was stimulated greatly by increasing the salt concentration during the reaction from the optimum for initiation (90 mM) to the optimum for elongation (155 mM). Only one initiation peptide could be identified, and a tryptic digest of the product contained mainly peptides that cochromatographed with peptides from authentic viral proteins. The RNA from a deletion mutant of poliovirus initiated protein synthesis at the same site used by standard RNA and programmed synthesis of an appropriately deleted set of polypeptides. The results strongly support the model of translation of poliovirus RNA from a single initiation site into a continuous polyprotein that is cleaved to form the functional proteins. It is suggested that uninfected HeLa cell extracts can carry out the cleavages of nascent polyprotein.**

The only viral messenger RNA found in poliovirus-infected cells is the 35S viral RNA (1, 2). Analysis of viral protein synthesis in infected cells has led to the hypothesis that the 35S RNA of poliovirus and other picornaviruses is translated from a single site near the 5′-end of the RNA into one continuous polypeptide (3). All the functional viral proteins would then be proteolytic cleavage products from this single polypeptide. The strongest evidence for this hypothesis is 2-fold: (*i*) the identification, in cells that were treated to inhibit proteolysis, of a virus-specific polypeptide that is larger than 200,000 daltons and could be the complete genome transcript (3) and (*ii*) the ability to map the viral genome using inhibitors of initiation of protein synthesis, a form of analysis which depends on the existence of only a single initiation site (4–6). The proteolytic cleavages responsible for synthesis of the viral proteins are of two forms, cleavages of nascent protein that occur soon after a given peptide bond has been formed and cleavages which have much longer half-times of occurrence (3, 5).

To test the hypothesis of a single initiation site for protein synthesis on a picornavirus messenger RNA requires analysis of cell-free protein synthesis programmed by the RNA. Poliovirus RNA has not been previously studied as a messenger RNA in eukaryotic cell-free systems, but RNA from another picornavirus, encephalomyocarditis (EMC) virus has been used successfully. *In vitro*, RNA of EMC virus is apparently translated from a single initiation site (7, 8) to generate a spectrum of polypeptides which appear to result from premature termination rather than from proteolytic cleavage (9–11). In most cases, only 60% of the EMC viral genome is expressed *in vitro;* however, complete translation of RNA of EMC virus in a cell-free system prepared from EMC virus-infected L cells occurs at a very low frequency (12).

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; EMC virus, encephalomyocarditis virus; NCVP, noncapsid viral protein; DI, poliovirus defective interfering.

* Present address: The Biological Laboratories, Harvard University, 16 Divinity Ave., Cambridge, Mass. 02138

MATERIALS AND METHODS

Virus Production. Poliovirus Mahoney Type I was grown in HeLa cells as described (15) except that virus was adsorbed to the cells for 30 min at 0°. Preparations of poliovirus defective interfering DI(1) were kind gifts of Dr. Charles Cole and Dr. Martinez Hewlett.

Preparation of Viral RNA. RNA was prepared from purified virions by the sodium dodecyl sulfate ($NaDodSO_4$)/acetic acid extraction method described (16, 17). The RNA was precipitated with ethanol, washed twice with ethanol, resuspended in water at a concentration of 250–500 μg/ml, and stored in small aliquots at −90°. Labeled RNA was prepared from purified virions, the RNA of which had been labeled with 10 μCi/ml of [^3H]uridine (New England Nuclear) from 30 min to 6 hr after infection.

Preparation of Poliovirus Proteins. HeLa cells infected in the presence of actinomycin D were harvested 3 hr after infection and resuspended in Earle's saline containing 12 μCi/ml of [^{35}S]methionine or 50 μCi/ml of [^{14}C]arginine. The large molecular weight precursors to the virus proteins were prepared by labeling the infected cells in the presence of amino acid analogs, fluorophenylalanine, canavanine, ethionine, and 2-azetidine carboxylic acid, as described by Jacobson, Asso, and Baltimore (3). Actinomycin D was the kind gift of Merck, Sharp and Dohme.

Preparation of Cell-Free Extracts. Extracts from HeLa cells were prepared as described (18, 19) except that cells were disrupted in a Dounce homogenizer in extract buffer [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.5; 120 mM KCl, 6 mM 2-mercaptoethanol; 5 mM $MgOAc_2$] without being preswollen in hypotonic buffer (18). Infected cell extracts were prepared from HeLa cells infected with poliovirus at a multiplicity of infection of 100 and harvested 2 hr after infection.

Cell-Free Protein Synthesis. Conditions for cell-free protein synthesis in preincubated HeLa extracts were as described by McDowell *et al.* (18) with the following modifications: the concentration of magnesium acetate varied from 2.0 to 3.0 mM; the concentration of creatine phosphokinase was 0.1 mg/ml; in some cases, the reaction mixture was incubated for 15 min with 92 or 112 mM KCl, then the KCl concentration was increased to 155 mM and the mixture incubated an additional 105 min. All reactions were at 30°.

Polyacrylamide Gel Electrophoresis. Cell-free reactions

Table 1. Incorporation of [^{35}S]methionine and formyl-[^{35}S]methionine (from f[^{35}S]Met-tRNA$_f^{Met}$) into polypeptides in preincubated HeLa cell extract

Source of S-30		cpm incorporated: [^{35}S]Met + RNA	[^{35}S]Met − RNA	f[^{35}S]Met-tRNA$_f^{Met}$ + RNA	f[^{35}S]Met-tRNA$_f^{Met}$ − RNA
Mock infected	(*a*)	8,876	4,146	494	71
HeLa cells	(*b*)	137,310	25,200	22,730	1,770*
Infected	(*a*)	ND	ND	481	97
HeLa cells	(*b*)	265,860	36,590	4,860	1,270†

Reaction mixtures (50 μl) containing poliovirus RNA and 250 μCi/ml of [^{35}S]methionine or f[^{35}S]Met-tRNA$_f^{Met}$ were incubated under optimal standard conditions for 50 min. Radioactivity incorporated in the reaction was determined by terminating the reaction with 0.1 M NaOH and incubating at 37° for 15 min. The products of the reaction were then precipitated with trichloroacetic acid; the precipitate was collected on ground glass filters and dried. Radioactivity was determined in a liquid scintillation counter in a toluene 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) scintillant. HeLa cell extracts designated (*a*) were resuspended in hypertonic buffer for 10 min before being disrupted. Extracts designated (*b*) were disrupted immediately as described in *Materials and Methods*. ND = not done.

* Salt concentration was increased after 15 min. Mixture was incubated for 120 min.

† Mixture was incubated for 120 min.

were terminated by adding pancreatic ribonuclease to 100 μg/ml and incubating the mixture at 37° for 30 min. The products of the reaction were then precipitated with 10 volumes of acetone and analyzed on polyacrylamide slab gels containing $NaDodSO_4$ (20). The gels were fixed in 10% acetic acid, 25% propanol, stained with Coomassie Blue, dried, and autoradiographed.

Preparation of f[^{35}S]Met-tRNA$_f^{Met}$. A preparation of tRNA from yeast containing about 33% tRNA$_f^{Met}$ and no detectable tRNA^{Met} was the kind gift of Dr. Uttam RajBhandary. The tRNA$_f^{Met}$ was charged with ^{35}Smethionine and formylated with an *E. coli* S100 as described (21).

RESULTS

Characteristics of cell-free protein synthesis

To determine the ability of poliovirus RNA to direct protein synthesis in preincubated extracts of mock-infected or poliovirus-infected HeLa cells, either [^{35}S]methionine or formyl-[^{35}S]methionyl-tRNA$_f^{Met}$ was used as precursor. The latter precursor selectively measures initiation of protein synthesis because NH_2-terminal formyl-methionyl residues are not cleaved from protein but NH_2-terminal [^{35}S]methionine is rapidly cleaved off (21). Table 1 shows that incorporation of both precursors was stimulated markedly but to variable extents by addition of 35S poliovirus RNA to either uninfected cell or infected cell extracts. Much more activity was evident if the extracts were made without a hypotonic swelling period for the cells.

The optimum conditions for synthesis were studied using [^{35}S]methionine as precursor. Because each extract had different characteristics, even when made and tested under apparently identical conditions, we optimized the Mg^{++} concentration for each extract preparation. The optimal Mg^{++} concentration varied from 2.0 to 3.0 mM. A broad KCl optimum from 92 to 112 mM was found. Incorporation increased with increasing poliovirus RNA concentration up to 30–60 μg of RNA per ml. The amount of RNA needed to saturate the reaction was determined for each extract since excess RNA inhibited synthesis. Addition of tRNA, hemin, or a reticulocyte ribosome salt wash did not enhance translation of poliovirus RNA in these preincubated extracts. Once optimal conditions had been determined, frozen aliquots of a given preparation maintained their characteristics for at least 6 months.

Protein synthesis *in vitro* under salt shift conditions

To study the size of the polypeptides synthesized under the direction of viral RNA, the products were separated by electrophoresis through a slab gel of polyacrylamide which was analyzed by autoradiography (Fig. 1). For comparison three marker preparations were analyzed in parallel: virion proteins (Fig. 1, lane O); the total proteins made in infected cells (lane P) and the polypeptides made in amino-acid analog-treated infected cells (lane Q). The polypeptide marked NCVP (noncapsid viral protein) 00 is the largest one found in amino-acid analog-treated cells and is thought to correspond to a translation product of the whole poliovirus genome (3). Polypeptides 0a and 0b derive from single cleavages of the nascent NCVP 00 (13). NCVP 1 is the capsid protein precursor and is encoded by the 5′-end of the RNA (4, 6).

Two conditions were used from amino acid incorporation in extracts incubated for 120 min. Either the K^+ concentration was maintained at the optimum for incorporation (92 mM in this extract) or it was shifted to 155 mM after 15 min of incubation. The salt shift was used because Mathews and Osborn (22) had found that the rate of polypeptide elongation in mouse ascites cell extracts was increased by the higher K^+ concentrations, but these concentrations inhibit initiation too severely to be used from the outset of incubation. In extracts incubated at a constant K^+ concentration for 120 min a small amount of polypeptide of exactly the size of NCVP 00 was evident (Fig. 1, lane M). It is not found until 120 min of incubation; at 60 and 90 min some NCVP Oa and much NCVP 1 was present (lanes K and L), while at 40 min some lower molecular weight polypeptides predominate.

When the salt shift was used, more of the largest polypeptides was evident. Lanes A–D of Fig. 1 show the products made by infected cell extracts incubated 40, 60, 90, and 120 min, respectively; lane E shows the product of an uninfected cell extract lacking added RNA; Lanes F–I show the products of uninfected cell extracts incubated 40, 60, 90, and 120 min, respectively, and lane N shows the products of an uninfected extract incubated 120 min in the absence of added RNA. It is evident that the added poliovirus RNA caused formation of a spectrum of polypeptides very similar to those made in infected cells treated with amino acid analogs. Proteins comigrating with NCVP 00, NCVP 0, NCVP 1, NCVP 1 ½, NCVP 2, NCVP 3b, and NCVP 4 are evident among the products of these cell-free systems. In the infected cell, all of the polypeptides smaller than NCVP 00 are thought to be generated by proteolysis.

Initiation of protein synthesis

Initiation of poliovirus protein synthesis appears to occur at only one site in virus-infected cells (3, 24). To obtain an estimate of the number of independent initiation sites on polio-

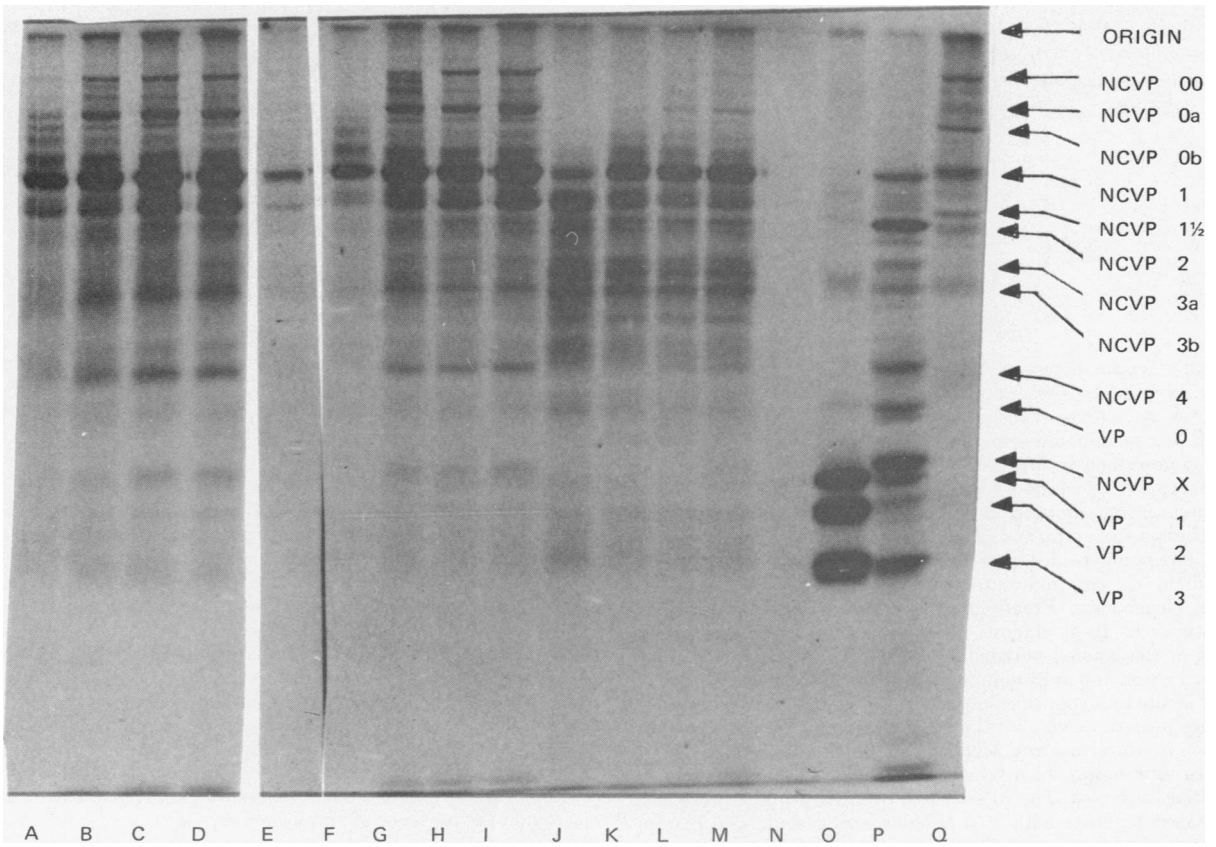

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of polypeptides synthesized in response to poliovirus RNA. Reaction mixtures (50 μl) containing either infected or mock-infected HeLa cell S-30, 250 μCi/ml of [^{35}S]methionine, and poliovirus RNA were either incubated for 15 min with 92 mM KCl, then for an additional 105 min with 155 mM KCl (gels A–I) or were incubated for 120 min with 92 mM KCl (reactions J–N). At the indicated times, 10-μl aliquots were withdrawn from the reaction and prepared and analyzed as described in *Materials and Methods*. NCVP, noncapsid viral protein; VP, viral protein. (A–D) Polypeptides synthesized in response to poliovirus RNA in an infected HeLa cell extract at 40, 60, 90, and 120 min, respectively. (E) Polypeptides synthesized in the absence of added RNA with a salt shift in an infected cell extract after 120 min of reaction. (F–I) Polypeptides synthesized in a mock-infected HeLa cell extract after 40, 60, 90, and 120 min, respectively. (J–M) Polypeptides synthesized in a mock-infected HeLa cell extract with a constant KCl concentration of 92 mM after 40, 60, 90, and 120 min of reaction, respectively. (N) Polypeptides synthesized in the absence of added RNA with a salt shift in a mock-infected cell extract after 120 min of reaction. (O) [^{35}S]Methionine-labeled proteins from purified virions. (P) [^{35}S]Methionine-labeled virus-specific proteins present in infected cells. (Q) Poliovirus proteins synthesized in the presence of amino-acid analogs as described in *Materials and Methods*.

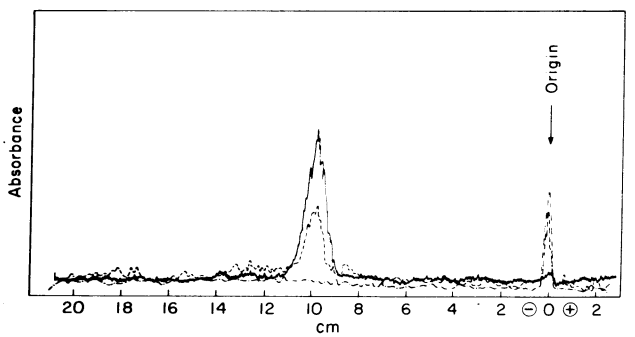

FIG. 2. Tryptic digest of protein labeled with f[^{35}S]Met-tRNA$_f^{Met}$ and synthesized *in vitro* under the direction of poliovirus RNA and DI RNA. Reaction mixtures (100 μl) containing f[^{35}S]methionine-tRNA$_f^{Met}$, unlabeled methionine, and 50 μg/ml of poliovirus RNA (—), or an unknown amount of DI-RNA (- - -), or no RNA (- · - ·) were incubated 60 min with a constant K^+ concentration of 112 mM. Products of the reaction were precipitated and digested with trypsin as described in *Materials and Methods*. The tryptic digests were subjected to ionophoresis at pH 3.5 as described (26). The paper was dried and used to expose Kodak RP Royal film for 3 days. The autoradiogram was scanned with a Joyce Loebl microdensitometer under conditions where full-scale pen deflection represented 1.49 *A* units.

virus RNA *in vitro*, the product directed by poliovirus RNA was labeled with f[^{35}S] Met-tRNA$_f^{Met}$, digested with trypsin, and analyzed by ionophoresis at pH 3.5. Only one f[^{35}S] methionine-containing tryptic peptide was found in the product synthesized in reactions using HeLa cell extracts (Fig. 2) or ascites cell extracts (not shown). As measured by its elution position on a Bio-Gel P-2 column, this peptide had a molecular weight of about 900 (data not shown).

The limited amount of radioactivity that could be incorporated from f[^{35}S] Met-tRNA$_f^{Met}$ made identification of the size of all products retaining the NH_2-terminal label difficult. We could, however, show that a protein migrating with NCVP 1 was labeled *in vitro* by f [^{35}S] Met-tRNA$_f^{Met}$, and therefore it must contain the NH_2-terminal peptide shown in Fig. 2.

Tryptic peptide analysis of the product synthesized in response to poliovirus RNA.

To determine if the polypeptides synthesized *in vitro* in response to poliovirus RNA represented virus-specific peptides, we compared a tryptic digest of the *in vitro* product synthesized under salt shift conditions to a tryptic digest of *in vivo* poliovirus-specific proteins (Fig. 3). Extensive over-

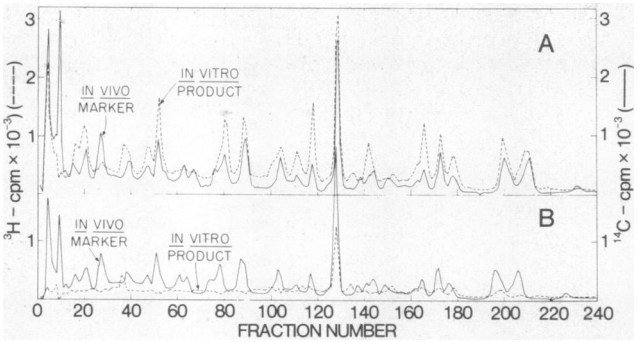

FIG. 3. Tryptic digest of [^{3}H]arginine-labeled peptides synthesized *in vitro* in the presence (A) or absence (B) of added poliovirus RNA. A cell-free reaction mixture (300 μl) incubated with a salt shift was terminated after 120 min by incubation with ribonuclease, as described in *Materials and Methods*. The product was precipitated with acetone and resuspended in 2% $NaDodSO_4$, 0.1 M ammonium bicarbonate, and 1% 2-mercaptoethanol. The sample was boiled for 5 min, and glycerol was added to a concentration of 20%. The polypeptides were layered onto a Sephadex G-150 column (25 cm × 1 cm) and eluted with 0.2% $NaDodSO_4$, 0.1 M ammonium bicarbonate. Fractions were collected by drops, and the radioactivity of 10-μl aliquots was determined to determine the location of the labeled polypeptides. The polypeptides chromatographed behind the void volume in a wide major peak. The fractions of labeled polypeptides representing the larger cell-free products were pooled. *In vivo* viral proteins that had been prepared as described in *Materials and Methods* were made 2% in $NaDodSO_4$, 0.1 M in ammonium bicarbonate, and 1% in 2-mercaptoethanol and boiled for 5 min. The *in vivo* and *in vitro* proteins were then precipitated together with 10% trichloroacetic acid. The precipitate was washed twice with 5% trichloroacetic acid, lyophilized repeatedly to remove residual acid, resuspended in 1% ammonium bicarbonate, and digested for 12 hr at 37° with 50 μg of TPCK-trypsin (Worthington Biochem.). Fresh trypsin (50 μg) was added and the sample digested an additional 4 hr. After lyophilization, the digest was resuspended in 0.2 M pyridine titrated to pH 3.1 with glacial acetic acid and analyzed on a column of Technicon type P chromobeads as described by Jacobson *et al.* (3). Fractions were added to 10 ml of Aquasol (New England Nuclear) or 10 ml of Handifluor (Mallinckrodt), and radioactivity was determined in a liquid scintillation counter.

lap of the two patterns was evident. The *in vitro* derived peptides that did not correspond to virus peptides appeared to be derived from polypeptides synthesized by the residual endogenous HeLa mRNA (Fig. 3B).

Translation of RNA from DI poliovirus particles

To test the notion that the polypeptides directed by poliovirus RNA in the cell-free system are identical to their apparent *in vivo* counterparts, we used the RNA from a mutant of poliovirus [poliovirus DI(1)] known to lack about one-third of the RNA encoding NCVP 1. In DI-infected cells, NCVP 1 and all of the precursors that contain it are about 30,000 daltons shorter than their normal counterparts (13). Therefore, translation of DI RNA *in vitro* should lead to appropriately deleted products if the *in vitro* system mimics the *in vivo* situation.

The products directed by DI RNA were compared with products directed by standard RNA (Fig. 4). The results confirm the fidelity of the system: no NCVP 00 was directed by DI RNA but a product (DI-NCVP 00) 12% shorter was formed; no NCVP 0a was made but the appropriately deleted form was found (DI-NCVP 0a); very little NCVP 1 was evident but the deleted form, which comigrates with NCVP 3, was formed [DI(P)]. Synthesis of NCVP 1½, NCVP 4, and

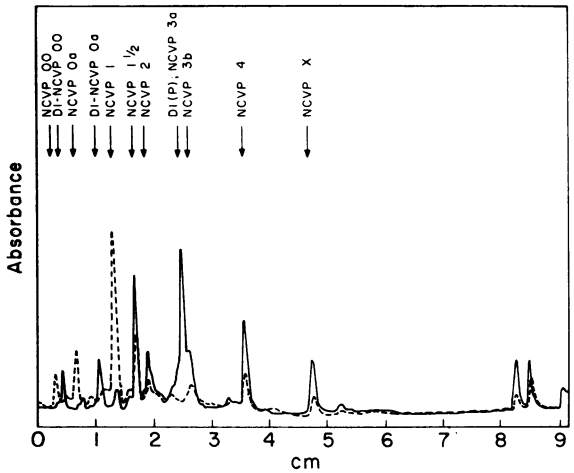

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of polypeptides synthesized in response to standard and DI RNA. Reaction mixtures (50 μl) containing infected HeLa cell S-30, 250 μCi/ml of [^{35}S]methionine, and either poliovirus RNA (- - -) or DI RNA (—) were incubated with a salt shift as described in the legend to Figure 1. After 120 min of incubation, 10 μl of the mixture was prepared and analyzed on $NaDodSO_4$/polyacrylamide slab gel as described in *Materials and Methods*. The dried gel was used to expose Kodak RP Royal x-ray film for 3 days. The autoradiogram was scanned with a Joyce Loebl microdensitometer under conditions where full-scale pen deflection represented 1.49 *A* units. The arrows indicate the position on the same gel in parallel slots of both standard and DI virus directed polypeptides synthesized *in vivo* in the presence of amino acid analogs (3).

NCVP X was directed by both RNAs. These results are consistent with the conclusion that faithful translation of standard and DI RNA occurs *in vitro* in extracts prepared from HeLa cells and that some processing of the protein products occurs.

To examine where on the DI RNA initiation of protein synthesis occurred, a tryptic digest of f[^{35}S] methionine-labeled products was submitted to ionophoresis. A single labeled peptide was found which comigrated at pH 3.5 with the initiation peptide formed under the direction of standard RNA (Fig. 2). The two peptides also comigrate at pH 1.9 (not shown). The apparent identity of initiation sites on the two RNAs suggests that DI RNA contains a deletion internal to the site of initiation.

DISCUSSION

Previous study of poliovirus-infected cells generated the hypothesis that the viral RNA is translated from a single initiation site into a single continuous chain of amino acids that is cleaved to form the functional proteins. The present analysis of protein synthesis *in vitro* directed by poliovirus RNA supports the hypothesis: the product is virus-specific by tryptic peptide analysis; only a single initiation site on the RNA was found; a product thought to be the translation product of the entire genome could be identified, and discrete products that comigrate with authentic viral proteins were evident. The nature of the products was verified by studying the translation of the RNA from a deletion mutant of poliovirus.

The incorporation of f[^{35}S] Met-$tRNA_f^{Met}$ into a single tryptic peptide (Fig. 2) indicates that initiation of protein synthesis *in vitro* occurs at only one site on the added poliovirus or DI RNA. Although there is no direct evidence that this site is the authentic initiation site, it seems likely that this is the case because of the correspondence in size of *in*

vitro and *in vivo* products. Similar results (and a similar conclusion) have been obtained in studies utilizing RNA of EMC virus (7–12).

In vivo, two classes of proteolytic cleavage of the poliovirus RNA translation product can be distinguished: nascent cleavages, which are rapid cleavages of the polypeptide while it is still in the process of elongation, and cytoplasmic cleavages, which occur more slowly and involve further processing of the nascent cleavage products. No evidence for cytoplasmic cleavages during cell-free synthesis has been found. However, our results are consistent with the notion that the HeLa cell-free system carries out the nascent cleavages with approximately 80% efficiency. This value is estimated from the ratio of NCVP 00 to the lower molecular weight products in Fig. 4. The evidence that the lower molecular weight products are formed by cleavage is as follows. (*i*) The various products appear to correspond to *in vivo* proteins both because of the exact correspondence of electrophoretic mobility and because these polypeptides containing NCVP 1 are all appropriately shifted in the *in vitro* product of DI RNA translation. (*ii*) The map of the poliovirus genome contains (5′ → 3′) NCVP 1 − NCVP X − NCVP 1½ (4). (*iii*) The products include proteins that comigrate with not only NCVP 1, which could be formed by a premature termination, but also with NCVP 1½ and NCVP X (see Fig. 4). Since it is difficult to envisage how these polypeptides, formed by both wild-type and DI RNA, could arise by premature termination, we assume that they are formed by either direct initiation or by proteolytic cleavage. (*iv*) Since only one initiation site is evident, we believe that nascent cleavages must be occurring.

The occurrence of nascent cleavages in extracts from uninfected cells would imply that either host cell enzymes are responsible or that the nascent polypeptides are proteases. Because the nascent cleavages occur so rapidly, both NCVP 1 and NCVP X would have to be proteases; this is unlikely, and we conclude that host cell enzymes carry out the cleavage. Our results differ from results obtained by others studying the translation of EMC virus RNA. The synthesis of a polypeptide larger than the capsid precursor in extracts from uninfected cells in response to EMC virus RNA and a polypeptide the same size as the capsid precursor in extracts from EMC virus-infected cells has been reported for cell-free systems derived from mouse L-cells and plasmacytoma cells (12, 25). Both groups suggest that the proteolytic activity necessary to process EMC viral proteins is activated in infected cells.

This work was supported by the following grants from the National Institutes of Health: AI-08814, AI-08388, AM-15929, and CA-12174. D.B. is an American Cancer Research Professor. H.F.L. is the recipient of Research Career Development Award no. GM-50175 from the National Institutes of Health.

1. Penman, S., Becker, Y. & Darnell, J. E. (1964) *J. Mol. Biol.* **8**, 541–555.
2. Spector, D. H. & Baltimore, D. (1975) *J. Virol.* **15**, 1418–1431.
3. Jacobson, M. F., Asso, J. & Baltimore, D. (1970) *J. Mol. Biol.* **49**, 657–669.
4. Tabor, R., Rekosh, D. & Baltimore, D. (1971) *J. Virol.* **8**, 395–401.
5. Rekosh, D. (1972) *J. Virol.* **9**, 479–487.
6. Summers, D. F. & Maizel, J. V. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2852–2856.
7. Oberg, B. F. & Shatkin, A. J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3589–3593.
8. Smith, A. E. (1973) *Eur. J. Biochem.* **33**, 301–313.
9. Boime, I. & Leder, P. (1972) *Arch. Biochem. Biophys.* **153**, 706–713.
10. Eggen, K. L. & Shatkin, A. J. (1972) *J. Virol.* **9**, 636–445.
11. Kerr, I. M., Brown, R. E. & Tovell, D. R. (1972) *J. Virol.* **10**, 73–81.
12. Esteban, M. & Kerr, I. M. (1974) *Eur. J. Biochem.* **45**, 567–576.
13. Cole, C. & Baltimore, D. (1973) *J. Mol. Biol.* **76**, 325–343.
14. Cole, C., Smoler, D., Wimmer, E. & Baltimore, D. (1971) *J. Virol.* **7**, 478–485.
15. Baltimore, D., Girard, M. & Darnell, J. E. (1966) *Virology* **29**, 179–189.
16. Mandel, B. (1962) *Cold Spring Harbor Symp. Quant. Biol.* **27**, 123–136.
17. Granboulan, N. & Girard, M. (1969) *J. Virol.* **4**, 475–479.
18. McDowell, M., Joklik, W. K., Villa-Komaroff, L. & Lodish, H. F. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2649–2653.
19. Villa-Komaroff, L., McDowell, M., Baltimore, D. & Lodish, H. F. (1974) "Nucleic acids and protein synthesis," in *Methods in Enzymology*, eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 30, Part F, pp. 709–723.
20. Maizel, J. V. (1971) *Methods Virol.* **V**, 180–247.
21. Housman, D., Jacobs-Lorena, M., RajBhandary, U. & Lodish, H. F. (1970) *Nature* **227**, 913–918.
22. Mathews, B. M. & Osborn, M. (1974) *Biochim. Biophys. Acta* **340**, 147–152.
23. Jacobson, M. F. & Baltimore, D. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 77–84.
24. Huang, A. & Baltimore, D. (1970) *J. Mol. Biol.* **47**, 275–291.
25. Lawrence, C. & Thach, R. E. (1974) *J. Virol.* **14**, 598–610.
26. Osborn, M., Weber, K. & Lodish, H. F. (1970) *Biochem. Biophys. Res. Commun.* **41**, 748–756.