Proc. Nat. Acad. Sci. USA
Vol. 71, No. 12, pp. 4773–4777, December 1974

Isolation of a Viral Polypeptide Associated with Poliovirus RNA Polymerase

(picornavirus/replication complex/replicase)

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Communicated by James E. Darnell, Jr., September 16, 1974

ABSTRACT **Poliovirus-infected HeLa cells were labeled with radioactive methionine or phenylalanine and subjected to a new purification procedure for the viral induced RNA polymerase activity. Detergent-solubilized polymerase activity was purified by precipitation with 2 M LiCl and sedimentation through sucrose gradients. Approximately 0.001% of the incorporated amino acid radioactivity sediments with the peak of polymerase activity. Gradient fractions comprising the polymerase activity peak were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and found to contain predominantly one virus-specific polypeptide. Polyacrylamide gel electrophoresis also reveals that this purified polypeptide migrates with a 58,000 molecular weight noncapsid poliovirus polypeptide.**

At least 14 virus-specific polypeptides can be detected in poliovirus-infected HeLa cells (1), all derived from post-translational proteolytic cleavage of a large precursor molecule (2–4). Although the general relationship of these polypeptides to the cleavage pattern is known (5), no enzymatic function has been assigned to any of the viral polypeptides. Poliovirus RNA polymerase activity is easily measured in crude extracts (6, 7), but the lack of a suitable purification procedure has prevented the identification of the viral polypeptides necessary for the enzymatic activity. Previous attempts to purify the polymerase activity from picornavirus-infected cells have shown that the polymerase activity sediments as a membrane-associated complex of 60–250 S (8–14) and is associated with many viral polypeptides (15).

We have found that precipitation with 2 M LiCl results in significant purification of the polio polymerase complex. Details of the purification procedure and properties of the purified polymerase will be reported elsewhere (Lundquist and Maizel, manuscript in preparation). Here, we demonstrate that only one virus-specific polypeptide remains with the polymerase activity and probably represents the viral component of the polio polymerase.

MATERIALS AND METHODS

Labeling of Infected Cells. S_3 HeLa cells were grown as previously described (1). Washed cells were suspended at 5×10^6 cells per ml and infected with 150 plaque-forming units per cell of purified Mahoney strain poliovirus type 1. Actinomycin D (4 μg/ml) was added 10 min after infection. The radioactive amino acid (25 μCi/ml) was added to infected cells incubated in medium containing normal levels of all other essential amino acids. The medium concentration of the radioactive amino acid was reduced to 2% of the concentration normally present in Eagle's minimum essential medium. The medium was supplemented with 5% fetal calf serum 30 min after infection.

Levels of guanidine that have no discernible effect on HeLa cells inhibit the replication of poliovirus, but viral inhibition of host cell protein synthesis is unaffected (1). Virus-specific proteins can then be labeled after reversal of guanidine inhibition. Guanidine (1 mM) was added 15 min after infection with poliovirus. After incubation at 37° for 1.5 hr, the infected cells were harvested by centrifugation, washed with Earle's isotonic salt solution, and resuspended in medium without guanidine, containing 5% fetal calf serum. The radioactive amino acid was added after 15 min, and the infection was stopped 2.5 hr after removal of guanidine.

The mechanism of guanidine inhibition is unknown (16), but some viral functions are expressed during guanidine inhibition (17). If a precursor to the poliovirus polymerase were synthesized during guanidine inhibition, it could escape labeling by the preceding procedure. Therefore, some preparations were made by the addition of radioactive amino acid 30 min after poliovirus infection in the absence of guanidine. Under these conditions, most (60%) amino-acid incorporation occurs before the shutoff of host protein synthesis. Labeled cells were harvested 3 hr after infection.

Poliovirus Polymerase Purification. The detergent-solubilized polymerase was purified from poliovirus-infected cells as previously described (12) and stored at −80°. Crude lysate refers to the infected cells after lysis by freezing and thawing three times. Centrifugation of detergent-solubilized polymerase at 100,000 × *g* for 90 min at 4° yielded a clear amber pellet. This ribosomal pellet was solubilized in one-tenth the volume of Tris buffer (0.01 M Tris·HCl, pH 8.0, 0.01 M NaCl). An equal volume of 4 M LiCl–Tris buffer was added and the solution was stored at −9° for 12–16 hr. The suspended RNA was sedimented (2500 × *g*, 10 min) and the supernatant discarded. The RNA pellet was resuspended in the same volume of 2 M LiCl–Tris buffer for several hours, and the washed pellet was collected by centrifugation and dissolved in Tris buffer. The sample was heated to 37° for less than a minute and any remaining insoluble material was re-

Abbreviations: EMC, encephalomyocarditis; NCVP, noncapsid viral polypeptide.

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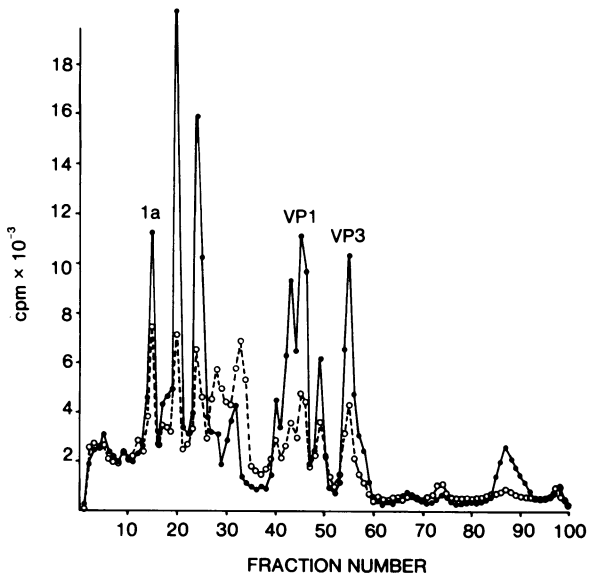

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of LiCl pellets of methionine-labeled poliovirus-infected HeLa cells. A sample of the solubilized LiCl pellet from 2×10^7 cells labeled with [^{3}H]methionine after guanidine reversal (●——●) was combined with the solubilized LiCl pellet from an equal number of cells labeled with [^{35}S]methionine immediately after infection (○- - -○). The combined sample was dissolved in sample buffer (18) and analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis. Here, as in Fig. 3, the anode is to the right. Poliovirus polypeptides are designated by numbers as previously described (1), except that the noncapsid viral polypeptide (NCVP) prefix has been omitted from the designation.

moved by centrifugation. The solubilized LiCl pellet contains the poliovirus polymerase activity.

The solubilized LiCl pellet from 3×10^8 infected cells (0.6 ml) was sedimented through 12-ml gradients of 15–30% (w/w) sucrose in 0.01 M Tris·HCl, pH 7.4, 0.1 M NaCl. Gradients were spun at 40,000 rpm for 6.5 hr in the Spinco SW 41 rotor at 4°. Fractions (0.55 ml) were collected from the bottom of each gradient. Sedimentation coefficients of gradient fractions with enzyme activity were estimated with the aid of radioactive HeLa cytoplasmic RNA spun in a separate gradient.

TABLE 1. *Recovery of in vivo incorporated radioactivity* during partial purification of poliovirus RNA polymerase activity*

Fraction	*In vivo* label		
	[^{35}S]Met†	[^{3}H]Met‡	[^{3}H]Phe†
Crude lysate	2,990	1,180	900
Detergent-soluble polymerase	1,040	598	390
Ribosomal pellet	131	108	62
Soluble LiCl pellet	12	12	7

* All values expressed as 10^6 hot trichloroacetic acid-precipitable cpm/10^9 cells.

† Radioactive amino acid was added 30 min after virus infection.

‡ Radioactive amino acid was added 15 min after removal of guanidine.

Separate aliquots of 60 μl from specific gradient fractions were analyzed for RNA polymerase activity and for *in vivo* incorporated radioactivity.

Assay of Gradient Fractions for RNA Polymerase Activity. Enzyme activity was assayed in a final volume of 100 μl. The assay mixture contained 60 μl of gradient fraction; 150 μM each of ATP, UTP, and CTP; 0.25% Nonidet P-40; 0.125% sodium deoxycholate; 10 μCi [^{3}H]GTP; 80 mM Tris·HCl, pH 8.0; and 8 mM $MgCl_2$. After 30 min of incubation at 37°, the reaction was stopped by the addition of 0.5 ml of saturated sodium pyrophosphate followed by 1 ml of cold 10% Cl_3CCOOH. Acid-insoluble material was collected on Whatman GF/C filters which were dried and counted in 5 ml of Aquasol (New England Nuclear Corp.).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Cylindrical 10% polyacrylamide gels, 20 cm in length, were prepared the day before use, using the sodium dodecyl sulfate-disc system (18). Sample volumes of up to 1 ml were applied to polyacrylamide gels containing 1.5 ml of stacking gel. After electrophoresis for 22 hr at 5 V/cm, gels were fractionated with the Savant Autogeldivider. The radioactivity of gel fractions was measured in a Triton X-100–toluene based scintillation mixture.

Materials. [^{35}S]Methionine (187 Ci/mmol), [^{3}H]methionine (6.25 Ci/mmol), [^{3}H]phenylalanine (5.4 Ci/mmol) and [^{3}H]-guanosine triphosphate (5.6 Ci/mmol) were purchased from New England Nuclear Corp. Actinomycin D was a generous gift from Merck and Co., Rahway, N.J. Ribonuclease-free sucrose from Schwarz/Mann was used for all sucrose gradients. Fisher Scientific Co. supplied the LiCl.

RESULTS

Our purification procedure for the poliovirus RNA polymerase was designed to isolate the enzyme bound to its endogenous template in an active ribonucleoprotein complex. Concentrations of monovalent salts that disrupt ribosomes and precipitate high-molecular-weight single-stranded nucleic acids (19) do not affect the polio polymerase activity (12). Under these salt conditions, the poliovirus polymerase activity is quantitatively precipitated, presumably due to its association with the RNA template. Thus, treatment of polio polymerase samples with 2 M LiCl dissociates contaminating ribosomes and precipitates ribosomal RNA and polymerase activity. After treatment with 2 M LiCl, the polio polymerase activity has an apparent sedimentation coefficient of 25 S. The activity found in the LiCl pellet is insensitive to Actinomycin D and is not found in preparations from uninfected HeLa cells (Lundquist and Maizel, manuscript in preparation).

Poliovirus-infected HeLa cells were labeled with a radioactive amino acid either 30 min after infection or immediately after guanidine reversal. Table 1 summarizes the recovery of radioactivity during LiCl purification of three different *in vivo*-labeled preparations. In contrast to the 1% or less recovery of radioactivity in the solubilized LiCl pellet, greater than 20% of the enzymatic activity of the crude lysate is found in the solubilized LiCl pellet of unlabeled infected HeLa cells.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to analyze the methionine-labeled polypeptides found in the solubilized LiCl pellet (Fig. 1). Despite a signifi-

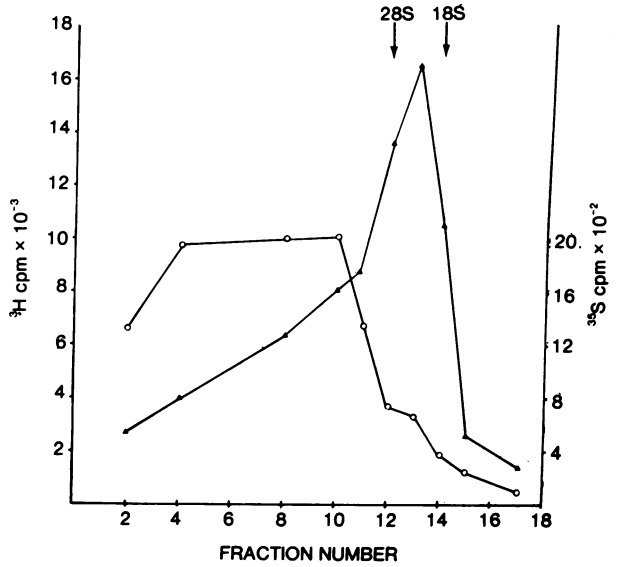

FIG. 2. Sucrose sedimentation profile of the solubilized LiCl pellet from cells labeled with [^{35}S]methionine immediately after infection. Aliquots (60 μl) of gradient fractions were analyzed for acid-precipitable [^{35}S]methionine incorporated *in vivo* (○). Separate 60-μl aliquots were assayed for RNA polymerase activity. Methionine cpm were subtracted from the polymerase assays to give the net incorporation of [^{3}H]GMP into acid-precipitable radioactivity (▲). Refer to *Materials and Methods* for additional details.

cant increase in the specific activity of the viral RNA polymerase, all viral polypeptides seen in the crude lysate of infected cells (compare Fig. 1 with Fig. 3) are also found in the LiCl pellet, albeit in different proportions. The labeled polypeptides in the solubilized LiCl pellet exist as large complexes in the undenatured state, since most (80%) of the amino-acid radioactivity is excluded from Sepharose 2B columns that include polio virus and polio RNA (data not shown).

Sucrose gradient centrifugation provides a simple method for further purification of the solubilized LiCl pellet. Fig. 2 illustrates that almost all the [^{35}S]methionine radioactivity from the solubilized LiCl pellet sedimented faster than marker 28S RNA. The poliovirus RNA polymerase activity of the [^{35}S]methionine solubilized LiCl pellet had a sedimentation coefficient of 25 S. Consistently, a small shoulder of *in vivo* amino-acid radioactivity sedimented with the RNA polymerase activity. Gradient fractions containing the peak of RNA polymerase activity were pooled and analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis. Fig. 3 demonstrates that predominantly one viral polypeptide is found in gradient fractions containing the peak of polymerase activity. This purified polypeptide migrated with noncapsid viral polypeptide (NCVP) 4 on dodecyl sulfate–polyacrylamide gel electrophoresis. NCVP 4 has previously been identified as a 58,000 molecular weight noncapsid viral polypeptide found in poliovirus-infected cells (20). Thus, the apparent molecular weight of the purified polypeptide is 58,000 (18).

The sucrose gradient isolation procedure has been repeated on LiCl-purified preparations from the [^{3}H]methionine- and [^{3}H]phenylalanine-labeled samples described in Table 1. In both cases, dodecyl sulfate–polyacrylamide gel electrophoresis of gradient fractions containing the peak of polymerase activity revealed one predominant labeled polypeptide that had an apparent molecular weight of 58,000. Table 2 summarizes the recovery of amino-acid radioactivity in this polypeptide. It has been estimated previously, based on the extent and kinetics of viral RNA synthesis, that the poliovirus-infected cell contains 3000 active viral polymerase molecules (21). If, as shown in Table 2, our results are extrapolated to the unfractionated infected cell, an estimate of 4,800 to 11,500 poly-

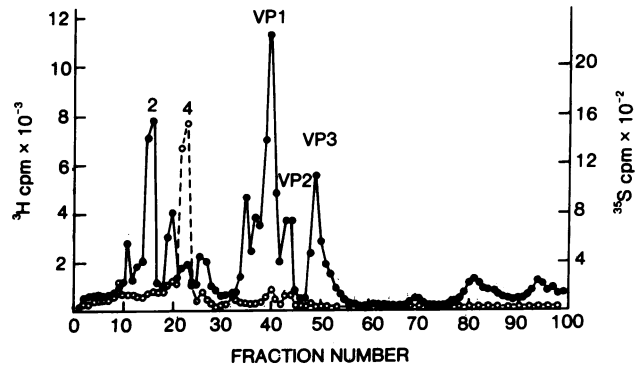

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel analysis of the LiCl pellet purified on a sucrose gradient. Gradient fractions containing the peak of polio polymerase activity were combined (0.4 ml each from fractions 12 and 13, Fig. 2), diluted with concentrated sample buffer minus glycerol, heated, and analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis (18). [^{3}H]Methionine-labeled crude lysate from infected cells labeled after guanidine reversal was included (●——●) for comparison with the *in vivo* incorporated [^{35}S]methionine (○- - -○).

TABLE 2. *Radioactive amino-acid incorporation into the purified putative poliovirus polymerase and estimated cellular concentration*

	In vivo label		
	[^{35}S]Met	[^{3}H]Met	[^{3}H]Phe
Isolated polymerase cpm/10^9 cells*	18,200	9,200	8,000
Total polymerase cpm/10^9 cells†	455,000	230,000	200,000
Polymerase polypeptides per cell‡	4,800	11,500	9,500

* Samples from the labeled preparations described in Table 1 were purified as described in the *text*. Isolated polymerase cpm were calculated from the radioactivity in the 58,000 molecular weight polypeptide isolated by dodecyl sulfate–polyacrylamide gel electrophoresis.

† Total polymerase cpm were calculated based on the assumption that recovery of enzyme activity was identical to the recovery of radioactivity in the 58,000 molecular weight polypeptide. Purification of unlabeled crude lysate demonstrated that 4% of the enzyme activity of the crude lysate was applied to the polyacrylamide gel. We therefore assumed that the isolated polymerase cpm represented 4% of the total polymerase cpm present in the crude lysate.

‡ The number of putative polymerase polypeptides per cell was estimated by assuming that the 58,000 molecular weight polypeptide had an average amino acid composition and contained 8 methionine and 14 phenylalanine residues (29).

merase polypeptides per cell is obtained. Thus, the small amount of *in vivo* radioactivity isolated with the purified polio polymerase is compatible with an independent estimate of the cellular concentration of polymerase molecules.

Radioactive material sedimenting faster than the 28S marker was also analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). The gel pattern of this material revealed a complex polypeptide pattern that resembled the pattern seen in the solubilized LiCl pellet (Fig. 1). In agreement with the enzyme activity profile (Fig. 2), the 58,000 molecular weight polypeptide was found in decreasing concentration towards the bottom of the gradient. In contrast, the minor polypeptides found in gradient fractions containing the peak of polymerase activity (Fig. 3) became more abundant in fractions containing faster sedimenting radioactivity.

DISCUSSION

The results reported here show that purification of the poliovirus RNA polymerase activity results in the isolation of an enzymatically active ribonucleoprotein complex which contains predominantly one virus-specific polypeptide. The association of the viral polymerase activity with this viral polypeptide suggests, but does not prove, that the 58,000 molecular weight polypeptide is the viral component of the polio polymerase. If this polypeptide is not responsible for the polymerase activity, then the polymerase activity must be due entirely to a modified host protein or to a viral polypeptide that does not incorporate significant radioactivity under our labeling conditions. The latter possibility is unlikely, since purification of infected cells labeled with methionine or phenylalanine, whether labeled immediately after infection or after guanidine reversal, results in the same simple polyacrylamide gel pattern.

We believe that the 58,000 molecular weight polypeptide is the viral component of the polio polymerase. Apparently no other viral polypeptide is required for the expression of the purified polymerase activity. The minor amounts of other labeled polypeptides seen in Fig. 3 are probably due to incomplete separation of polymerase activity from nonessential labeled polypeptides. Identity between the polymerase-associated viral polypeptide and NCVP 4 is suggested by coelectrophoresis of the two polypeptides on dodecyl sulfate–polyacrylamide gels. Previously reported results support the conclusion that NCVP 4 is the viral component of the polio polymerase. Genetic analysis of poliovirus mutants revealed that the region responsible for RNA synthesis was located at the opposite end of the genome from that coding for capsid proteins (22). Subsequent mapping of the poliovirus genome demonstrated that the capsid proteins are derived from NCVP 1a, which is translated from the 5′ end of the genome (23). NCVP 1b is translated from the 3′ end of the genome and undergoes the cleavage sequence NCVP 1b → NCVP 2 → NCVP 4 (5). The conclusion that NCVP 4 is the polio polymerase is consistent with the genetic analysis and indicates that the polio polymerase results from the final cleavage of precursor polypeptides translated from the 3′ end of the poliovirus RNA.

Not all NCVP 4 polypeptides are involved in viral RNA synthesis. On the basis of the estimates given in Table 2, we calculate that only 1% of the NCVP 4 polypeptides in the crude lysate are associated with the poliovirus replication complex. It, therefore, appears that proteolytic cleavage to form NCVP 4 is necessary, but not sufficient, in the assembly of an active poliovirus replication complex.

A polycytidylate-dependent RNA polymerase activity has been isolated from encephalomyocarditis (EMC) virus-infected baby hamster kidney cells (24). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of this purified activity, believed to be derived from the EMC replicase, revealed five major polypeptides, one of which had an apparent molecular weight of 57,000. The EMC equivalent of NCVP 4 is polypeptide E, which has a molecular weight of 56,000 in HeLa cells (5). This EMC preparation therefore contains a polypeptide analogous to the polypeptide believed to be the viral component of the polio polymerase.

Similarities in the replication of picornaviruses and the RNA bacteriophages, exemplified by polio and Qβ, respectively, have been previously noted (25). Qβ replicase has been extensively purified and exists as a complex of three host polypeptides and one viral polypeptide (26, 27). No comparable purification has been reported for a picornavirus polymerase, and it is not known whether the replication complex from picornavirus-infected animal cells contains polypeptides analogous to those found in Qβ replicase. The results reported here suggest that poliovirus polymerase, like Qβ replicase (28), contains one viral polypeptide that is translated from the 3′ end of the viral genome. The viral component of Qβ replicase has a molecular weight of 65,000 and is, therefore, slightly larger than the viral component of polio polymerase. Further experiments are required to determine if host proteins are an essential component of the polio polymerase.

We are grateful to Dr. D. F. Summers for encouragement and many helpful discussions. This work was supported by grants from the National Institutes of Health AI 10216, National Science Foundation GB 18026, American Cancer Society VC-33 and from a National Institute of Health Center Grant 613-4707. J. V. M. is the recipient of an American Cancer Society Award PRA-69 and E. E. is supported by Public Health Service Career Development Award IK04 AI 70020.

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