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10. We used the transfection procedure described in (*8*) with the following modification: $4.5 \times 10^6$ cells attached to 600 $cm^2$ tissue culture flasks (Singletray Unit, Nunc) containing 180 ml of medium were treated with a 20-ml calcium phosphate–DNA precipitate of 240 μg of cosmid DNA and 24 μg of pSVtkneoβ.
11. As described in (*8*). Briefly, 1,500 CTL specific for E were added to $4 \times 10^4$ transfected cells in a microwell. After 24 hours, 50 μl of the supernatant were added to $3 \times 10^4$ cells of cell line WEHI 164 clone 13. This TNF-sensitive cell line was developed by T. Espevik and J. Nissen [*J. Immunol. Methods* **95**, 99 (1986)]. The mortality of WEHI cells was estimated 24 hours later by a colorimetric assay described by M. B. Hansen, S. E. Nielsen, and K. Berg [*ibid.* **119**, 203 (1989)].
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20. pTZ18R and pTZ19R were used as cloning vectors in order to produce double-stranded DNA for transfection and single-stranded DNA for sequencing.
21. RNA isolation was performed as in L. G. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986), pp. 130–135. Northern blot analysis was performed as by B. Van den Eynde *et al.* in (7).
22. For cDNA synthesis, total RNA (1 μg) was diluted to a total volume of 20 μl with 2 μl of 10× buffer from the GENEAmp kit (Perkin Elmer-Cetus), 2 μl each of 10 mM dNTP, 1.2 μl of 25 mM $MgCl_2$, 1 μl of a 80-μM solution of oligonucleotide primer CHO-9, 20 units of RNAsin (Promega), and 200 units of MoMLV reverse transcriptase (BRL). This mixture was incubated at 42°C for 40 min. For PCR amplification, 8 μl of 10× buffer, 4.8 μl of 25 mM $MgCl_2$, 1-μl of a 80-μM solution of primer CHO-8, 2.5 units of Taq polymerase, and water were added to a total volume of 100 μl. Amplification was performed for 30 cycles (1 min at 94°C, 2 min at 52°C, and 3 min at 72°C). Each reaction (10 μl) was size-fractionated in agarose gels, blotted on nitrocellulose, and hybridized with $^{32}P$-labeled oligonucleotides. Hybridization and washing conditions were as in (*14*).
23. We thank P. Coulie and F. Lehmann for contributing to CTL lysis assays; P. Hainaut, P. Weynants, F. Brasseur, G. Parmiani, L. Old, E. Stockert, and L. Suardet for providing tumor cell lines; F. Lejeune for melanoma tumor samples; A. M. Lebacq, J. J. Cassiman, P. Marijnen, and E. Bellefroid for RNA of normal and tumor cells; A. Authom for technical assistance; and S. Khaoulali for secretarial assistance. C.T. is supported by a postgraduate fellowship for Cancer Research Training of the Commission of the European Communities.

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# Cell-Free, De Novo Synthesis of Poliovirus

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**Cell-free translation of poliovirus RNA in an extract of uninfected human (HeLa) cells yielded viral proteins through proteolysis of the polyprotein. In the extract, newly synthesized proteins catalyzed poliovirus-specific RNA synthesis, and formed infectious poliovirus de novo. Newly formed virions were neutralized by type-specific antiserum, and infection of human cells with them was prevented by poliovirus receptor-specific antibodies. Poliovirus synthesis was increased nearly 70-fold when nucleoside triphosphates were added, but it was abolished in the presence of inhibitors of translation or viral genome replication. The ability to conduct cell-free synthesis of poliovirus will aid in the study of picornavirus proliferation and in the search for the control of picornaviral disease.**

VIRUSES ARE COMPLEX AGGREGATES of organic macromolecules that assemble from multiples of either a few or many distinct building blocks (*1*). Cell-free morphogenesis of infectious virus particles, in contrast to de novo synthesis, has been reported with some plant viruses and bacteriophages (*2*). In these cases, the viral structural components were isolated from virions or from infected cells. All previral attempts to assemble animal viruses, including members of the Picornaviridae family, have been unsuccessful.

The prototype picornavirus, poliovirus, is a non-enveloped, icosahedral particle consisting of a single-stranded RNA genome that is surrounded by 60 copies each of capsid polypeptides VP1 and VP3, 58 to 59 copies each of VP2 and VP4, and one to two copies of VP0, the precursor to VP2 and VP4 (*3–5*). Poliovirion formation involves the proteolysis of the capsid polypeptide precursor P1 and the formation of "immature" protomers $[(VP0,VP1, \text{ and } VP3)_5]$, procapsids (an empty shell consisting of 12 immature protomers) and, possibly, "provirions" (*3*). Provirions are noninfectious RNA-containing particles whose VP0 polypeptides have not been cleaved to yield the mature virion (*6*). The formation of provirions in a cell-free extract of poliovirus-infected HeLa cells has been described but, for unknown reasons, these particles did not "mature" to virions (*7*). Palmenberg (*8*) and Grubman *et al.* (*9*) have succeeded in assembling capsid intermediate structures by translating, in rabbit reticulocyte lysates, RNAs of the picornaviruses encephalomyocarditis virus and foot-and-mouth disease virus. Formation of infectious particles in these lysates was not reported, and it is conceivable that the rabbit reticulocyte lysate lacks components essential for morphogenesis (*10*).

Numerous problems concerning the structure and replication of picornaviruses remain unsolved. These include the function of virus-encoded polypeptides, the mechanism of initiation of polyprotein synthesis, the mechanism of genome replication, specific steps in morphogenesis, and the involvement of cellular components in viral proliferation. Here we describe a procedure for de novo synthesis of infectious poliovirus in a cell-free system. We provide evidence not only for the formation and proteolytic processing of the polioviral polyprotein in vitro, a result reported previously (*11*), but also for viral RNA synthesis and assembly.

We have prepared a cell-free extract from uninfected HeLa cells (*12*) capable of translating RNA of poliovirus type 1 (Mahoney) [PVI(M)] with great accuracy (Fig. 1). All viral proteins were visible, with the exception of capsid proteins VP2 and VP4. On longer exposure of gels (approximately 96 hours) a band migrating to the position of VP2 emerged (*13*). However, there was too little material to confirm the identity of the polypeptide by immunoprecipitation with

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antibodies to VP2. The small capsid protein VP4 could not be detected because it migrated off the gel under the conditions of gel electrophoresis. VP4 is difficult to detect even in [$^{35}$S]methionine-labeled extracts of infected cells because it contains only one methionine (*14*). In some translations, polypeptide 3CD, the precursor to proteinase 3C$^{pro}$ and RNA polymerase 3D$^{pol}$ (*3, 5*), appeared relatively resistant to proteolytic processing. We cannot explain this phenomenon at present.

To test whether the incubation mixture contained infectious virus, six aliquots (between 1 and 20 μl) of an incubation mixture were added to separate monolayers of HeLa cells. In several experiments, in which independently prepared extracts were used for 15 hours of in vitro translation, plaques appeared [on the average at 600 plaque-forming units (PFUs) per milliliter] whose morphology was characteristic of poliovirus. Compared with the roughly $10^{11}$ full-length poliovirus mRNA molecules contained in the incubation mixture, the number of observed plaques represents a minute fraction, even if the ratio of particles to PFUs were as unfavorable as 1000 to 1. (Depending on differences in virus preparations from different infected cell cultures, the particle/PFU ratio varies widely for poliovirus.)

The formation of plaques observed here could have been the result of transfection of monolayer cells with the mRNA used for translation, although the specific infectivity of plus-stranded poliovirus RNA is low (*15*). To test this possibility, we assayed for PFUs in the complete incubation mixture (cell extract, translation mixture, and template RNA) before and after 15 hours of translation; in each case the mixtures were treated with ribonuclease (RNase) A and RNase T1 before being added to the cells. No plaques were generated from the incubation mixture at the start of the experiment, an observation confirming that the mixture was not contaminated with poliovirus. Similarly, no plaques were observed if viral RNA was incubated for 15 hours with the cell extract alone (Fig. 2). This suggests that translation is required for the formation of PFUs, as plaques were reproducibly formed from the 15-hour incubation mixture in spite of RNase treatment (Fig. 2). These results were obtained regardless of the source of the RNA [transcripts of plasmid DNA (*16*) or genomic RNA isolated from virions] (Fig. 2).

The preparation of the HeLa cell extract includes a dialysis step (*12*) that removes most of the endogenous nucleoside triphosphates (NTPs) from the mixture. As protein synthesis requires ATP and GTP (*17*), ATP (1 mM) and GTP (50 μM) were part of the complete incubation mixture. To test whether virus-specific RNA synthesis occurred during poliovirus RNA translation in the HeLa cell extract, we examined the effects of addition of CTP, UTP, and additional GTP. The number of PFUs per milliliter increased about 70-fold (from ~600 to ~41,000) at 1 mM of ATP and 125 to 250 μM each of GTP, CTP, and UTP (*18*), an observation suggesting RNA synthesis–dependent virus formation. Further increase in the concentration of GTP, CTP, and UTP reduced the number of plaques, and at 1 mM each, plaque formation was completely abolished. This can be explained by the inhibitory effect of the high NTP concentration on translation; at 1 mM of each of the four NTPs, no poliovirus proteins were synthesized (Fig. 3A, lane 8). The reason for the inhibition of translation is probably a reduction of available $Mg^{2+}$ ions for protein synthesis. Indeed, an increase of the $Mg^{2+}$ concentration at high NTP concentrations restored translation (Fig. 3A, lanes 9 and 10) and PFU formation (*19*).

At 125 μM each of GTP, CTP, and UTP and 1 mM ATP, the first PFUs were detected between 5 and 10 hours of incubation, while maximal virus production (~41,000 PFUs per milliliter) was achieved at 15 to 20 hours. If edeine, an inhibitor of in vitro translation (*20*), was added after 9 hours of incubation, the increase in PFUs between 9 and 15 hours was markedly inhibited (from

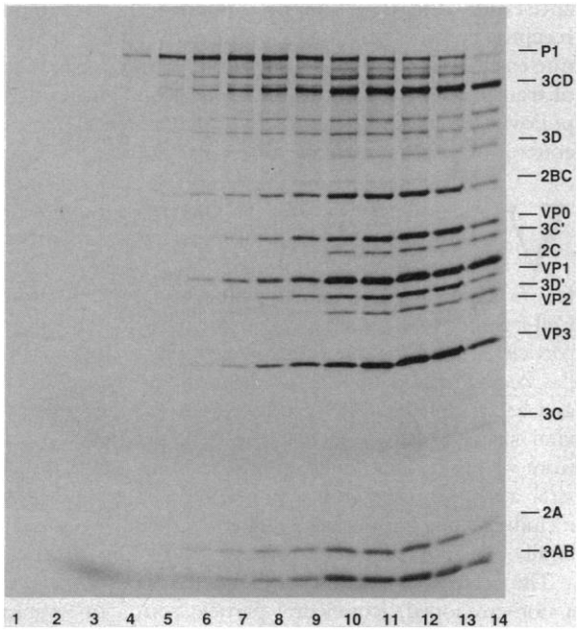


**Fig. 1.** Time course of synthesis of poliovirus polypeptides in a HeLa S10 extract (*12*) complemented with a translation mixture and viral mRNA. The in vitro incubation mixtures (250 μl) contained 93 μl of S10 extract with the following additions: 1 mM ATP, 50 μM GTP, 10 mM creatine phosphate, 6 μg creatine phosphokinase, 2 mM DTT, 6 μg of calf liver tRNA, 12 μM each of all amino acids except methionine, 300 μCi of [$^{35}$S]methionine, 18 mM Hepes (pH 7.4), 240 μM spermidine, 0.1 M K acetate, 0.35 mM Mg acetate, 0.75 mM Mg chloride and 2 μg of PV1(M) RNA [the $K^+$ and $Mg^{2+}$ ions were added in addition to those contributed from the dialysis buffer (*12*)]. Incubation was for 18 hours at 30°C. At each time point samples (20 μl) were removed, mixed with RNAse A (to 20 μg/ml), RNAse T1 (to 100 U/ml), and cyclohexamide (to 5 μg/ml), incubated for 5 min at 30°C, and frozen immediately. Portions (6 μl) were analyzed on 12.5% SDS-polyacrylamide gels. Gels were fixed, treated with EN$^3$HANCE, dried, and exposed for 15 to 24 hours to x-ray film at −70°C. Lane 1, no RNA; lanes 2 to 13, incubation at 0, 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 15, and 18 hours, respectively; lane 14, cytoplasmic extract of [$^{35}$S]methionine-labeled PV1(M) infected HeLa cells as marker.

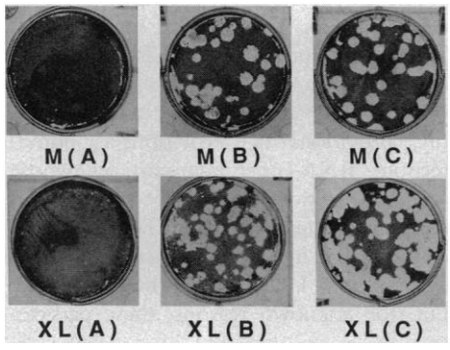


**Fig. 2.** Synthesis of poliovirus in the cell-free incubation mixture. Messenger RNAs were either derived from plasmid DNA such as pT7PVXL2 that was transcribed with phage T7 RNA polymerase (*16*), or from purified PV1(M). Translation reactions were identical to those described in Fig. 1, except that 2 μl of 1 mM cold methionine and 300 ng each of pT7PVXL2 RNA and PV1(M) RNA were incubated in a total incubation volume of 37.5 μl. Incubation was for 15 hours at 30°C. The samples were then treated with RNAse A (20 μg/ml) and RNAse T1 (100 U/ml) for 30 min at room temperature, diluted to 200 μl with PBS, and added to HeLa cell monolayers. Plaques were scored after 48 hours of incubation as described (*22*). XL(A) and M(A), pT7PVXL2 and PV1(M) RNAs, respectively, were incubated with HeLa extract without addition of the translation mixture. XL(B) and M(B), complete incubation mixtures programmed with pT7PVXL2 and PV1(M) RNAs, respectively. XL(C) and M(C), HeLa monolayers were infected with virus stocks derived from pT7PVXL2 RNAs or from PV1(M), respectively.

**Table 1.** Effect of antibodies and inhibitors on the synthesis of poliovirus in the incubation mixture. Conditions were as in Fig. 2 except that concentrations of GTP, CTP, and UTP were adjusted to 125 μM each; different batches of HeLa extracts were used; and PFUs were measured after 15 hours of incubation. The "−" and "+" refer to the absence or presence of the indicated treatment, except as noted. ND, not determined. MAb D171 is a poliovirus receptor–specific antibody. Anti-PV1(M) and anti-PV3(L) are neutralizing antisera specific for types 1 and 3 poliovirus, respectively.

| RNA | PFU per milliliter | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Guanidine HCl (2 mM) | | MAb D171 | | Anti-PV1(M) | | Anti-PV3(L) | | $Mg^{2+}$* | | Edeine (4 μM) | |
| | − | + | − | + | − | + | − | + | − | + | − | + |
| PV1(M)$g^s$ | 24,948 | 0 | 24,965 | 0 | 21,868 | 0 | 15,932 | 15,924 | 0 | 12,000 | 12,000 | 0 |
| PV1(M)$g^r$ | 400 | 2,200 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

*: "−", lysate contained 1.5 mM $Mg^{2+}$; and "+" lysate contained 2.6 mM $Mg^{2+}$.

40-fold in the absence of edeine to 16-fold in its presence). We conclude that translation-dependent PFU formation continues beyond 9 hours, but that the maturation of infectious virus lags behind protein synthesis.

The stimulation of PFU formation by addition of NTPs suggested the involvement of RNA replication. Infectious virions might also have been formed, in part, by encapsidation of mRNA into capsid proteins that were synthesized de novo. Guanidine hydrochloride (at 2 mM) is an inhibitor of poliovirus RNA replication in HeLa cells (*21, 22*). At this concentration, the drug has no adverse effect on the metabolism and growth of HeLa cells for several divisions (*21*). Addition of guanidine HCl (to 2 mM) to the incubation mixture had no apparent effect on the translation of RNA derived from wild-type poliovirus (Fig. 3B, lane 6) but it completely prevented the formation of PFUs [PV1(M)$g^s$ (Table 1)]. In contrast, when RNA from guanidine-resistant poliovirus [PV1(M)$g^r$] (*23*) was used as template for translation (Fig. 3B, lane 4), infectious virus was produced in the presence of the drug (Table 1).

In several experiments it appeared as if synthesis of PV1(M)$g^r$ in vitro was guanidine-dependent to some degree (Table 1). We cannot explain this result at present, particularly since the PV1(M)$g^r$ isolate used in these experiments was not drug-dependent in vivo.

In poliovirus, virion RNA is, by definition, plus-stranded RNA because it is of messenger-sense polarity (*24*), although plus and minus strands are synthesized during viral replication (*25*). It is unlikely that poliovirions contain minus strands (*26*) and, therefore, the detection of such strands would be indicative of virus-specific RNA synthesis. We have analyzed total RNA of the incubation mixture for poliovirus-specific minus strands by cDNA synthesis and amplification with the polymerase chain reaction (PCR) (*27*). Two primers chosen (*27*) were predicted to yield a DNA product of 178 nucleotides if poliovirus-specific RNA is used as a template; the reaction is specific for the amplification of minus strands if the plus strand primer is used for cDNA synthesis. A DNA product of the predicted length was observed when pT7PVXL2 DNA, containing poliovirus infectious cDNA, was used as a template (Fig. 4, lane 1). A similar band was obtained with total RNA of the incubation mixture after 15 hours of translation (Fig. 4, lane 2). The identity of this band was confirmed by sequence analysis (*19*). No band was found when the procedure was carried out at 0 hours of incubation (Fig. 4, lane 4), a control confirming that virion RNA used in this translation does not contain minus strands detectable by this method (*28*). Similarly, no band was seen when the in vitro reaction was carried out with viral RNA in the absence of the translation mixture (Fig. 4, lane 5). A band of the expected size was observed when virion RNA was used as a template for cDNA synthesis, but this required priming with the minus strand prim-

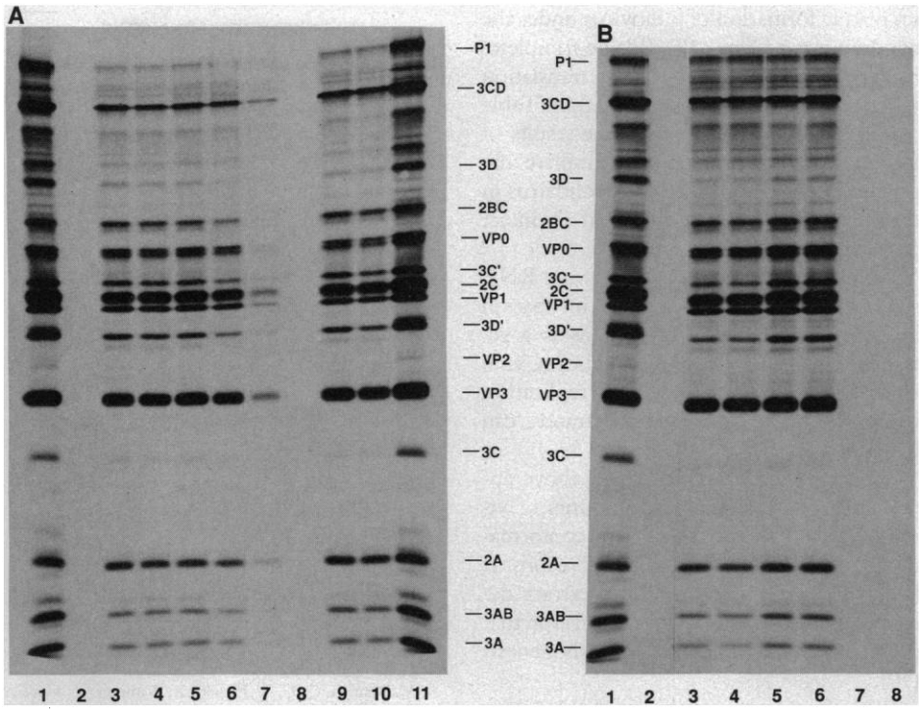


**Fig. 3.** Translations of poliovirus RNA in the presence of added ingredients. (**A**) Effect of NTPs on translation that was carried out as described in Fig. 1 except 100 ng of PV1(M) RNA were incubated in a total volume of 12.5 μl. Incubation was for 15 hours at 30°C. All samples contained 1 mM ATP. Lanes 1 and 11, marker polypeptides; lane 2, no RNA; lane 3, 50 μM GTP but no CTP and UTP. Samples of lanes 4 to 8 contained 62.5, 125, 250, 500, and 1000 μM CTP, UTP, and GTP each, respectively. Samples in lanes 9 and 10 contained, in addition to 500 and 1000 μM NTPs, 3.1 mM $Mg^{2+}$ and 3.35 mM $Mg^{2+}$, respectively. The products were analyzed on 15% polyacrylamide gels. (**B**) Effect of inhibitors and salt on translation that was carried out as described in (A), lane 5. Lane 1, marker polypeptides; lane 2, no RNA; lanes 3 and 4, translation of RNA of PV1(M)$g^r$ in the absence and presence of guanidine HCl, respectively. Lanes 5 to 8, translation of PV1(M) RNA in the absence (lane 5) and presence (lane 6) of 2 mM guanidine HCl; lane 7, in the presence of 1.5 mM $Mg^{2+}$ (total $Mg^{2+}$ concentration); lane 8, in the presence of 4 μM edeine. The products were analyzed as in Fig. 3A.

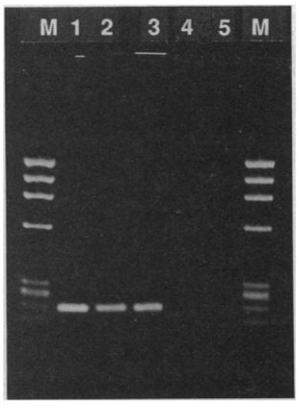


**Fig. 4.** Detection by polymerase chain reaction (PCR) of PV1(M)-related RNAs on an agarose gel. Synthesis of cDNA and PCR was as described (*27*). M, ØX174 DNA, digested with Hae III; lane 1, pT7PVXL2 DNA as a template; lanes 2 and 4, PCR product of cDNA synthesized with primer 1 from total RNA of the complete incubation mixture [translation programmed with PV1(M) RNA as template, as in Fig. 1] after 15 and 0 hours of incubation, respectively; lane 3, PCR product of cDNA synthesized from PV1(M) RNA with primer 2; lane 5, PCR product of cDNA synthesized with primer 1 from total RNA of the HeLa cell extract to which PV1(M) RNA, but no translation mixture, was added, and which was incubated for 15 hours at 30°C. Samples (10 μl) of the amplification reactions (*27*) were separated on an 0.8% agarose gel containing ethidium bromide and the fluorescent DNA bands were visualized on a transilluminator.

er (Fig. 4, lane 3). These results show that poliovirus-specific minus strands were synthesized during translation of viral RNA in vitro, an observation suggesting that active poliovirus RNA polymerase was generated. When very low concentrations of total RNA from the incubation mixture were used for cDNA synthesis and PCR, the yield of minus strand RNA was estimated to be 5 μg per milliliter of incubation mixture. Because of the methodology used, however, this can only be a rough estimate.

To analyze the nature of the infectious material formed in the HeLa cell-free extract, we tested the effect of the receptor-specific monoclonal antibody (MAb) D171 and of type-specific hyperimmune sera on plaque formation (Table 1). MAb D171 binds to the cellular receptor of poliovirus, thereby completely blocking infection with all three serotypes of poliovirus (*29*). Treatment of HeLa cells with MAb D171 prior to addition of the incubation mixture completely inhibited plaque formation. Similarly, no plaques were observed when the incubation mixture was treated with poliovirus type 1–specific rabbit hyperimmune serum [anti-PV1(M)]. Neutralization of the in vitro synthesized virus was type-specific: hyperimmune serum to poliovirus type 3 (Leon) did not reduce the titer in the incubation mixture (Table 1). These results suggest that intact poliovirions of serotype 1 were synthesized in the cell-free extract, and that on subsequent incubation with HeLa cells, these virions entered the cells by a poliovirus receptor-mediated pathway.

It is highly unlikely that even a small fraction of intact HeLa cells remained in the HeLa cell extract after several cycles of centrifugation, dialysis, and freeze-thawing. If 0.01% of the cells survived, 5 μl of the extract would contain 100 cells. To exclude the possibility that the infectious virus found after translation grew in such a residual fraction of HeLa cells, we prepared an incubation mixture that contained 1.5 mM $Mg^{2+}$ rather than 2.6 mM $Mg^{2+}$. The lower $Mg^{2+}$ concentration does not allow poliovirus RNA translation, but is sufficient for the propagation of HeLa cells in tissue culture. Neither poliovirus proteins (Fig. 3B, lane 7) nor infectious virus (Table 1) was found after 15 hours of incubation in the presence of 1.5 mM $Mg^{2+}$. Furthermore, we could not detect intact cells when cell extracts were inspected microscopically (*19*). Finally, we added edeine to the incubation mixture, a drug that inhibits translation in vitro, but not in vivo (*19, 20*), and which has no effect on plaque formation of poliovirus under the conditions used here (*19*). Edeine completely inhibited in vitro poliovirus translation (Fig. 3B, lane 8) and virus formation (Table 1). These data, together with the results of PCR and NTP stimulation, eliminate the possibility that the synthesis of poliovirus in the incubation mixture occurred in intact cells.

Our data suggest that de novo viral RNA synthesis occurs in the cell-free incubation mixture, and that RNA synthesis is a requirement for assembly. Possibly only viral strands newly released from a replication complex, or even only nascent strands, can be encapsidated.

The cell-free extract described above apparently provides sufficient amounts of virus-specific capsid proteins in a conformation suitable for the formation of virions. It is also possible that the HeLa extract described here contains a host cell factor that participates in poliovirus morphogenesis (*10*).

It has been assumed that viral RNA synthesis requires cellular membranes (*30, 31*). Purified poliovirus RNA polymerase $3D^{Pol}$, on the other hand, is active in the absence of membranes and it is also not inactivated by mild detergent (*32*). It is possible that the HeLa cell extract described here contains sufficient amounts of membranous material to allow viral RNA synthesis and assembly. Whether or not addition of membrane vesicles will stimulate virus production in vitro remains to be seen. We have observed considerable covariation in translation efficiency of viral RNA and virus yield (500 to 45,000 PFUs per milliliter of incubation mixture) from different cell extracts, a fluctuation which we cannot explain at present. Apart from an extreme sensitivity of the efficiency of translation to small differences in poliovirus mRNA and $Mg^{2+}$ concentrations, it is possible that limiting factors for translation or for viral replication (or both) in the extract may vary in their abundance and activity, dependent on extract preparation.

Our results demonstrate the in vitro synthesis of a self-replicating entity whose living or nonliving nature has been debated since the discovery of viruses (*33*). For the chemist, poliovirus is a highly ordered aggregate of five species of macromolecules (*5, 34*) with an empirical formula of its organic matter of $C_{332,652}$ $H_{492,388}$ $N_{98,245}$ $O_{131,196}$ $P_{7501}$ $S_{2340}$ (*5, 35*). The test tube synthesis of this chemical, starting with viral RNA as a template for protein synthesis, allows the study of the biochemical and genetic properties of the virus, and traits of its replication, without the barrier of the cell membrane.

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(S10 lysate) was dialyzed for 2 hours against 100 volumes of 10 mM Hepes, 90 mM potassium acetate, 1.5 mM Mg acetate, and 2.5 mM DTT, pH 7.4. The dialyzed S10 lysate was again centrifuged for 10 min (10,000 rev/min, SS34 rotor), and the supernatant was stored at −80°C for at least 24 hours. The S10 lysate was thawed and centrifuged at 4°C for 10 min at 10,000 rev/min in an Eppendorf centrifuge. The supernatant (S10 extract) was treated with micrococcal nuclease (15 μg per milliliter of extract) in the presence of $CaCl_2$ (7.5 μl of 0.1 M $CaCl_2$ per milliliter of extract) at 20°C for 15 min. The reaction was terminated by the addition of 200 mM EGTA (15 μl per milliliter of extract). The S10 extract was adjusted to 10% glycerol and stored in small portions at −80°C. Stringent control of the pH of all buffers during the preparation of the extract was found necessary. The most important parameters for the efficiency of translation were the concentrations of viral mRNA and $Mg^{2+}$, as small deviations from maximal conditions led to drastically reduced protein synthesis.

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# Intrasubunit Signal Transduction by the Aspartate Chemoreceptor

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**Receptors that transmit signals across cell membranes are typically composed of multiple subunits. To test whether subunit interactions are required for transmembrane signaling by the bacterial aspartate receptor, dimers were constructed with (i) two full-length subunits, (ii) one full-length subunit and one subunit lacking the cytoplasmic domain, or (iii) one full-length subunit and one subunit lacking both the cytoplasmic and the transmembrane domains. Methylation of the cytoplasmic domain of all three receptor constructs was stimulated by the binding of aspartate. These findings demonstrate that transmembrane signaling does not require interactions between cytoplasmic or transmembrane domains of adjacent subunits and suggest that signaling occurs via conformational changes transduced through a single subunit.**

SPECIFIC TRANSMEMBRANE RECEPTORS mediate the detection of a wide variety of extracellular ligands, such as growth factors, hormones, nutrients, and neurotransmitters (*1*). Binding of a ligand to the extracellular domain of such a receptor results in a change in the signaling properties of the cytoplasmic domain of the protein. The aspartate receptor from *Salmonella typhimurium* is a member of a family of transmembrane receptors in which globular extracellular and cytoplasmic domains are separated by one or two membrane-spanning segments (*2*). They form a class of receptors distinct from the receptors with seven transmembrane segments, such as rhodopsin and the β-adrenergic receptor (*3*). Transmembrane signal transduction by the aspartate receptor occurs within a stable dimeric receptor (*4*), but the question of how a conformational change is transduced across the membrane remains unanswered. Two possible mechanisms for signal transduction are: (i) an intersubunit mechanism in which a change in the relative position of the ligand-binding domains of the dimeric receptor leads to a change in the relative positions of the cytoplasmic domains of the receptor, or (ii) an intrasubunit mechanism in which a conformational change occurs independently within each subunit of the dimer. To differentiate between these possibilities, we constructed a series of hybrid receptors (Fig. 1) that contain subunits lacking one or two of the three domains of the receptor. The first hybrid, the 1 + 2/3 receptor, contained one full-length subunit and one truncated subunit consisting of the ligand-binding domain and the transmembrane domain (residues 1 through 259). A second hybrid, the 1 + 1/3 receptor, contained one full-length subunit and one subunit consisting of the ligand-binding domain alone. The wild-type receptor, 1 + 1 receptor, was included as a positive control in each experiment. To evaluate the requirement for interaction between subunits during signal transduction, we tested each of these receptors for its ability to transmit a transmembrane signal.

We constructed hybrid aspartate receptors by disulfide cross-linking full-length and

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