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Identification of Poliovirus Polypeptide P63 as a Soluble RNA-Dependent RNA Polymerase

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A poliovirus-specific RNA-dependent RNA polymerase was isolated from a cytoplasmic extract of infected HeLa cells and was shown to copurify with a single virus-specific protein. The polymerase was isolated from cells labeled with [^{35}S]-methionine and was fractionated from other soluble cytoplasmic proteins by ammonium sulfate precipitation, phosphocellulose chromatography, gel filtration on Sephacryl S-200, and chromatography on hydroxylapatite. The activity of the enzyme was measured by using either polyadenylic acid or poliovirion RNA as a template in the presence of an oligouridylic acid primer. A single virus-specific protein that had an apparent molecular weight of 63,000 (p63) was found to copurify with this activity. Host-coded proteins were present in reduced molar amounts relative to p63. Noncapsid viral protein 2 (NCVP2) and other viral proteins were clearly separated from p63 by gel filtration on Sephacryl S-200. Polymerase activity coeluted from the column precisely with p63. NCVP2 was totally inactive as an RNA polymerase and did not stimulate the polymerase activity of p63. The purified enzyme sedimented at about 4S on a glycerol gradient and thus appeared to be a monomer of p63. Two-dimensional gel electrophoresis of the polymerase protein indicated that it had an isoelectric point of about 7.5. Thus, the viral polypeptide, p63, as defined by the above physical parameters, is an RNA-dependent RNA polymerase that can copy poliovirion RNA when oligouridylic acid is used as a primer.

Poliovirus contains a single-stranded RNA genome of positive polarity which replicates in the cytoplasm of the host cell. The molecular mechanism of this replication is not well understood. Studies in vivo as well as in vitro indicate that the major intermediate in replication is a ribonucleoprotein complex consisting of a polymerase and one full-length minus-strand RNA molecule complexed to several nascent positive strands (2, 17, 21). It was in this form that the poliovirus RNA-dependent RNA polymerase was first isolated (1). Purification of the polymerase-template complex suggested that only one virus-specific protein was required for elongation activity in the complex (7, 13, 20). We found that this protein had an apparent molecular weight of 62,500 (p63) as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (13).

The polymerase was isolated as a soluble enzyme, first by using polyadenylic acid [poly(A)] as a template and oligouridylic acid [oligo(U)] as a primer (12). The polyuridylic acid [poly(U)] polymerase was partially purified from the soluble portion (i.e., in a 200,000 × *g* supernatant) of an infected cytoplasmic extract and was shown by us (15) and by Dasgupta et al. (9) to use poliovirion RNA as a template to synthesize a heteropolymeric and complementary RNA product. Two major viral proteins (p63 and noncapsid viral protein 2, NCVP2) and several host proteins were found to copurify with polymerase activity (12). Several lines of evidence have suggested that both of the viral proteins might be required components of the soluble poliovirus RNA polymerase (5, 13, 19, 20, 22).

To determine whether both p63 and NCVP2 are required for polymerase activity, we further purified the soluble polymerase and analyzed the viral proteins associated with the purified enzyme. The in vitro polymerase assay used in this study used an oligo(U) primer and specifically measured elongation activity on a poliovirion RNA template. We report that p63 was the only viral protein that copurified with this activity after gel filtration on Sephacryl S-200. No polymerizing activity was found to be associated with NCVP2 when separated from p63. All host proteins still present were recovered in submolar amounts relative to p63. The purified enzyme sedimented at about 4S on a glycerol gradient, and thus appears to be a monomer of p63 and to be synonymous with the polymerase associated with the endogenous replication complex.

MATERIALS AND METHODS

Preparation of labeled cell extracts. Suspension cultures of HeLa cells were infected with poliovirus type 1 as previously described (27). The infected cells (100 ml at 4×10^6 cells/ml) were collected by centrifugation at 3 h postinfection, washed once in Earle saline solution at 37°C, and resuspended in 100 ml of Earle saline supplemented with 3.5% dialyzed calf serum, 3.5% dialyzed fetal calf serum, 1/20 the standard concentration of Eagle minimum essential medium amino acids (GIBCO Laboratories), 2 mM glutamine, and 1 mCi of [^{35}S]methionine. The cells were collected at 5 h postinfection, washed once in Earle saline, and suspended at 8×10^7 cells/ml in 10 mM Tris-hydrochloride (pH 8.0)-10 mM NaCl (TN buffer). After swelling for 10 min, the cells were broken with 10 strokes of a Dounce homogenizer, and the nuclei were removed by centrifugation at $900 \times g$ for 5 min.

Enzyme purification. A high-speed supernatant of the cytoplasmic extract was prepared by centrifugation at $200,000 \times g$ for 2 h at 4°C. Ammonium sulfate was added to the supernatant until it was 35% saturated, and the resulting precipitate was collected by centrifugation as described (15). The precipitate was suspended in 1 ml of TN buffer-20% glycerol, diluted with 4 ml of 50 mM Tris (pH 8.0)-20% glycerol-0.1% Nonidet P-40-2 mM dithiothreitol (DTT)-10 μg of ovalbumin per ml (buffer A), and chromatographed on phosphocellulose as described (15). Peak fractions of polymerase activity were pooled, concentrated approximately fourfold against solid sucrose, and loaded onto a Sephacryl S-200 column (either 1.6 by 63 cm or 1.6 by 180 cm) that was previously equilibrated with buffer A-0.2 M KCl. The column was developed at a flow rate of 5 ml/h, using buffer A-0.2 M KCl and by collecting 2-ml fractions. Peak fractions of activity were pooled, dialyzed against 50 mM Tris (pH 8.0)-50% glycerol-2 mM DTT-0.1 M KCl (buffer B), and loaded onto a 2-ml hydroxylapatite column. The column was washed with 10 ml of 0.1 M KPO_4 (pH 7.5)-2 mM DTT-10% glycerol, and the polymerase was then eluted in 1-ml fractions, using 0.5 M KH_2PO_4 (pH 7.5)-2 mM DTT-10% glycerol. Peak fractions of polymerase activity were pooled, dialyzed against buffer B, and stored at −20°C. All operations were carried out at 0 to 4°C. Polymerase activity was assayed at each step, using a poly(A) template and an oligo(U) primer as described (12, 15). All buffers were treated with 0.01% diethylpyrocarbonate and autoclaved before use.

Polymerase assays. Polymerase activity was measured by using either poly(A) (Miles Laboratories) or purified poliovirion RNA as a template. When poly(A) served as the template, the enzyme was assayed in a 60-μl solution containing 50 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 8.0), 3 mM $Mg(CH_3COO)_2$, 13 mM [5,6-^{3}H]UTP (8.6×10^3 cpm/pmol), poly(A) (2.5 μg), oligo(U)$_{20}$ (0.63 μg), actinomycin D (10 μg/ml), and 10 mM DTT. Reactions were carried out at 30°C for 30 min. When poliovirion RNA was used as a template, the reaction conditions were as described (15), except that 3 mM $Mg(CH_3COO)_2$ was used and phosphoenolpyruvate was omitted. The ratio of oligo(U)$_{20}$ to poliovirus RNA molecules was 16:1 when oligo(U) was used in the reaction. It should be noted that it was essential to treat some commercial preparations of oligo(U) with alkaline phosphatase before use in order to achieve maximal enzyme activity in these assays. Presumably, a 3'-phosphate remained on enough molecules to inhibit polymerase activity. The amount of poliovirion RNA used and the incubation time varied as indicated in the figure legends. The reactions were stopped by adding 1 ml of cold 7% trichloroacetic acid-2% sodium pyrophosphate and 100 μg of carrier RNA. The labeled product was collected on membrane filters (Gelman GN-6, 0.45 μm) and counted as described (15).

Polyacrylamide gel electrophoresis. The preparation of protein samples and their electrophoresis in 10% polyacrylamide gels were as described (13). Electrophoresis was carried out at 150 V (constant voltage) for 3 h. The gels were fixed, stained with Coomassie brilliant blue, dried, and autoradiographed at −70°C, using Dupont Cronex X-ray film. To increase the sensitivity of detection, the fluorography procedure described by Bonner and Laskey (4) was used for most gels. The molecular weight markers used to calibrate the gels were phosphorylase b (94,000), catalase (60,000), bovine serum albumin (67,000), and ovalbumin (46,000).

Two-dimensional analysis of proteins. The ^{35}S-labeled viral proteins were analyzed by isoelectric focusing in the first dimension and by SDS-polyacrylamide gel electrophoresis in the second dimension, using methods similar to those described by O'Farrell (25) and Horst and Roberts (18). The labeled viral proteins were first treated with RNase A (15 μg) for 1 h at 25°C to remove any residual RNA. The samples were then made 9.5 M in urea, 2% in carrier ampholytes (1.2%, pH range 5 to 7; and 0.8%, pH range 3 to 10), and 5% in β-mercaptoethanol. In the first dimension, the samples were subjected to isoelectric focusing after loading onto a 4.3% polyacrylamide gel crosslinked with *N,N'*-diallyltartardiamide (Bio-Rad Laboratories). The gels contained 9 M urea, 2% Nonidet P-40, 1.2% carrier ampholytes, pH 5 to 7, and 0.8% carrier ampholytes, pH 3 to 10. The upper chamber was filled with degassed 0.04 M NaOH, and the lower chamber was filled with 0.06 M H_2SO_4. The gels were run at 75 V for 30 min, 150 V for 1 h, 300 V for 15 h, and 450 V for 1 h. The temperature was maintained at 18°C. After focusing, the gels were equilibrated in 0.06 M Tris (pH 6.8)-1% SDS-1% β-mercaptoethanol containing bromophenol blue for 1 to 2 h. For the second dimension, the tube gels were fixed in position above a 10% polyacrylamide slab gel, using 1% agarose in equilibration buffer, and were subjected to electrophoresis as described above. Molecular weights were determined by electrophoresing a reference sample in the same gel. The pH gradient formed in the first dimension was determined by measuring the pH of 5-mm gel slices in 0.4 ml of degassed 9.2 M urea.

Iodination of proteins. Protein samples were iodinated by the method of Bolton and Hunter (3). The ^{125}I-labeled Bolton-Hunter reagent (4,000 Ci/mmol) was dried with a stream of N_2 before use and suspended in the original volume with 0.1 M sodium borate buffer, pH 8.5. The molecular weight markers (5 μg, total) were precipitated with 10 volumes of

acetone. Pellets were dried in vacuo, resuspended in 10 μl of Bolton-Hunter reagent (100 μCi), and incubated on ice for 1.5 h. One volume of 1 M Tris buffer, pH 7.5, was added, and the proteins were precipitated twice with 10 volumes of acetone and 20 μg of carrier cytochrome *c*.

Poliovirus RNA preparation. Poliovirus RNA was extracted from purified virions as previously described (15).

Protein determination. Protein concentrations were measured by the method of Bradford (6), using bovine serum albumin as a standard.

Materials. [5-^{3}H]UTP was obtained from Schwarz/Mann in a 50% ethanol solution. The ethanol was removed, and the solution volume was reduced fivefold. [^{35}S]methionine was obtained from New England Nuclear Corp. Oligo(U)$_{20}$ (lot 683-36F) was from Collaborative Research, Inc. Carrier ampholytes were purchased from Bio-Rad Laboratories. Poly(U)-Sepharose 4B and Sephacryl S-200 were from Pharmacia Fine Chemicals, Inc. The sources for other materials were as previously described (15).

RESULTS

Polymerase purification. To determine which virus-specific polypeptides were required for polymerizing activity on an exogenously added poliovirion RNA template, we purified the soluble poliovirus RNA polymerase from infected cells grown in the presence of added [^{35}S]methionine. The virus-specific proteins present at each stage of purification were examined by SDS-polyacrylamide gel electrophoresis and autoradiography. Because poliovirus infection inhibits the synthesis of host proteins, only virus-specific proteins were labeled and detected in these experiments (11). Thus, purification of the polymerase allowed us to specifically determine which virus-specific protein copurified with polymerase activity. The enzyme was purified as a poly(U) polymerase, using a poly(A) template complexed to an oligo(U) primer. At various stages of purification the enzyme was tested for its ability to copy a poliovirion RNA template complexed to an oligo(U) primer.

Initial fractionation of the poliovirus RNA polymerase from other soluble proteins in the cytoplasm of infected cells was achieved by ammonium sulfate precipitation and by phosphocellulose chromatography (Table 1). This resulted in about a 75-fold purification of the enzyme (fraction III, Table 1). Polyacrylamide gel electrophoresis showed that four major viral proteins were still present at this stage of purification (Fig. 1, lane 4). During this initial purification of the polymerase, the percentage of the total radioactivity recovered in NCVP2 and p63 increased significantly, whereas the amount of radioactivity present in the two smaller proteins (p45 and NCVPY) remained about the same

TABLE 1. *Purification of poliovirus RNA polymerase*[a]

Purification step	Vol (ml)	Total U[b]	Protein (mg/ml)	Sp act (U/mg)[b]	Yield (%)
I. Supernatant (200,000 × *g*)	5.6	14.4	2.3	1.1	100
II. Ammonium sulfate	1.0	35.3	2.9	12.2	246
III. Phosphocellulose	1.15	24.1	0.25	83.4	168
IV. Sephacryl S-200 (1.6 by 180 cm)	28.0	0.54	ND[d]	ND	3.8[c]
V. Hydroxylapatite	0.9	0.34	ND	ND	2.3

[a] Purification represented here is the same that is analyzed in Fig. 1.

[b] One unit of activity is equivalent to the amount of enzyme required to incorporate 1 nmol of labeled substrate into acid-insoluble product in 30 min at 30°C under the reaction conditions specified in the text. Poly(A) and oligo(U) were used to measure polymerase activity at each stage of purification.

[c] In this experiment, the yield from the Sephacryl column was significantly lower than usual. The yield of input activity in two other experiments was 40 and 31%. The Sephacryl column used in this experiment was developed in 0.5 M KCl instead of 0.2 M KCl. KCl is known to inhibit the activity of the enzyme (15) and may also affect its stability.

[d] ND, Not determined.

(Fig. 1, lanes 1–4). The smallest protein, Y, is a noncapsid viral protein that migrates at about the same position as VP2 (8). Because virions would not be present in a high-speed supernatant, VP2 (found only in mature virions) would not be present. Although our previous study suggested that p63 was required for polymerase activity (13), it was not clear whether the other three proteins were also required for activity, and therefore additional purification of the enzyme was required.

Affinity chromatography on poly(U)-Sepharose was previously shown by Dasgupta et al. (9) to be a useful procedure for purifying the poliovirus RNA polymerase. Adsorption of the fraction III enzyme to poly(U)-Sepharose and batch elution with 0.25 M KCl resulted in an additional twofold purification of the polymerase (data not shown). When each fraction containing polymerase activity was analyzed for viral proteins by SDS-gel electrophoresis, the same four proteins (NCVP2, p63, p45, and NCVPY) were found to have coeluted with the activity (data not shown). The relative amounts of each viral protein were essentially the same as those found after phosphocellulose chromatography (Fig. 1, lane 4). Thus, chromatography on poly(U)-Sepharose

did not prove useful in fractionating these four proteins relative to each other and to the peak of polymerase activity.

Further purification of polymerase activity by a combination of gel filtration in Sephacryl S-200 and by hydroxylapatite chromatography resulted in a fractionation of these four viral proteins (Fig. 1, lanes 5 and 6). The major viral protein component of the purified enzyme was p63 (Fig. 1, lane 6). In addition, it was clear that the fraction of the total radioactivity recovered in p63 relative to the other viral proteins increased at each purification step (Fig. 1, lanes 1–6). Thus, out of the four viral proteins, it appeared that only p63 was copurifying with polymerase activity.

The purified enzyme (Table I, fraction V) was tested for its ability to copy a poliovirion RNA template. The enzyme actively incorporated labeled substrate into product RNA in the presence of poliovirion RNA, oligo(U), and all four ribonucleoside triphosphates (Fig. 2). Omission of the virion RNA, oligo(U), or the three unlabeled ribonucleoside triphosphates resulted in a total loss of activity. The enzyme was active on a poliovirion RNA template complexed to an oligo(U) primer at all stages of purity (data not shown).

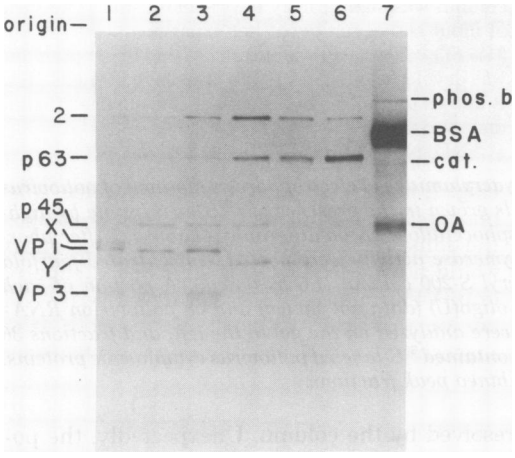

FIG. 1. *Polyacrylamide gel electrophoresis of poliovirus RNA polymerase at each stage of purification. The polymerase was purified from infected cells grown in the presence of [^{35}S]methionine (Table 1). The labeled proteins present at various stages of purity were analyzed by SDS-polyacrylamide gel electrophoresis as follows: lane 1, cytoplasmic extract; lane 2, 200,000 × g supernatant (fraction I); lane 3, $(NH_4)_2SO_4$ precipitate (fraction II); lane 4, phosphocellulose (fraction III); lane 5, Sephacryl (fraction IV); lane 6, hydroxylapatite (Fraction V). A constant amount of labeled protein (900 cpm of acid-insoluble material) was loaded in lanes 1 through 6. Lane 7 contained ^{125}I-labeled phosphorylase b (phos b), bovine serum albumin (BSA), catalase (cat), and ovalbumin (OA).*

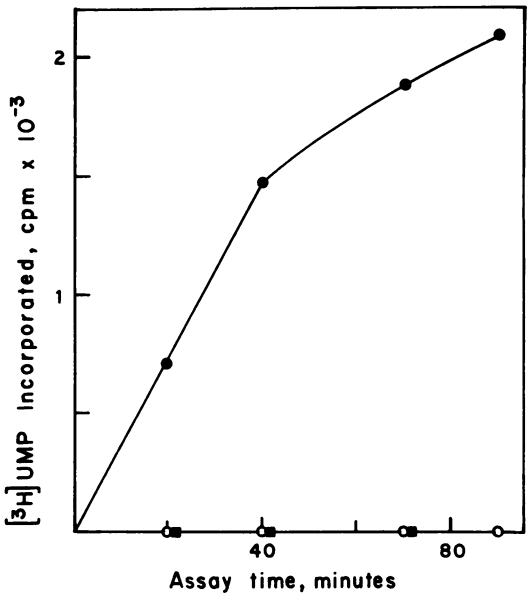

FIG. 2. *Activity of poliovirus RNA polymerase on a poliovirion RNA template. The polymerase (fraction V, Table 1) was assayed for activity by measuring the incorporation of [^{3}H]UMP into an acid-insoluble product. Incorporation was measured with poliovirion RNA, oligo(U), and all four nucleoside triphosphates (●); poliovirion RNA, oligo(U), and UTP only (○); and poliovirion RNA and all four nucleoside triphosphates (■).*

Gel filtration on Sephacryl S-200. Although the purification described above yielded a polymerase preparation rich in p63, a small amount of NCVP2 and a trace of NCVPY were still present. Thus, it was not possible to definitively state that p63 was the only viral protein responsible for the purified activity. Because gel filtration enriched only p63 and diminished the quantity of NCVP2 and the other viral proteins, we analyzed the relative elution positions of the polymerase and the labeled viral proteins after filtration on Sephacryl S-200. The column fractions collected were assayed for poly(U) polymerase as well as for elongation activity on poliovirion RNA, and the two activities were found to copurify in the same position. To determine which of the viral proteins eluted with these activities, an equal portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis for the presence of ^{35}S-labeled proteins (Fig. 3). The only viral protein that precisely eluted with the peak of polymerase activity was p63. NCVP2 eluted ahead of p63, as did p45 and NCVPY, in a region where no polymerizing activity could be detected (Fig. 3). In addition,

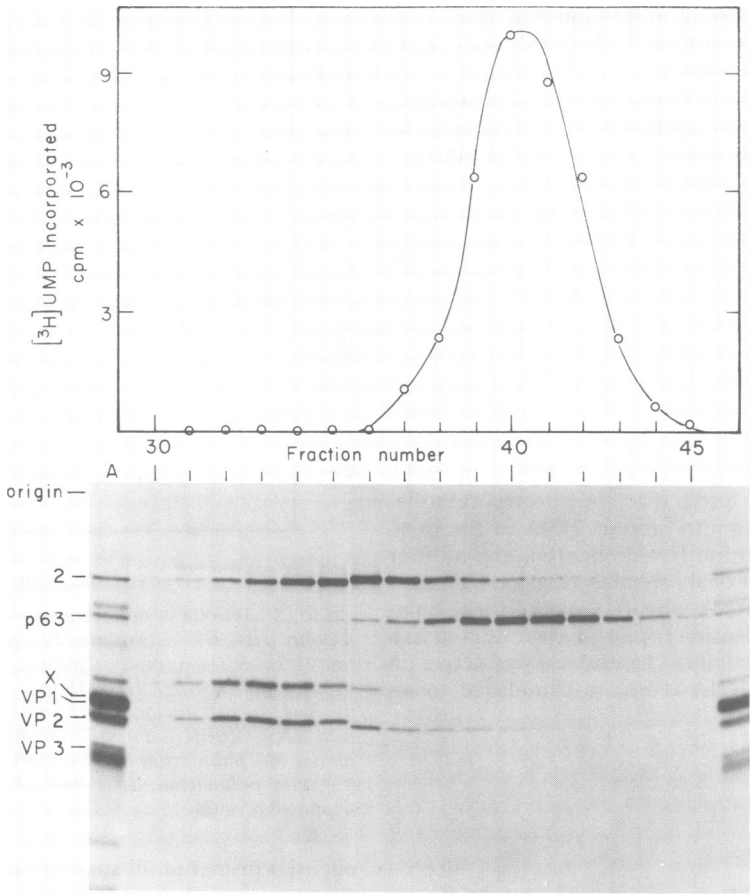

FIG. 3. *Gel filtration on Sephacryl S-200 (top) and polyacrylamide gel electrophoresis (bottom) of poliovirus RNA polymerase. The polymerase was isolated from cells grown in the presence of [^{35}S]methionine by high-speed centrifugation, $(NH_4)_2SO_4$ precipitation, and phosphocellulose chromatography (Table 1). After phosphocellulose chromatography, the peak fractions of polymerase activity were pooled, concentrated fourfold with solid sucrose, and chromatographed on a Sephacryl S-200 column (1.6 by 63 cm). A portion of each fraction was assayed for polymerase activity on poly(A)·oligo(U) (data not shown) and on poliovirion RNA·oligo(U) (top). The labeled proteins in fraction 30 to 35 were analyzed on the gel to the left, and fractions 36 through 45 were analyzed on the gel to the right. Lane A contained ^{35}S-labeled poliovirus cytoplasmic proteins. About 40% of the input activity was recovered in the combined peak fractions.*

polymerase activity was not enhanced by mixing equal amounts of fraction number 36 (the peak of NCVP2) with fraction number 40 (the peak of p63 and polymerase activity) (data not shown). Consistent with this result is the fact that the peak of activity was symmetrical. These results argue that the viral polypeptide associated with elongation activity is p63 and not NCVP2, and that NCVP2 alone has no polymerase activity.

Size of the polymerase. We attempted to estimate the size of the polymerase by determining its relative elution volume during gel filtration. A Sephacryl S-200 column (1.6 by 180 cm) was prepared and was calibrated with five protein molecular weight standards which were well resolved by the column. Unexpectedly, the polymerase eluted very late from the column in a position that corresponded to a molecular weight of 20,000 (data not shown). Increasing the KCl concentration in the column buffer to 0.5 M had no effect on this profile. Because the enzyme was expected to elute at a position corresponding to a molecular weight of 63,000 or larger, the polymerase may have had a special affinity for the Sephacryl resin which retarded its mobility.

Because the poliovirus polymerase ran anomalously on Sephacryl S-200, it was not possible to determine its size by this method. For this reason we analyzed the size of the polymerase at various stages of purity by glycerol gradient

centrifugation. Before chromatography on phosphocellulose, the polymerase sedimented broadly at about 6S (Fig. 4A; 15), and this suggested that the polymerase was not simply a monomer of p63 at this stage of purity. In contrast, the polymerase sedimented as a sharp peak at 4S after phosphocellulose chromatography (Fig. 4B), which suggested that a monomer of p63 was responsible for the activity. We do not yet understand the composition of the 6S polymerase, nor do we know exactly what causes its conversion to a 4S form. We do know that the conversion is a result of chromatography on phosphocellulose and is not a function of the salt concentration, the protein concentration, the presence of Nonidet P-40, or treatment with the reducing agent, DTT (data not shown).

Host-coded polypeptides and polymerase activity. To determine whether any host-coded proteins had copurified with the polymerase in similar quantities to p63, we analyzed the fraction IV polymerase on an SDS-polyacrylamide gel and stained the protein bands with Coomassie brilliant blue. The gel was dried and autoradiographed to compare labeled viral proteins with stained bands. The major labeled viral protein present was p63, although small amounts of NCVP2 and NCVPY were present (Fig. 5, lane 5). When the same gel was stained, only three bands were detected (Fig. 5, lane 4). One band migrated at the exact same position as p63. The other bands were ovalbumin and its breakdown product, which were present in the column buffer as carrier protein. The staining procedure was not sensitive enough to detect the small amount of NCVP2 that was present. All of the host proteins that were present at the early stages of purification (Fig. 5, lanes 1-3) could not be detected in the purified enzyme (Fig. 5,

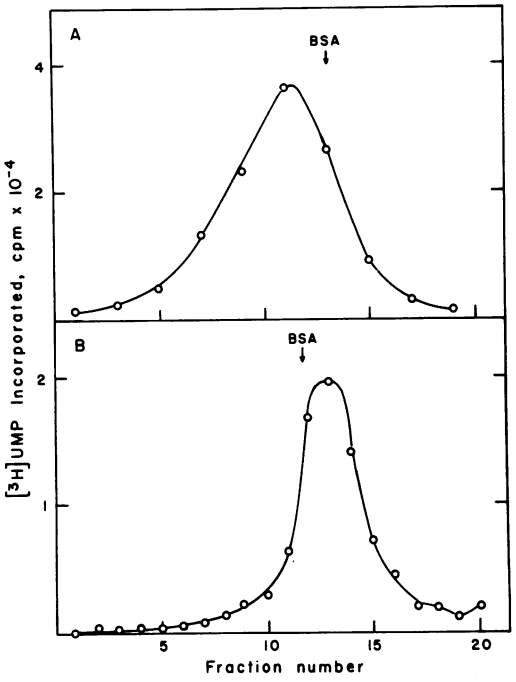

FIG. 4. *Glycerol gradient centrifugation of the poliovirus RNA polymerase before (A) and after (B) phosphocellulose chromatography. The polymerase was analyzed by centrifugation on a 15 to 30% glycerol gradient before (A, fraction II, Table 1) and after (B, fraction III, Table 1) chromatography on phosphocellulose. The samples were centrifuged at 40,000 rpm for 28 h at 4°C in a Beckman SW41 rotor. The gradient was collected in 0.5-ml fractions by pumping from the bottom of the tube. Each fraction was assayed for activity with poly(A)·oligo(U). Bovine serum albumin (BSA) was run as a sedimentation marker in a parallel gradient with both samples.*

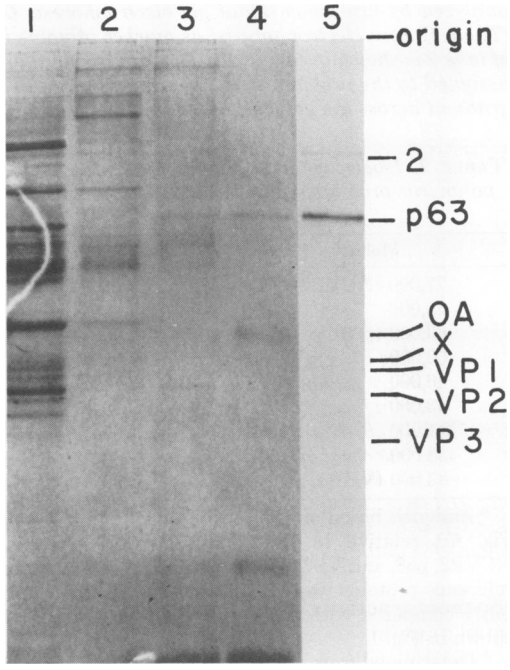

FIG. 5. *Protein composition of poliovirus RNA polymerase determined by staining and autoradiography after polyacrylamide gel electrophoresis. The polymerase was isolated from infected cells grown in the presence of [^{35}S]methionine (Table 1). The proteins present at various stages of purity were analyzed by SDS-polyacrylamide gel electrophoresis and by staining with Coomassie brilliant blue. Polymerase fractions were analyzed as follows: lane 1, 200,000 × g supernatant (fraction I); lane 2, $(NH_4)_2SO_4$ precipitate (fraction II); lane 3, phosphocellulose (fraction II); lane 4, Sephacryl S-200 (fraction IV). The labeled proteins present in the fraction IV enzyme (lane 4 above) were detected by autoradiography of the same area of the gel (lane 5).*

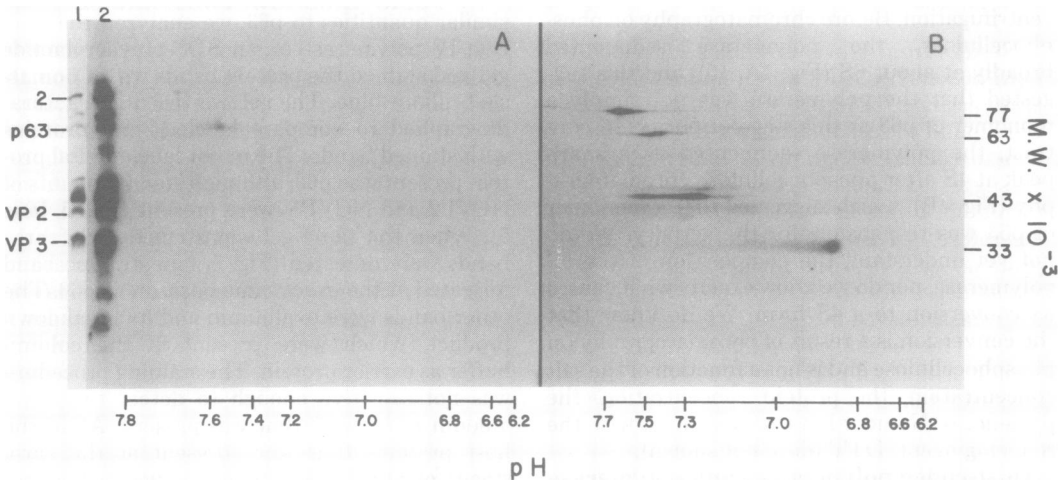

FIG. 6. *Two-dimensional gel electrophoresis of poliovirus RNA polymerase. The purified polymerase (fraction V, Table 1) was isolated from infected cells grown in the presence of [^{35}S]methionine and was analyzed by two-dimensional gel electrophoresis (A). ^{35}S-labeled poliovirus proteins present in fraction II (Table 1) were electrophoresed as marker proteins in the second dimension only (lane 2). A lighter exposure of lane 2 is shown in lane 1. (B) Two-dimensional analysis of the fraction II proteins. Molecular weights were assigned to the proteins in (B) by using the values determined for NCVP2, p63, and VP3 in Fig. 1. The pH gradient across the gel was determined as described in the text.*

TABLE 2. *Isoelectric point and molecular weight of poliovirus proteins separated by two-dimensional gel electrophoresis*

Mol wt[a]	Isoelectric point[b]
77,000 (NCVP2)	7.65
65,000	7.1
63,000 (p63)	7.45
60,000	7.05
46,000	7.1–7.5
43,000	7.15–7.55
41,000	7.05–7.65
38,000	7.35
33,000 (VP3)	6.95

[a] Assigned based on the position of the proteins in Fig. 6B relative to the identified viral proteins, NCVP2, p63, and VP3. The molecular weight of these reference proteins was calculated based on their mobility compared with molecular weight standards as shown in Fig. 1.

[b] Determined from position in the two-dimensional gel shown in Fig. 6B.

lane 4). Thus, we concluded from this that there were no host proteins present in the same molar amount as p63 in the purified enzyme.

Two-dimensional gel electrophoresis. We felt it was important to analyze the structure of the polymerase by two-dimensional gel electrophoresis in order to characterize the polymerase protein in terms of its isoelectric point as well as its molecular weight. In addition, we wanted to determine whether more than one viral protein might be migrating at a molecular weight of 63,000 during polyacrylamide gel electrophoresis. Therefore, the purified polymerase was analyzed by isoelectric focusing in the presence of urea in the first dimension and by SDS-polyacrylamide gel electrophoresis in the second dimension. When the purified polymerase (Table 1, fraction V) was analyzed by this method, only one protein was found in the 63,000-dalton region of the gel, and this protein had an isoelectric point of 7.5 (Fig. 6A). A trace amount of NCVP2 was also detected with an isoelectric point of 7.7. The position of p63 relative to other viral proteins in the two-dimensional gel was also determined (Fig. 6B). This served to compare the isoelectric point of p63 relative with that of the other viral proteins (Table 2).

DISCUSSION

These results indicate that poliovirus polypeptide p63 is a soluble RNA-dependent RNA polymerase that is present in the cytoplasm of poliovirus-infected cells. The soluble polymerase (fraction III, Table 1) copies poliovirion RNA into a full-genome-length product RNA that is covalently linked to an oligo(U) primer (R. J. Rickles and J. B. Flanegan, unpublished data). The purified polymerase did not require the presence of any detectable virus-coded or host-coded proteins for activity and was found to sediment at about 4S on a glycerol gradient. These findings suggest that p63 alone, in monomer form, can function as an RNA polymerase in vitro.

Results from previous studies suggested that both p63 and NCVP2 were required for polymerase activity (5, 13, 19). Because both proteins were found to cosediment on a glycerol gradient and to cochromatograph on phosphocellulose, we suggested that the active form of the enzyme might be a complex of NCVP2 and p63 (13). Therefore, to determine whether one or both proteins were required for polymerase activity, it was necessary to separate p63 from NCVP2. This was achieved by gel filtration on Sephacryl S-200. The results showed that NCVP2 alone had no associated polymerase activity, and that it was not required by p63 for elongating activity in vitro. These findings were consistent with the previous work in which it was demonstrated that p63 was the only virus-specific protein associated with the endogenous RNA replication complex (13). p63 is apparently the same protein that was designated as NCVP4 by Lundquist et al. (20) and Butterworth et al. (7) in their studies on the endogenous RNA replication complex. In other reports, however, viral proteins that may not be the polymerase protein have been designated as NCVP4 (5, 26). To help clarify this situation, we have analyzed the polymerase protein by two-dimensional gel electrophoresis. This provides two physical parameters for the identification of the polymerase protein. The protein's isoelectric point was about 7.5 in the presence of urea, and its apparent molecular weight was 63,000. In addition, the position of this protein in the two-dimensional gel relative to NCVP2 and other viral proteins should prove useful in identifying this protein in future studies.

We have made no conclusions about which proteins may be required to initiate poliovirus RNA replication. In this study, we assayed for elongation activity on a poliovirion RNA template only when oligo(U) was used as a preformed primer. Initiation of RNA synthesis in vivo may require the presence of additional virus- or host-coded proteins. Before chromatography on phosphocellulose, the polymerase sediments at about 6S. This suggests that p63 is complexed with macromolecular factors before chromatography on phosphocellulose. The nature of this factor is not yet defined and is clearly not required for elongating activity, but it may be required to initiate RNA synthesis. One protein factor that has been related to the initiation of viral RNA synthesis is a host-coded protein that was originally described by Dasgupta et al. (10). This protein stimulated poliovirus RNA polymerase activity in vitro. Recent evidence suggests that this host-coded protein may play a role in initiating RNA synthesis because the protein stimulates polymerase activity in the absence of oligo(U) but not in its presence (10). Additional studies are required to define the mechanism by which this protein stimulates polymerase activity. In addition, it has been proposed that the protein which is covalently linked to the 5'-terminal end of poliovirion RNA (i.e., VPg) may play a role in initiating viral RNA synthesis (14, 24). No evidence, however, for this model is now available. Further studies are required to define all the factors that may be required to initiate poliovirus RNA synthesis in vitro in the absence of an oligo(U) primer.

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