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Coupled Translation and Replication of Poliovirus RNA In Vitro: Synthesis of Functional 3D Polymerase and Infectious Virus

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Poliovirus RNA polymerase and infectious virus particles were synthesized by translation of virion RNA in vitro in HeLa S10 extracts. The in vitro translation reactions were optimized for the synthesis of the viral proteins found in infected cells and in particular the synthesis of the viral polymerase $3D^{pol}$. There was a linear increase in the amount of labeled protein synthesized during the first 6 h of the reaction. The appearance of $3D^{pol}$ in the translation products was delayed because of the additional time required for the proteolytic processing of precursor proteins. $3D^{pol}$ was first observed at 1 h in polyacrylamide gels, with significant amounts being detected at 6 h and later. Initial attempts to assay for polymerase activity directly in the translation reaction were not successful. Polymerase activity, however, was easily detected by adding a small amount (3 μl) of translation products to a standard polymerase assay containing poliovirion RNA. Full-length minus-strand RNA was synthesized in the presence of an oligo(U) primer. In the absence of oligo(U), product RNA about twice the size of virion RNA was synthesized in these reactions. RNA stability studies and plaque assays indicated that a significant fraction of the input virion RNA in the translation reactions was very stable and remained intact for 20 h or more. Plaque assays indicated that infectious virus was synthesized in the in vitro translation reactions. Under optimal conditions, the titer of infectious virus produced in the in vitro translation reactions was greater than 100,000 PFU/ml. Virus was first detected at 6 h and increased to maximum levels by 12 h. Overall, the kinetics of poliovirus replication (protein synthesis, polymerase activity, and virus production) observed in the HeLa S10-initiation factor in vitro translation reactions were similar to those observed in infected cells.

Poliovirus is a member of the *Picornaviridae* family of RNA viruses and has a single-stranded polyadenylated genome of positive polarity. Poliovirus RNA has a virus-encoded protein, VPg, covalently linked to the 5′-terminal nucleotide (22, 36), a large 5′ noncoding region involved in the cap-independent initiation of translation (11, 24, 29, 49, 50), one long open reading frame encoding the viral proteins, and a 3′ noncoding region of 71 nucleotides followed by a poly(A) tail of variable length. Translation of virion RNA results in the synthesis of a polyprotein, which is processed by three viral proteases ($2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$) into the mature viral proteins (27). Proteins from the P2 and P3 coding regions of the genome have been shown to be required for poliovirus RNA replication (3, 6, 13, 25, 30, 33, 34, 38, 54). In particular, $3D^{pol}$ has been identified as the poliovirus RNA-dependent RNA polymerase (57).

$3D^{pol}$ has been purified from infected HeLa cells (21, 23, 39, 52, 57) and from both prokaryotic (44, 51) and eukaryotic (45, 46) cells expressing the cloned $3D^{pol}$ gene. The purified polymerase is a template- and primer-dependent enzyme which requires either an oligonucleotide primer (4, 23, 46) or a host factor from uninfected cells (5, 14, 15). Various enzymatic activities have been associated with different preparations of host factor (e.g., protein kinase [43], terminal uridylyltransferase [1, 2], and endonuclease [28]); however, the role of these host factors in RNA replication in vivo has not been determined. The purified $3D^{pol}$ catalyzed the synthesis of genome-length minus-strand RNA in reactions containing poliovirion RNA as a template and oligo(U) as a primer (58). In reactions containing host factor, $3D^{pol}$ catalyzed the synthesis of product RNA twice the size of the poliovirion RNA template (61). Despite these in vitro experimental observations, many details concerning the mechanism of poliovirus RNA replication in vivo remain unknown. Poliovirus RNA replication occurs naturally in the cytoplasm of infected cells by using both newly translated viral proteins and various host components. RNA replication occurs in replication complexes found associated with smooth membranes and the cytoskeleton (8, 9, 12, 37). Physicochemical techniques have shown these replication complexes to contain 2B, 2C, VPg, $3D^{pol}$, and viral RNA, yet their exact composition is not clear. At some point in the replication of poliovirus RNA, VPg is covalently linked to the 5′ end of both minus-strand and plus-strand RNAs (22). Although various models have been proposed, the precise mechanism by which VPg is linked to poliovirus RNA has not yet been elucidated.

Translation of poliovirus RNA is mediated by a *cis*-acting region in the 5′ noncoding region called the internal ribosome entry site or ribosome landing pad (11, 19, 24, 29, 40, 47, 49, 50). This region of the genome interacts with specific factors from the host cell to support the cap-independent initiation of translation (16, 19, 24, 41). Rabbit reticulocyte lysate and cytoplasmic extracts of HeLa cells were shown to contain the cellular components necessary to translate picornavirus mRNAs in vitro (55, 59, 60). Translation of poliovirus RNA in a reticulocyte extract, however, resulted in the synthesis of some poliovirus proteins which did not correspond in molecular weight to those found in vivo (55). A ribosomal salt wash from HeLa cells was shown to stimulate poliovirus protein synthesis in reticulocyte extracts and to promote the synthesis of poliovirus proteins corresponding in molecular weight to those found in vivo (10, 18). Appar-

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ently, reticulocyte extracts are deficient in one or more factors found in a salt wash of HeLa cell ribosomes that are necessary for translation of poliovirus RNA. The ability to express poliovirus proteins in vitro has been used extensively to study the poliovirus proteases which are functional after translation in vitro (32, 35, 56). In addition, subviral particles are formed after expression of the poliovirus capsid proteins in vitro, indicating functional folding of these proteins (31). Recently, Molla et al. (42) demonstrated the ability of HeLa cell extracts to support the de novo synthesis of infectious poliovirus. Despite these important technological achievements, no work which biochemically characterized functional 3D polymerase after its expression by translation of virion RNA in vitro has been published.

Because of the importance of understanding the molecular mechanisms of picornavirus RNA replication, we characterized the synthesis of poliovirus $3D^{pol}$ in cell extracts of HeLa cells. In this study we demonstrated that biochemically functional $3D^{pol}$ can be synthesized and assayed after translation of poliovirion RNA (vRNA) in vitro. We used a coupled translation-RNA replication system to characterize the activity of $3D^{pol}$ synthesized in vitro. Genome-length minus-strand RNA was synthesized in a standard poliovirus polymerase assay containing vRNA and oligo(U). In the absence of oligo(U), product RNA about twice the size of vRNA was synthesized. In addition, we confirmed and extended the primary finding by Molla et al. (42) that infectious poliovirus can be synthesized in vitro. The kinetics of viral protein synthesis, polymerase activity, and assembly of infectious virus in the HeLa S10-initiation factor (IF) in vitro translation reaction were found to be very similar to the same events observed in vivo during one cycle of viral replication.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa S3 cells were maintained at 2×10^5 to 4×10^5 cells per ml in Joklik's modified Eagle medium (ICN Flow, Costa Mesa, Calif.) supplemented with 5% calf serum and 2% fetal calf serum. Cells were infected with poliovirus type 1 (Mahoney strain) as previously described (60).

RNA preparation. vRNA was prepared from CsCl-banded virus by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. vRNA was quantitated by determination of its A_{260}. Encephalomyocarditis (EMC) virus P3 RNA was purchased from Novagen, Madison, Wis. In some experiments, proteinase K-treated vRNA was used. vRNA was treated twice with 300 μg of proteinase K per ml in 0.5% sodium dodecyl sulfate (SDS) buffer (0.5% SDS [Sigma], 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl) at 37°C for 45 min to remove VPg. The proteinase K-treated vRNA was phenol-chloroform-isoamyl alcohol extracted and ethanol precipitated.

HeLa S10 Preparation. HeLa S10 cell extracts were prepared essentially as described by Brown and Ehrenfeld (10). Briefly, 2.5 liters (4×10^5 cells per ml) of uninfected HeLa cells was harvested by centrifugation, washed three times with isotonic buffer (35 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4], 146 mM NaCl, 11 mM glucose), and collected by centrifugation in a 50-ml conical tube. The pellet was resuspended in 2 volumes of hypotonic buffer [20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $Mg(CH_3CO_2)_2$, 1 mM dithiothreitol (DTT)] and incubated on ice for 10 min. The cells were broken with 25 strokes of a glass Dounce homogenizer, using a tight-fitting pestle. Then 0.1 volume of 10× buffer [0.2 M HEPES (pH 7.4), 1.2 M KCH_3CO_2, 40 mM $Mg(CH_3CO_2)_2$, 50 mM DTT] was added to the homogenate, and the nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4°C in a Beckman JS 4.0 rotor. The supernatant was poured into a 30-ml Corex tube and centrifuged at 10,000 rpm for 15 min at 4°C in a Beckman JA-20 rotor. The S10 supernatant was transferred to a 50-ml conical tube, adjusted to 1 mM $CaCl_2$ by addition of 0.01 volume of 0.1 M $CaCl_2$, and treated with 75 U of S7 nuclease (Boehringer Mannhiem, Indianapolis, Ind.) per ml of S10 supernatant at 20°C for 15 min. The nuclease was inactivated by addition of 0.1 M ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) to 2 mM. The S10 supernatant was centrifuged again at 10,000 rpm for 15 min at 4°C in a JA-20 rotor, and 400-μl aliquots were frozen at −70°C. This S10 extract contained 12.8 mg of protein per ml as measured by using a Bradford assay (Bio-Rad) and had 65 A_{260} U/ml.

HeLa cell IF preparation. HeLa cell IFs were prepared essentially as described by Brown and Ehrenfeld (10). Uninfected HeLa cells were harvested and homogenized as described above for the preparation of the S10 supernatant. After homogenization, the nuclei were removed by centrifugation without the addition of any buffer or salt. The postnuclear supernatant was centrifuged for 15 min at 10,000 rpm at 4°C in a JA-20 rotor. The S10 supernatant was centrifuged at 60,000 rpm for 1 h at 4°C in a Beckman 70 Ti rotor to pellet the ribosomes. The supernatant was discarded, and the ribosomal pellet was resuspended in hypotonic buffer at 240 A_{260} U/ml. The ribosomes were made 0.5 M KCl by the addition of 4 M KCl and stirred for 15 min on ice. The salt-washed ribosomes were centrifuged at 60,000 rpm for 1 h in a 70 Ti rotor at 4°C, and the supernatant was removed and dialyzed for 2 h at 4°C against 5 mM Tris-HCl (pH 7.5)–100 mM KCl–0.05 mM EDTA–1 mM DTT–5% glycerol. This ribosomal salt wash IF preparation was frozen in 50-μl aliquots at −70°C.

In vitro translation. HeLa S10 translation reactions (20 to 200 μl) contained 50% (by volume) HeLa S10 extract, 20% (by volume) HeLa cell IFs, 1 mM ATP, 0.2 mM GTP, 120 mM KCH_3CO_2, 2.75 mM $Mg(CH_3CO_2)_2$, 3 mM DTT, 35 mM HEPES (pH 7.4), 400 U of RNasin (Promega, Madison, Wis.) per ml, 25 mM creatine phosphate (Boehringer Mannheim), 400 μg of creatine phosphokinase (Boehringer Mannheim) per ml, 20 μM amino acids (Promega), and 50 μg of vRNA per ml or 25 μg of EMC virus P3 RNA per ml as indicated. The concentration of vRNA used in the reactions was optimized for protein synthesis. Concentrations of virion RNA from 1 to 500 μg/ml were tested, and 50 to 200 μg/ml was found to be optimal. Reactions involving rabbit reticulocyte extract (Promega) were performed as specified by the manufacturer. Briefly, rabbit reticulocyte translation reaction mixtures contained 70% (by volume) rabbit reticulocyte extract, 400 U of RNasin per ml, 20 μM amino acids, 50 μg of vRNA per ml or 25 μg of EMC virus P3 RNA per ml as indicated, and 20% (by volume) HeLa cell IFs as indicated. Reactions were incubated at 30°C for the times indicated. The reaction mixtures also contained [^{35}S]methionine (1,200 Ci/mmol; Amersham) when indicated. ^{35}S-labeled proteins were solubilized in Laemmli sample buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.8], 0.5% 2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol) and analyzed by SDS-gel electrophoresis at a constant current of 30 mA in 10% polyacrylamide gels (acrylamide/bisacrylamide ratio, 29:1) containing 0.1% SDS and 187.5 mM Tris-HCl (pH 8.8). Gels were fixed in 50% trichloroacetic acid, fluorographed

by using 22% 2,5-diphenyloxazole [PPO] in dimethyl sulfoxide, dried, and exposed to XAR-5 film (Kodak) at −70°C.

Polymerase assays. Reactions mixtures (30 μl) containing 50 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each unlabeled nucleoside triphosphate (NTP) (ATP, GTP, and UTP), 10 μCi [α-^{32}P]CTP (410 Ci/mmol; Amersham) adjusted to a final concentration of 5 μM, and the indicated amount of in vitro translation product or purified poliovirus polymerase were incubated at 30°C for 90 min. The reaction mixtures also contained 2 μg of vRNA and 45 ng of oligo(U) when indicated. The reactions were terminated by the addition of 170 μl of 0.5% SDS buffer, and the labeled product RNA was phenol-chloroform-isoamyl alcohol extracted and ethanol precipitated. The RNAs were then analyzed by CH_3HgOH–1% agarose gel electrophoresis as previously described (61). An RNA molecular weight ladder (Bethesda Research Laboratories) was used to determine the sizes of labeled products. The gels were dried, and labeled RNA was detected by autoradiography. Control reactions contained poliovirus polymerase purified to homogeneity after expression in *Escherichia coli* by using plasmid pT7POL (generously provided by Steve Plotch, American Cyanamid Co., Lederle Laboratories, Pearl River, N.Y.).

RESULTS

Translation of poliovirion RNA in vitro. Translation reactions containing either a rabbit reticulocyte extract, a reticulocyte extract plus HeLa cell IFs, or a HeLa cell extract supplemented with IFs were used to compare poliovirus protein synthesis in vitro. EMC virus P3 RNA was used as a control because it is very efficiently translated in reticulocyte extracts (48). A reaction mixture containing both reticulocyte extract and HeLa cell IFs was used because previous work showed that the initiation factors provide components necessary for efficient translation of poliovirus RNA (10, 18). The proteins synthesized in the in vitro translation reactions were compared with poliovirus proteins synthesized in infected cells (Fig. 1). The EMC virus P3 mRNA was translated efficiently in the rabbit reticulocyte extract, yielding the expected protein pattern (Fig. 1). In particular, a significant amount of EMC virus polymerase ($3D^{pol}$) was evident. As expected, translation of vRNA in the rabbit reticulocyte extract yielded numerous proteins that did not correspond well to the poliovirus proteins synthesized in vivo (Fig. 1). The presence of HeLa cell IFs in the reticulocyte extract had only a small effect on the pattern and amount of the proteins synthesized from EMC virus P3 RNA (Fig. 1). However, the presence of the HeLa cell IFs significantly altered the pattern of poliovirus proteins synthesized in the rabbit reticulocyte extract (Fig. 1). The proteins synthesized in the reticulocyte reaction containing the HeLa cell IFs corresponded well to those synthesized in vivo. However, the amount of poliovirus $3D^{pol}$ synthesized in the reticulocyte extract containing the HeLa cell IFs was small relative to the amount of the other poliovirus proteins. As in the reticulocyte translation reactions, the EMC virus P3 mRNA was translated efficiently in the HeLa S10-IF translation reaction and yielded the expected pattern of EMC virus proteins (Fig. 1). Translation of poliovirus vRNA in the HeLa S10-IF reaction resulted in the synthesis of poliovirus proteins that clearly corresponded to those synthesized in vivo (Fig. 1). There was, however, little or no poliovirus $3D^{pol}$ evident in the products of the HeLa S10-IF translation reaction. The comparison of the HeLa extract with the commercially available and widely used reticulocyte extract-IF translation

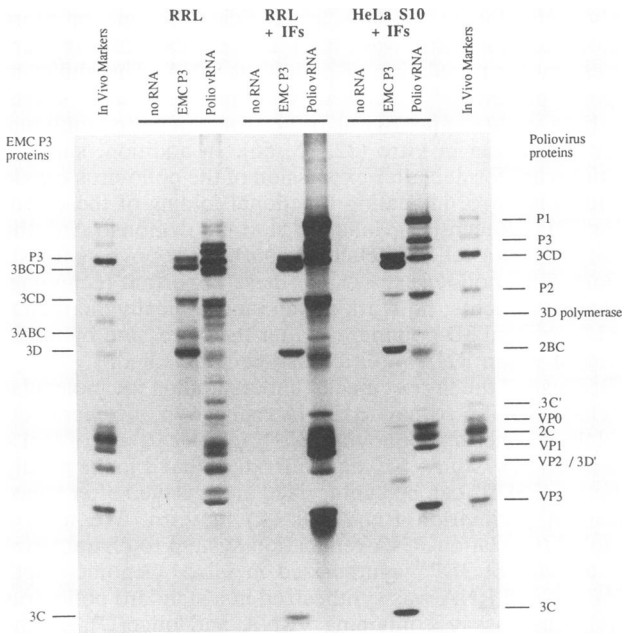

FIG. 1. In vitro translation of poliovirion RNA and EMC virus P3 RNA in rabbit reticulocyte (RRL) and HeLa cell extracts. The reactions were 20 μl in total volume and contained reticulocyte lysate, reticulocyte lysate supplemented with HeLa cell IFs, or HeLa S10 extracts supplemented with HeLa cell IFs as indicated. The reactions also contained the indicated RNAs and 200 μCi of [^{35}S]methionine per ml and were incubated at 30°C for 2 h. A portion was solubilized in Laemmli sample buffer, and the labeled proteins were analyzed on an SDS–10% polyacrylamide gel. Twice as much product was loaded from the HeLa S10-IF reactions as from the reticulocyte reactions. The outside lanes were loaded with ^{35}S-labeled poliovirus proteins from infected HeLa cells. The mobility of poliovirus proteins is indicated on the right side of the figure, and the mobility of EMC virus proteins is indicated on the left. The mobilities of poliovirus 3C′, 3D′, 3D, and 3C were determined by immunoprecipitation with antibodies to 3C and 3D. The mobilities of VP0, VP1, VP2, and VP3 were determined by using purified virus.

reaction demonstrated that both the HeLa S10 extract and the HeLa cell initiation factors were accurately translating the poliovirus proteins.

The protein products of the in vitro translation reactions described in Fig. 1 were analyzed for viral RNA polymerase activity in a standard polymerase assay containing poliovirion RNA and oligo(U) (23, 58). Polymerase activity was not assayed directly in the in vitro translation reactions because the high-salt conditions used in these reactions strongly inhibits the activity of the polymerase (23). A detectable amount of poliovirus polymerase activity was found only in the protein products of the rabbit reticulocyte-IF vRNA translation reaction (Fig. 2). This was consistent with the small amount of $3D^{pol}$ detected in this reaction (Fig. 1). Surprisingly, there was no polymerase activity in the translation products of any of the reactions in which EMC virus P3 mRNA was translated, even though large amounts of EMC virus $3D^{pol}$ were apparently synthesized in these reactions (Fig. 1). This suggested that the EMC polymerase was inactive on poliovirion RNA under conditions that were optimal for the poliovirus polymerase. We found that full-length product RNA was synthesized in control assays containing purified polymerase and 5 μl of the mock (no RNA) translation products, although there was some inhibi-

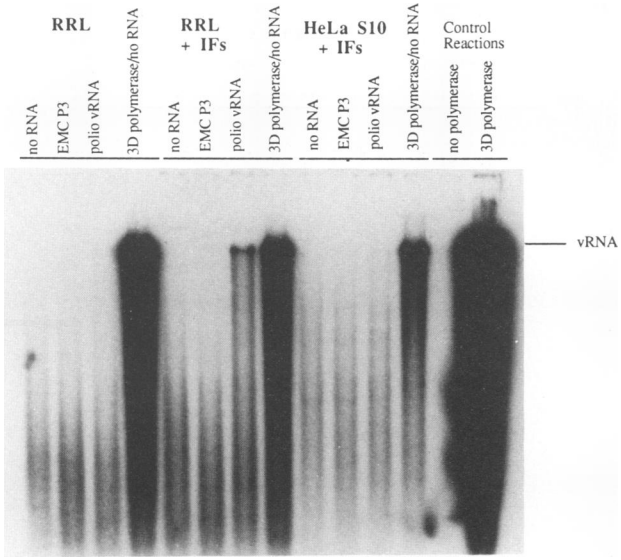

FIG. 2. RNA polymerase activity of viral proteins translated in vitro. RNA polymerase assays were performed in vitro as described in Materials and Methods, using 5 μl of the products of the translation reactions described in the legend to Fig. 1. Control reactions contained either no polymerase or 3 μl of purified $3D^{pol}$. In addition, 3 μl of purified $3D^{pol}$ was added to reactions containing 5 μl of the mock translation reactions to demonstrate the inhibitory effect of the components in the translation reactions (3D polymerase/no RNA). The mobility of vRNA was as indicated.

tion in the amount of product RNA synthesized compared with that in assays not containing the mock translation products (Fig. 2). This indicated that components in the translation mixture partially inhibited polymerase activity but that the polymerase was still able to synthesize large amounts of full-length product RNA under the conditions used in these assays.

Synthesis of poliovirus polymerase in HeLa S10 extracts. Since polymerase activity was not detected in our S10 extracts after 2 h of translation, we optimized these reactions for the synthesis of $3D^{pol}$. We determined that one of the critical factors for detecting $3D^{pol}$ in the products of in vitro translation reactions was that of providing sufficient time for proteolytic processing in the translation reactions. We also found that addition of exogenous rabbit liver tRNA to the in vitro translation reactions was not necessary for optimum protein synthesis and, in fact, inhibited protein synthesis at >40 μg/ml (data not shown). A time course of protein synthesis was performed by using a HeLa S10 translation reaction with and without HeLa cell IFs (Fig. 3). Incorporation of radiolabeled methionine into acid-precipitable proteins increased linearly for the first 6 h in both reactions, and the presence of HeLa cell IFs resulted in about a threefold increase in the incorporation of [^{35}S]methionine (Fig. 3A). Processing of the poliovirus precursor proteins began at the earliest times examined and continued throughout the time course (Fig. 3B). In fact, significant processing occurred after the incorporation of [^{35}S]methionine had ceased. $3D^{pol}$ was not detected in significant amounts until 6 h, and then the level continued to increase until 21 h (Fig. 3B). However, overexposed films showed that trace amounts of $3D^{pol}$ could be detected as early as 1 h (data not shown). Thus, the amount of $3D^{pol}$ increased over the entire time course. To determine whether polymerase activity correlated with the increased synthesis of $3D^{pol}$, we assayed for polymerase activity by using a standard polymerase reaction containing poliovirion RNA and oligo(U). A 3-μl sample of the proteins synthesized in a HeLa S10-IF translation reaction was assayed at each time point. Polymerase activity (i.e., full-length minus-strand RNA) was detected as early as 2 h in Fig. 3C and 1 h in an overexposed film (data not shown). Thus, in general agreement with the increase in $3D^{pol}$ accumulation throughout the reaction, there was a similar increase in polymerase activity over the 21-h time course (Fig. 3C).

Coupled translation-RNA replication in vitro. Poliovirus RNA replication was characterized in a standard polymerase assay by using the viral proteins synthesized in a HeLa S10 translation reaction. The polymerase assays were run with and without exogenous vRNA and with and without an oligo(U) primer. Two HeLa S10-IF translation reactions containing [^{35}S]methionine were incubated at 30°C for 20 h to generate the products for the RNA replication reactions: a mock translation (no RNA) and a reaction containing poliovirus vRNA. No detectable proteins were synthesized in the mock control, whereas all the poliovirus proteins, including $3D^{pol}$, were synthesized in the reaction containing vRNA (Fig. 4A). No virus-specific RNA products were observed in the polymerase assays containing [^{32}P]UTP in the absence of exogenous vRNA (Fig. 4B). The polymerase assay containing products from the mock translation control and exogenous vRNA demonstrated the presence of host activity with the ability to incorporate some label into vRNA with [^{32}P]UTP (Fig. 4B) but not with [^{32}P]CTP (Fig. 4C). This may be the result of terminal uridylyltransferase activity previously described (1, 2). The same reaction containing viral proteins synthesized in the vRNA translation reaction synthesized labeled product RNA that was about twice the size of the vRNA template (Fig. 4B and C). Full-length template-size minus-strand RNA was synthesized in the reactions containing viral proteins, exogenous vRNA, and oligo(U) (Fig. 4B and C). Dimer-sized product RNA, however, was also synthesized in the reactions containing oligo(U) and vRNA (Fig. 4B and C). It is noteworthy that the use of [^{32}P]CTP resulted in more labeled poliovirus RNA than that found in reactions containing equal amounts of [^{32}P]UTP. The reason for this is not known, but using [^{32}P]CTP clearly increased the sensitivity of these assays. Finally, it should be noted that a small amount of labeled product RNA was synthesized in the polymerase assays containing [^{32}P]CTP and the viral translation products on the endogenous vRNA (Fig. 4). A very light band of template-sized product RNA and smaller RNAs were detected in this reaction (Fig. 4C).

Stability of input vRNA in translation reactions. The stability of poliovirion RNA in HeLa S10-IF translation reactions was important because of the longer times required for $3D^{pol}$ synthesis and because we wished to examine poliovirus RNA replication on both endogenous (i.e., the mRNA template) and exogenous RNA templates. The stability of poliovirion RNA 3′ end labeled with [^{32}P]pCp and uniformly labeled with $^{32}P_i$ was examined in a HeLa S10-IF translation reaction. After 2 h, about 40% of the uniformly labeled RNA and about 20% of the 3′ end-labeled RNA was still acid precipitable (Fig. 5A). Only small decreases in the amount of labeled RNA were observed after 2 h. Despite the significant loss of acid-precipitable vRNA in the first 2 h of the reaction, intact vRNA was still present at 6 h (Fig. 5B). In fact, infectious vRNA was still present in the translation reaction after more than 20 h at 30°C (Fig. 6, with tRNA). Thus, a

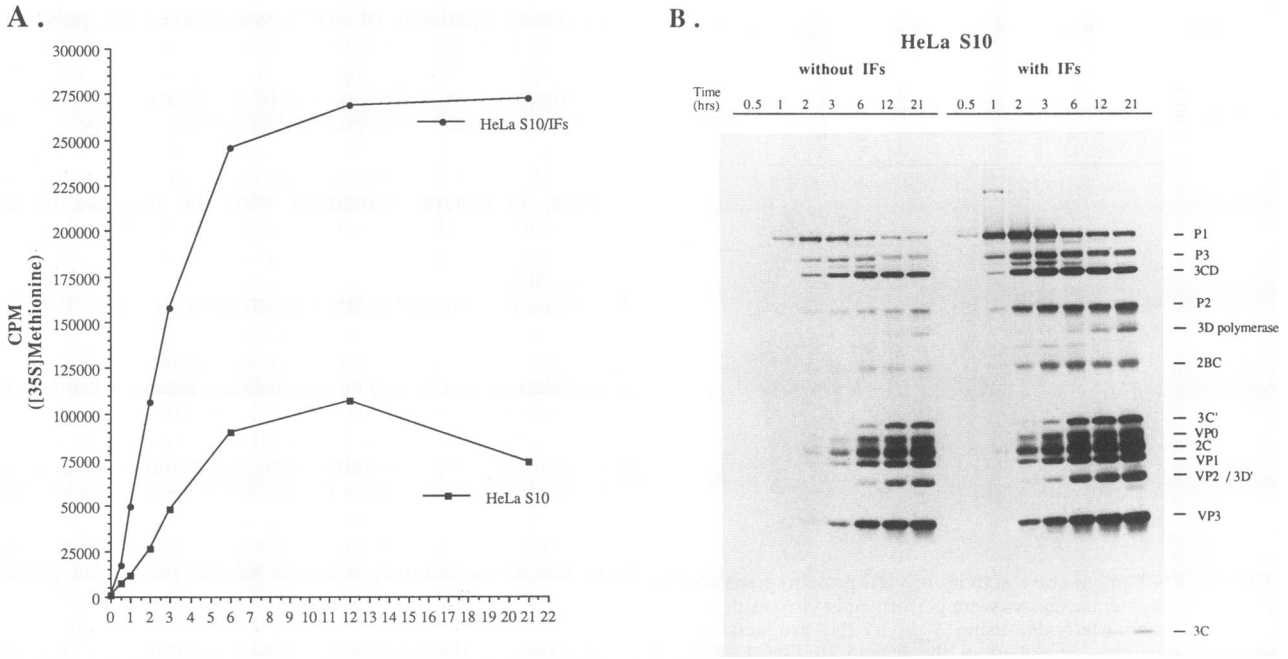

FIG. 3. Time course of protein synthesis and RNA polymerase activity in a HeLa S10 and a HeLa S10-IF translation reaction. (A) Translation reactions containing HeLa cell S10 extract or HeLa S10 extract and HeLa cell IFs (HeLa S10/IFs) were incubated at 30°C. The reactions also contained 800 μCi of [^{35}S]methionine per ml and 50 μg of vRNA per ml. At the indicated times, 1-μl samples were removed from the reactions, precipitated with trichloroacetic acid, filtered, and counted in a scintillation counter. (B) Samples (1 μl) were removed at the indicated times, solubilized in Laemmli sample buffer, analyzed by electrophoresis on an SDS–10% polyacrylamide gel, and visualized by fluorography. (C) A second translation reaction containing both S10 and initiation factors was run without [^{35}S]methionine, and 20-μl samples were removed and stored on ice at the indicated times. Standard polymerase assays were performed with 3 μl of the translation products at the indicated time points. The polymerase reactions also contained 2 μg of vRNA, 45 ng of oligo(U), and 10 μCi of [α-^{32}P]CTP as described in Materials and Methods. The products of the reactions were analyzed by CH_3HgOH–1% agarose gel electrophoresis and visualized by autoradiography. The mobility of vRNA was as indicated.

large fraction of the input RNA was rapidly degraded in the translation reactions, but 10 to 20% of the RNA was protected and remained intact and infectious for more than 6 h.

Synthesis of infectious virus in vitro. The small amount of RNA replication that was observed above on the endogenous vRNA suggested that the viral RNA may also be replicating in the original translation reactions, as previously reported (42). To test this hypothesis, we assayed for the presence of virus at the end of the in vitro translation reactions. Virus production in the HeLa S10 translation reactions was examined by using a plaque assay. Two reactions were run to examine the effect of exogenous tRNA on virus production: a reaction with 200 μg of rabbit liver tRNA per ml and a reaction without exogenous tRNA. In addition, identical reactions were incubated at 0 and 30°C to demonstrate the effectiveness of the postincubation RNase treatment in removing vRNA from the reactions. Plaques formed when aliquots of the reactions incubated at 0°C were placed on the cell monolayers (Fig. 6). If the aliquots of the 0°C reactions were treated with RNases A and T_1, however, no plaques were detected (Fig. 6). The presence of these nuclease-sensitive plaques indicated that intact vRNA was still present at the end of the translation reactions. The translation reactions incubated at 0°C did not allow for the synthesis of any viral proteins or virus. The translation reaction incubated at 30°C with tRNA yielded only 40 PFU per ml after nuclease treatment. In contrast, the translation reaction that did not contain additional tRNA yielded 2,400 PFU per ml (Fig. 6). The presence of HeLa cell IFs in the translation reaction boosted virus production twofold (Table 1). In addition, the production of virus was twofold higher in reactions containing all four exogenous NTPs as opposed to only ATP and GTP (Table 1). Our protocol for the preparation of S10 extract does not include a dialysis step. Therefore, significant amounts of endogenous NTPs are probably present in the S10 extract. Addition of HeLa IFs to the

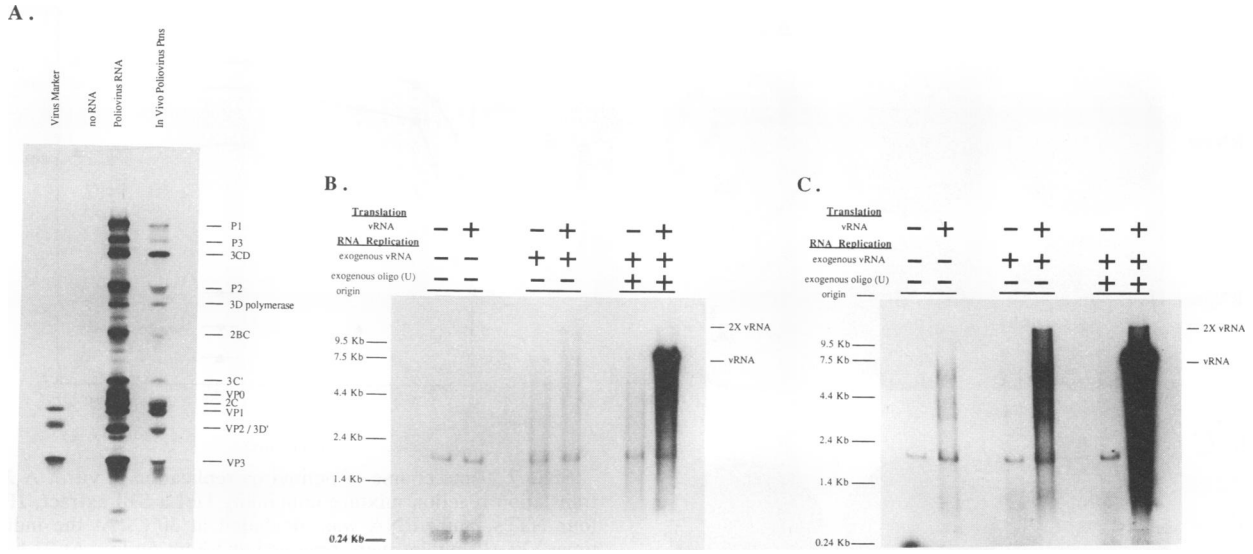

FIG. 4. Poliovirus RNA synthesized in vitro by poliovirus proteins synthesized in HeLa S10-IF translation reactions. (A) Two HeLa S10-IF translation reactions (100 μl) were incubated at 30°C for 24 h in the presence of 800 μCi of [^{35}S]methionine per ml: a mock reaction (no RNA) and a reaction containing 50 μg of vRNA per ml. The labeled viral proteins were analyzed by electrophoresis in an SDS–10% polyacrylamide gel and visualized by autoradiography. Purified ^{35}S-labeled poliovirions (lane Virus Marker) and ^{35}S-labeled poliovirus proteins from infected cells (lane In Vivo Poliovirus Ptns) were used as markers. (B) In vitro RNA replication reactions were performed by using 5 μl of the above translation products in 30-μl polymerase assays as described in Materials and Methods. The reactions contained 10 μCi of [α-^{32}P]UTP and vRNA and oligo(U) as indicated. The products of the reactions were characterized in a CH_3HgOH–1% agarose gel and visualized by autoradiography. (C) In vitro RNA replication reactions with [α-^{32}P]CTP in place of labeled UTP.

translation reaction and use of our most recent and intact preparations of proteinase K-treated vRNA increased the yield of infectious virus to >100,000 PFU/ml (data not shown). Thus, significant amounts of virus were synthesized in the long term-translation reactions that were optimized for protein synthesis.

The kinetics of infectious-virus production in the in vitro translation reaction were determined in a time course exper-

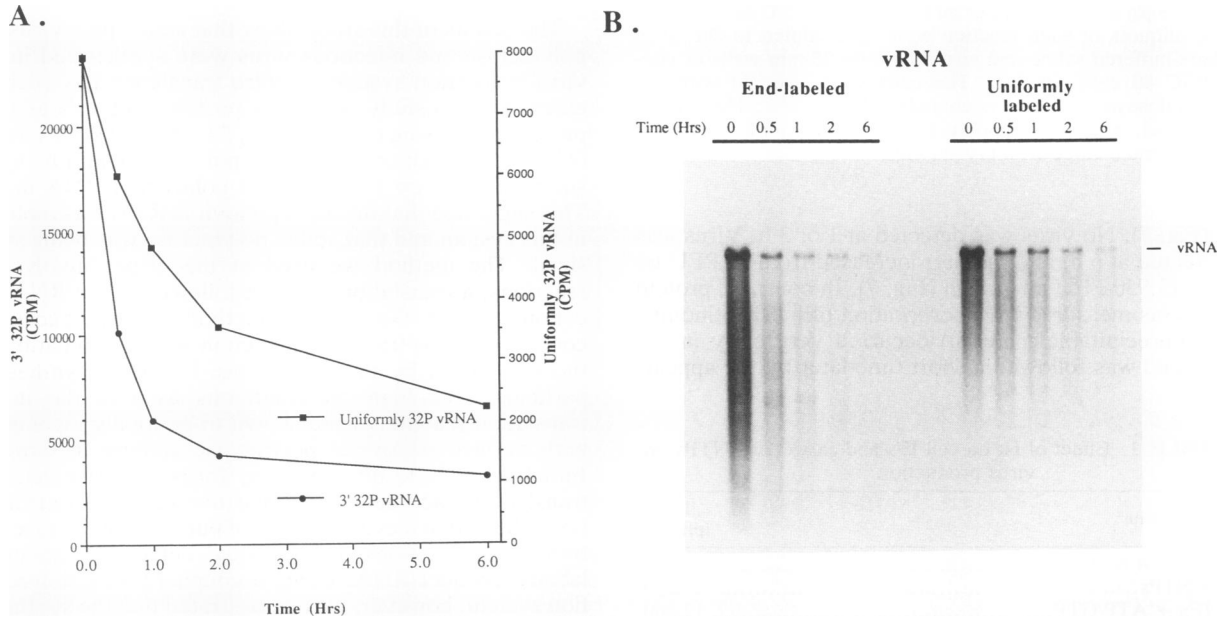

FIG. 5. Stability of poliovirion RNA in HeLa S10-IF translation reactions. (A) vRNA 3′ end labeled with [^{32}P]pCp and vRNA uniformly labeled with $^{32}P_i$ were incubated in a HeLa S10-IF translation reaction. At the indicated times, samples were removed from the reaction, precipitated with trichloroacetic acid, filtered, and counted in a scintillation counter. (B) Samples were also phenol-chloroform-isoamyl alcohol extracted, ethanol precipitated, and separated by electrophoresis in a CH_3HgOH–1% agarose gel. The gel was dried, and the RNA was located by autoradiography. The mobility of vRNA was as indicated.

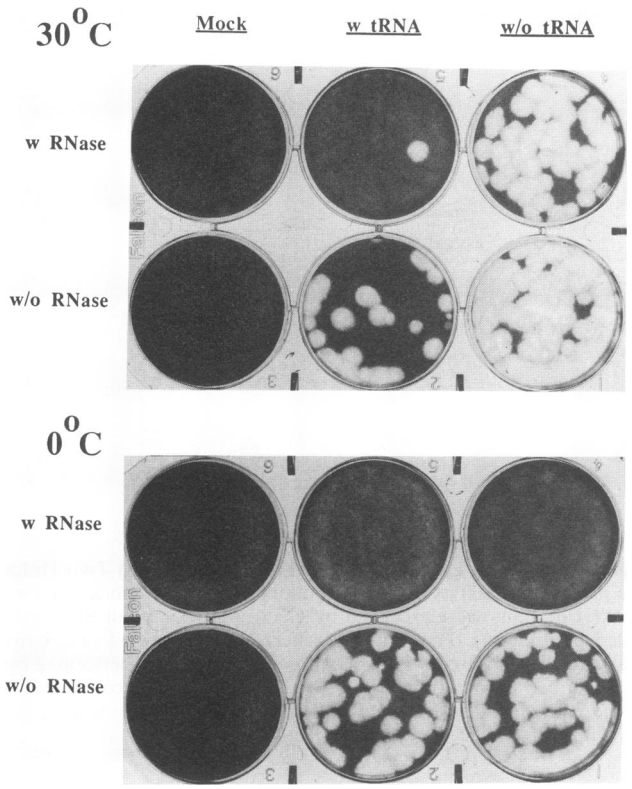

FIG. 6. Synthesis of infectious poliovirus in vitro. HeLa S10 translation reactions (50 μl) were incubated at 0 and 30°C as indicated. The reactions contained 0.2 mM CTP and 0.2 mM UTP in addition to the ATP and GTP. The reactions were run with and without 200 μg of exogenous rabbit liver tRNA per ml as indicated. No HeLa IFs were used in these reactions. After incubation for 20 h, the reactions were divided into two 25-μl aliquots. One aliquot was untreated, and the second was treated with 20 μg of RNase A per ml and 8 μg of RNase T_1 per ml for 25 min at 25°C as indicated. The two aliquots of each reaction were then diluted to 200 μl in phosphate-buffered saline and adsorbed onto 35-mm wells of confluent BSC 40 cells for 1 h. The cells were overlaid with 1% methylcellulose medium and incubated for 48 h at 37°C. The overlay was removed, and the monolayers were fixed with methanol-oxalacetate and stained with crystal violet.

iment (Fig. 7). No virus was detected at 1 or 3 h. Virus was first detected at 6 h, and the titer increased from 55 PFU/ml at 6 h to 15,950 PFU/ml at 12 h (Fig. 7). In contrast, protein synthesis (as measured by incorporation of [^{35}S]methionine into acid-precipitable material) occurred very early in the reaction and was followed a short time later by the appear-

TABLE 1. Effect of HeLa cell IFs and exogenous NTPs on virus production

Reaction[a]	Titer (pfu/ml)[b]
S10 + IFs + 4 NTPs	22,400
S10 + 4 NTPs	11,200
S10 + IFs + ATP/GTP	12,800
S10 + ATP/GTP	5,200

[a] Translation reactions containing the indicated components and vRNA were incubated at 30°C for 20 h as described in Materials and Methods.

[b] The translation products were treated with RNases A and T_1 and allowed to form plaques as described in the legend to Fig. 6.

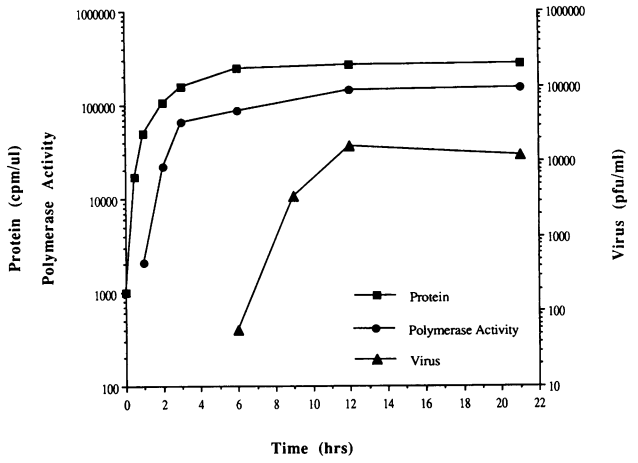

FIG. 7. Time course of poliovirus replication in vitro. A 200-μl translation reaction mixture containing HeLa S10 extract, IFs, all four NTPs, and vRNA was incubated at 30°C. At the indicated times, 20-μl samples were removed and kept on ice. At 21 h, each sample was treated with RNases A and T_1 and allowed to form plaques as described in the legend to Fig. 6. The titer of nuclease-resistant plaques was plotted against time. Also plotted against time are the synthesis of poliovirus proteins and the polymerase activity from Fig. 3. The relative amount of polymerase activity at each time point was determined by densitometry of full-length product RNA in Fig. 3C.

ance of functional $3D^{pol}$ (Fig. 7). Thus, there was a delay of several hours between the synthesis of viral proteins and polymerase and the synthesis of infectious virus particles. Overall, the kinetics of poliovirus replication in the in vitro translation reaction were similar to those observed in vivo.

DISCUSSION

The results of this study show that active poliovirus RNA polymerase and infectious virus were synthesized in an in vitro translation system. In vitro translation has been used extensively to study poliovirus protein synthesis and polyprotein processing (7, 10, 17, 20, 24, 26, 41, 62). Molla et al. (42) recently demonstrated that poliovirus could be synthesized de novo by translation of poliovirion RNA in vitro. This suggested that the input poliovirus RNA was replicating in this system and that active polymerase was being synthesized. The method we used in this paper involves two reactions, a translation reaction followed by an RNA replication reaction. We used this method primarily because the conditions of the translation reaction are not compatible with those required by the polymerase for RNA synthesis. In particular, the high-salt conditions used in the in vitro translation reactions were shown to be totally incompatible with purified poliovirus polymerase activity in vitro (23). Initial attempts to directly assay for polymerase activity in translation reactions yielded negative results. Control reactions showed that even addition of purified polymerase to the translation reactions did not yield detectable amounts of labeled product RNA. Using a coupled translation-replication system, however, we demonstrated that the synthesis of poliovirus $3D^{pol}$ in vitro correlated with the appearance of elongation activity on poliovirion RNA in standard polymerase assays. That is, the polymerase was functional. On the other hand, the EMC virus $3D^{pol}$ synthesized in the in vitro translation reactions did not have detectable poly-

merase activity. Sankar and Porter (53), using an EMC virus RNA-oligo(U) template primer, reported that EMC virus $3D^{pol}$ was able to synthesize full-length product RNA. Our reactions used poliovirus RNA instead of EMC virus RNA, but we have not yet determined whether this is the explanation for our negative results in trying to demonstrate EMC virus polymerase activity. This striking difference between the EMC virus polymerase and poliovirus polymerase activities will be very interesting if confirmed by additional studies.

A number of reaction conditions had to be optimized for detection of poliovirus polymerase elongation activity. In particular, adding HeLa cell IFs significantly increased the amount of protein synthesized in vitro and, consequently, the amount of $3D^{pol}$ (Fig. 3). Additional tRNA was not required for optimal protein synthesis and was inhibitory at more than 40 μg/ml. The most important parameter for detecting polymerase activity was the length of time for which the translation reactions were incubated. Significant amounts of polymerase accumulated after 6 h at 30°C, and only low levels of activity were detected at earlier times. Thus, a significant period was required for proteolytic processing of the polyprotein precursors to yield active $3D^{pol}$. This result was consistent with our previous studies, which showed that only mature $3D^{pol}$, and not its precursors (e.g., 3CD), possesses elongation activity (57).

There appeared to be two populations of vRNA in the HeLa S10-IF translation reactions, a population of vRNA that was rapidly degraded and a population of vRNA that remained intact and infectious. We believe that factors in the HeLa cell extracts protected a percentage of the vRNA in the reactions while the remainder was completely degraded by nucleases in the cell extracts. Addition of less HeLa cell extract to the translation reactions actually resulted in smaller amounts of protected vRNA, indicating that the factors that protect the vRNA from degradation were probably required in stoichiometric amounts whereas only catalytic amounts of the nucleases were required to degrade the RNA (data not shown).

The viral proteins synthesized in the in vitro translation reactions catalyzed the synthesis of full-length minus-strand RNA when exogenous poliovirion RNA and oligo(U) were added to the polymerase assays. Not only was polymerase elongation activity readily detected, but also the product RNAs were full-length intact copies of the template RNA. We found that both the template and the product RNAs were relatively stable in these assays. In both the presence and absence of oligo(U), a significant amount of dimer-sized product RNA was synthesized on poliovirion RNA. This was very similar to the dimer-sized product RNA which is synthesized by the purified polymerase on virion RNA in the presence of various host factor preparations including those which contain terminal uridylyltransferase (TUTase) activity (1, 2, 61). The HeLa S10 extracts which were used in this study appeared to have TUTase activity since virion RNA and other cellular RNAs were apparently end labeled in the presence of [^{32}P]UTP. Additional studies are now required to further characterize this activity.

Viral plaques were formed on BSC 40 cells after the cells had adsorbed RNase-treated HeLa S10 translation products synthesized at 30°C. The vRNA in the translation reactions incubated at 0°C also formed plaques on the BSC 40 cells; however, plaque formation in this case was completely sensitive to digestion with RNases A and T_1. Thus, the synthesis of infectious virus was dependent on the synthesis of viral proteins in the in vitro translation reaction at 30°C. Reaction conditions that were optimal for the synthesis of viral proteins and active polymerase were also optimal for the synthesis of virus. The results in this paper indicated that the virus titer in a 20-h translation reaction under optimal conditions was more than 100,000 PFU/ml. Molla et al. (42) demonstrated the synthesis of infectious virus by translation of both vRNA and infectious transcript RNA in vitro and performed a number of control experiments to demonstrate that the RNase-resistant plaque-forming activity in their in vitro translation reactions was due to infectious virus particles. Our results confirm their primary finding that poliovirus can be synthesized de novo in an in vitro translation reaction and show that even higher virus titers can be achieved by adding HeLa cell initiation factors to the reactions.

The kinetics of poliovirus replication observed in the in vitro translation reaction are consistent with those observed in one-cycle growth curves. Obviously, this in vitro system of poliovirus replication circumvents the attachment, penetration, and uncoating steps of normal virus infection. The in vitro reactions begin with the translation of the input vRNA into the viral polyprotein, which is subsequently co- and posttranslationally processed into the functional viral proteins. After the accumulation of significant amounts of the functional viral proteins, virus production begins and continues for a number of hours. The ability to reproduce these steps of viral replication in an in vitro reaction provides direct access to these processes, which are normally shielded from experimental manipulation by the cellular membrane. This in vitro system thus provides direct access to the events of poliovirus replication occurring in the cytoplasm of infected cells.

Overall, the coupled translation and replication of either virion RNA or viral RNA transcripts synthesized in vitro should provide a powerful new method for studying the molecular mechanisms involved in poliovirus RNA replication. The interaction of the polymerase with different RNA templates and with the other viral proteins involved in RNA replication can be examined by using this general strategy. This approach should also be very useful in characterizing polymerase mutants. The method eliminates the need for preparing a stock of mutant virus and the purification of the mutant polymerase from infected cells. Mutations in other viral replication proteins could also be examined for their effect on viral RNA replication. By using the coupled translation-RNA replication system, it should be possible to perform experiments with mutant RNAs in both the translation and the RNA replication reactions. This method, in combination with the ability to synthesize infectious virus in vitro, should make it possible to significantly advance our knowledge of the unique RNA replication strategies used by poliovirus and other positive-strand RNA viruses.

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